Role of the Lectin Pathway of Complement Activation in Septic and Anaphylactic Shock

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Michaela Windbichler

Institute of Immunology

University of Regensburg, and

Department of Infection, Immunity and Inflammation

University of Leicester

September 2005

Role of the Lectin Pathway of Complement Activation in Septic and Anaphylactic Shock

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Michaela Windbichler

Institute of Immunology

University of Regensburg, and

Department of Infection, Immunity and Inflammation

University of Leicester

Role of the Lectin Pathway of Complement Activation in Septic and Anaphylactic Shock

PhD Thesis by Michaela Windbichler

The complement system constitutes an important part of innate host defence. Complement activation can be initiated via the classical, lectin or alternative pathway. This work focuses on the role of the lectin pathway in mouse models of septic and anaphylactic shock.

As a model of septic shock cecal ligation and puncture (CLP) was used. Complement activation is crucial for survival as mouse strains deficient in C1q and factors B and C2 show a higher mortality than complement-sufficient controls. *H2-Bf/C2*$^{-/-}$ mice also demonstrate an impaired bacterial clearance. The involvement of the lectin pathway can be seen in a rapid and long lasting decrease of serum MBL levels. mRNA expression was not altered during the course of infection. Furthermore, MBL deposition was demonstrated on the abscess that forms after peritoneal infection.

Activation of the complement system also occurs during anaphylactic shock. It is believed that the classical pathway is responsible for complement activation, as serum C1q levels decrease during shock.

This work shows also an involvement of the lectin pathway with serum MBL levels being diminished after antigen challenge. The mechanism leading to this decrease was investigated.

To find out whether complement activation is of pathophysiological consequence in anaphylactic shock, several mouse strains with selective deficiencies in complement components, as well as mast-cell deficient mice were tested. Only mast cell deficient mice were protected from anaphylaxis and showed no decrease in serum MBL levels, indicating that cell derived mediators and not the complement system are crucial for the induction of anaphylaxis.

Taken together, these results demonstrate a clear need for the complement system in combating bacterial infections during septic shock. In the case of anaphylaxis, however, complement activation is not causative of the symptoms.

Abbreviations

APS	ammonium persulfate
BSA	bovine serum albumin
C1-INH	C1-inhibitor
C4bp	C4 binding protein
C5aR	C5a receptor
CCP	complement control protein
CD	cluster of differentiation
cDNA	complementary DNA
CFU	colony forming unit
CL-L1	collectin liver-1
CLP	cecal ligation and puncture
CL-P1	collectin placenta-1
Con A	concanavalin A
CR	complement receptor
CRD	carbohydrate recognition domain
Crry	complement receptor 1-related gene/protein y
CUB	C1r/C1s/Uegf/bone morphogenetic protein 1
CVF	cobra venom factor
DAF	decay-accelerating factor
dCTP	2'-deoxy-cytidin-5'-triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2'-deoxy-nucleosid-5'-triphosphate
EACA	ε-amino-n-caproic acid
ECL	enhanced chemoluminiscence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cell
Ig	immunoglobulin

IL	interleukin
IPTG	isopropyl-β-D-thiogalactoside
LB medium	Luria-Bertani medium
MAC	membrane attack complex
MAp19	MBL-associated plasma protein of 19 kDa
MASP	MBL associated serine protease
MBL	mannan binding lectin
MCP	membrane cofactor protein
MOPS	3-[N-morpholino]propanesulfonic acid
NMS	normal mouse serum
OD	optical density
OVA	Ovalbumin (albumin from chicken egg white)
PAF	platelet activating factor
PAGE	polyarcylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
RA	rheumatoid arthritis
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythematosus
SP-A	surfactant protein A
SP-D	surfactant protein D
SSC	sodium-sodium-citrate buffer
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	tumor necrosis factor
TRIFMA	time resolved immunofluorometric assay
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Table of contents

1. Introduction

1.1. The complement system

1.1.1. Complement activation

The complement system consists of more than 30 plasma proteins and a number of associated cell surface proteins that act as receptors and regulators of complement activation. It is part of the innate immune system and provides a critical first line of defence against invading microorganisms, as well as mediating a large variety of cellular and humoral interactions within the immune response.

The complement system can be activated via three distinct pathways, namely the classical, the lectin, and the alternative pathway. The classical pathway is activated when its recognition molecule C1q binds to the Fc portions of IgG1, IgG2, IgG3, and IgM in antigen-antibody complexes. Then the associated proenzyme C1r is activated, which then activates the serine esterase C1s. Activated C1s cleaves complement components C4, liberating C4a, and C4-bound C2 to generate the C3 convertase C4b2b, which in turn cleaves native C3 into C3b and C3a. This exposes a thioester bond through which C3b is covalently bound to nearby surfaces. Deposition of multiple C3b molecules in close proximity of the C3 convertase complex changes its substrate specificity, forming the C5 convertase complex, $C4b2b(3b)_n$, which converts native C5 to C5b and C5a. These enzymatic reactions are accompanied by the production of potent anaphylatoxins (C5a, C3a and C4a). C5b then binds C6 and C7 to form C5b67. This complex can detach from C3b and insert itself into the lipid bilayer of microbial surfaces, where it binds one C8 and multiple C9 molecules, thereby forming a transmembrane pore called the membrane attack complex (MAC) (Whaley and Schwaeble, 1997).

The recognition molecules of the lectin pathway are mannan-binding lectin (MBL) and ficolins. They recognise carbohydrate patterns on the surface of bacteria. When they are

bound, the MBL-associated serine protease 2 (MASP-2) cleaves C4 and C4-bound C2, thus activating the complement cascade similar to the classical pathway (Holmskov *et al.*, 2003).

The alternative pathway acts as powerful amplification loop of complement activation. C3b binds factor B, which then is cleaved by factor D to form the alternative pathway C3 convertase, C3bBb. This convertase has a very short half-life unless it is stabilised by properdin to form the C3bBbP convertase complex, which cleaves more C3 (Whaley and Schwaeble, 1997).

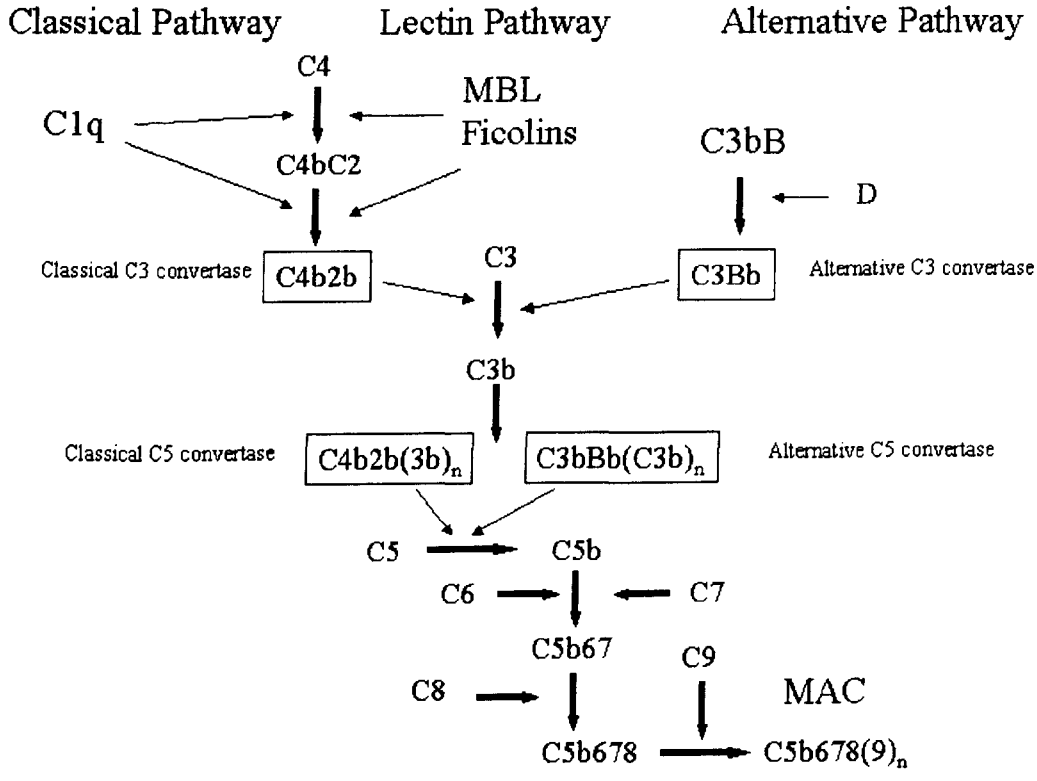

Fig. 1.1: Diagram of the complement activation pathways.
Narrow arrows denote enzymatic cleavage.

1.1.2. Complement inhibition

To avoid excessive complement activation and damage to host tissue, soluble and membrane-bound inhibitors tightly regulate the activation.

Soluble regulators are C1-inhibitor (C1-INH), C4 binding protein (C4bp), factor I, factor H, clusterin, and S-protein/vitronectin. C1-INH binds and inactivates C1r and C1s. C4bp disassembles the C4b2b complex, prevents binding of C2 to C4b, and acts as a cofactor for factor I in the inactivation of C4b. The serine protease factor I cleaves C3b and C4b, thus converting them to hemolytically inactive forms. Factor H accelerates the decay of the alternative convertases by displacing Bb from the complex and also acts as cofactor for factor I (Whaley and Schwaeble, 1997; Turnberg and Botto, 2003). Clusterin and S-protein bind to the terminal complement complexes and prevent their insertion into cell membranes (Meri and Jarva, 1998).

Membrane-bound inhibitors include complement receptor 1 (CR1, CD35), decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and CD59 (protectin). DAF accelerates the decay of the C3 and C5 convertases. MCP, which is ubiquitously expressed in primates, but only in testes in rodents, acts as cofactor for factor I. Ubiquitously expressed CD59 limits the insertion of C9 into the MAC, thus protecting cells from lysis. CR1 also serves as a cofactor for factor I (Whaley and Schwaeble, 1997; Turnberg and Botto, 2003). The mouse expresses another transmembrane protein with complement regulatory activities, the Complement receptor 1-related gene/protein Y (Crry), also called complement regulatory protein Crry/p65. It inhibits both the classical and the alternative pathway and possesses potent decay-accelerating and membrane cofactor activities (Kim *et al.*, 1995).

1.1.3. Consequences of complement activation

Complement activation results in opsonisation and lysis of microorganisms, recruitment and activation of inflammatory cells, regulation of antibody production, and the elimination of immune complexes.

Opsonisation is the coating of targets like unwanted cell debris or bacteria with antibodies and complement components. Thus they are marked for destruction by phagocytic cells. Polymorphonuclear leukocytes, monocytes and macrophages recognise their targets via Fc-receptors, and via complement receptor 1 (CR1, CD35), binding C3b and C4b, and CR3 (CD11a/CD18), binding iC3b (Frank and Fries, 1991). C1q and collectins like MBL are also able to enhance phagocytosis. It has been shown that binding of these molecules to cell surface calreticulin/CD91 is important for the clearance of apoptotic cells (Stuart *et al.*, 1997; Ogden *et al.*, 2001; Vandivier *et al.*, 2002). CR1 also binds C1q (Klickstein *et al.*, 1997) and MBL (Ghiran *et al.*, 2000).

In some cases (e.g. gram-negative bacteria) the complement system can directly kill the target cell by formation of the MAC, which forms pores in the cell wall, thereby lysing the target cell. Other cells (e.g. gram-positive bacteria or fungi) are resistant to the MAC. Host cells are protected from MAC-induced lysis through a number of regulatory proteins on their surface (Nauta *et al.*, 2004). Lack of inhibitors or excessive complement activation, however, can override this protection. The MAC also causes a number of non-lytic effects, namely cell activation and proliferation, release of inflammatory mediators and expression of adhesion molecules (Nauta *et al.*, 2004).

Binding of C1q and deposition of C3b support the formation of soluble immune complexes. These complexes are then bound by CR1 on erythrocytes in primates and on platelets in other species and transported to the liver and spleen, where they are cleared by resident macrophages (Miller and Nussenzweig, 1974; Whaley and Schwaeble,

1997). When this mechanism is impaired, immune complexes are deposited in tissues, resulting in immune complex diseases and tissue damage.

During complement activation small cleavage products of complement components termed anaphylatoxins (C3a, C4a, and C5a) are released and promote inflammation. C3a induces smooth muscle contraction and enhances vascular permeability. Receptors for C3a exist on mast cells, eosinophils, neutrophils, basophils and monocytes and are directly involved in triggering proinflammatory effects (Frank and Fries, 1991). C5a is much more potent than C3a. In addition to enhancing smooth muscle contraction and vascular permeability, it is also a powerful chemotactic factor for basophils, eosinophils, neutrophils and mononuclear phagocytes and stimulates neutrophil oxidative metabolism and the production of toxic oxygen species (Frank and Fries, 1991). Both C3a and C5a have been shown to directly stimulate mast cell degranulation (Johnson *et al.*, 1975; Schulman *et al.*, 1988; el Lati *et al.*, 1994). C3a, however, also seems to have anti-inflammatory properties, as C3aR-deficient mice were more susceptible to endotoxin shock and exhibited elevated levels of IL-1β, TNF and IL-6 (Kildsgaard *et al.*, 2000).

Other complement-derived chemotactic factors are C5b67, which attracts neutrophils and eosinophils, and Bb, which attracts neutrophils (Tizard, 2000a).

Complement activation also plays a role in regulating the humoral immune response. Mice deficient for C3 or C4 show an impaired immune response against T-cell-dependent antigens (Carroll and Fisher, 1997). CR2 (CD21, binding the breakdown product C3d) is expressed on B cells and on follicular dentritic cells. On B cells CR2 acts as co-receptor for the B cell-receptor. Activation of the co-receptor lowers the threshold for B cell activation and is important for survival and expansion of naive B cells, survival of B cells in germinal centres, but also for negative selection of self-reactive B cells (Carroll, 2004).

1.2. Collectins and ficolins

The collectins belong to the Ca^{++}-dependent (C-type) lectin superfamily, which includes MBL, the lung surfactant proteins SP-A and SP-D, collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, CL-43, and CL-46. They are built of structural subunits composed of three identical polypeptide chains, consisting of a short N-terminal cysteine-rich region, a collagenous region, the length of which differs for each collectin, a short neck region and a C-terminal carbohydrate recognition domain (CRD). Through this CRD they recognise a wide range of sugar residues on the surface of microbes including bacteria, fungi and viruses (Lu *et al.*, 2002; Holmskov *et al.*, 2003). They act as a first line of defence by interacting with phagocytes or, in the case of MBL, activating the lectin pathway of complement activation.

Conglutinin, CL-43 and CL-46 are serum proteins only found in bovids (Holmskov *et al.*, 2003). CL-P1, identified in humans and mice, is a type II membrane protein on endothelial cells which mediates phagocytosis (Ohtani *et al.*, 2001). CL-L1, recently cloned from human liver, is expressed in most tissues (except skeletal muscle). The gene is present in mammals and birds (Ohtani *et al.*, 1999). SP-A and SP-D are mainly produced by alveolar type II cells in the lung. They promote clearance of microbial wall components and protect the lung from inflammatory injury. SP-A also inhibits influenza infectivity; SP-D agglutinates viruses (Holmskov *et al.*, 2003). Both interact with monocytes, macrophages and neutrophils (Lu *et al.*, 2002) and bind apoptotic neutrophils (Schagat *et al.*, 2001).

Ficolins are structurally similar to the collectins, but they have a fibrinogen-like domain instead of the CRD and bind to sugar residues in a calcium-independent manner. The human ficolins are L-ficolin (ficolin/P35), H-ficolin (Hakata antigen) and M-ficolin (ficolin/P35-related protein). In the mouse only two ficolins have been identified, termed ficolin-A and ficolin-B (Matsushita and Fujita, 2001; Holmskov *et al.*, 2003).

L-ficolin (Matsushita *et al.*, 2000a) and H-ficolin (Matsushita *et al.*, 2002) can activate the lectin pathway; M-ficolin is detected on the surface of monocytes, where it mediates phagocytosis (Teh *et al.*, 2000).

Mouse ficolin-A is a serum protein expressed in liver and spleen, while ficolin-B is expressed in bone marrow and spleen (Matsushita and Fujita, 2001).

1.3. MBL

1.3.1. Structure and function of MBL

Mannan-binding lectin (MBL), also called mannose-binding protein, is the best-studied member of the collectin family. In humans, the MBL gene encodes for a 32 kDa glycoprotein, showing the typical collectin structure consisting of an N-terminal cysteine-rich region, a collagen-like region followed by a neck region and a C-terminal carbohydrate recognition domain (CRD) (Turner and Hamvas, 2000). They form homotrimers composed of a collagenous triple helix subunit and several such homotrimers assemble to form higher order oligomers. There is evidence that full biological function requires assembly to at least the tetrameric level (Yokota *et al.*, 1995).

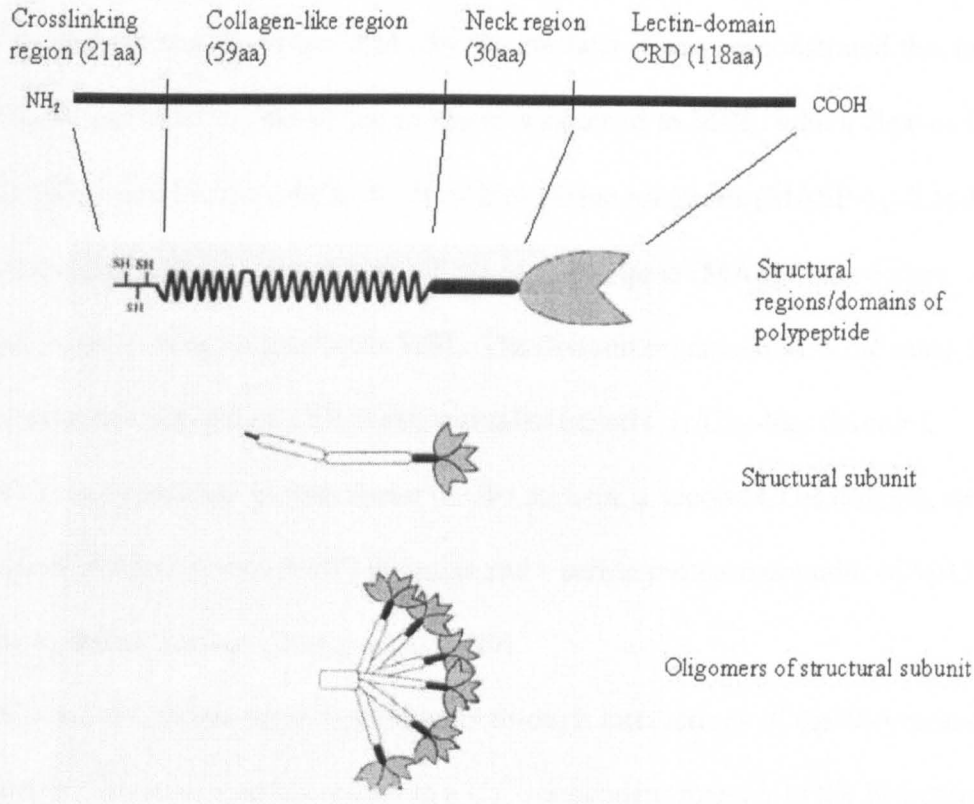

Fig. 1.2: Domain organisation and structure of the mature MBL polypeptide chain, a structural subunit and a hexameric oligomer (Petersen *et al.*, 2001b).

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

MBL activates the lectin pathway. Initially it was believed that MBL associates with the $C1r_2$-$C1s_2$ complex to do so (Lu *et al.*, 1990), but later it was demonstrated that not C1r and C1s, but a novel C1s-like serine protease is attached to MBL, which cleaves C4 and C2 (Matsushita and Fujita, 1992). To date three serine proteases (MASP-1, -2 and -3) and an enzymatically inactive product of the MASP-2 gene (MAp19) have been identified, which are associated with MBL. The domain organisation is the same for all four proteins and consists of a CUB domain (also called C1r/C1s-like domain), followed by an epidermal growth factor (EGF) domain, a second CUB domain, two complement control protein (CCP) domains and a serine protease domain. MAp19 lacks the serine protease domain (Wong *et al.*, 1999).

MASPs form Ca^{++}-independent homodimers through interactions of the N-terminal CUB domain. These homodimers bind in a Ca^{++}-dependent manner to the N-terminal portion of the collagenous domain of MBL (Wallis, 2002). For this interaction, the N-terminal CUB1-EGF-CUB2 domains are important (Chen and Wallis, 2001). Map19 binds to MBL with a lower affinity, as it lacks the second CUB domain (Chen and Wallis, 2001).

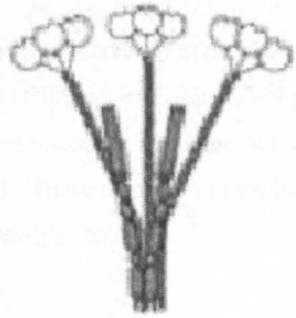

Fig. 1.3: Proposed structural organisation of the MBL/MASP complex.
This is based on the known biophysical properties of the interacting proteins, in which the N-terminal three domains of each MASP protomer bind to a separate MBL subunit (Wallis, 2002).

MBL dimers form complexes with a single MASP dimer, whereas higher oligomers are able to interact with more MASP dimers. Each MASP protomer probably interacts with a separate MBL subunit (Wallis, 2002).

MASP-2 cleaves C4 and C2, thereby activating the lectin pathway (Thiel *et al.*, 1997; Vorup-Jensen *et al.*, 2000). In circulating complexes MASP-2 is inactive, but upon binding of MBL to its target, a conformational change exposes an accessory C4 binding site and MASP-2 is activated (Chen and Wallis, 2004).

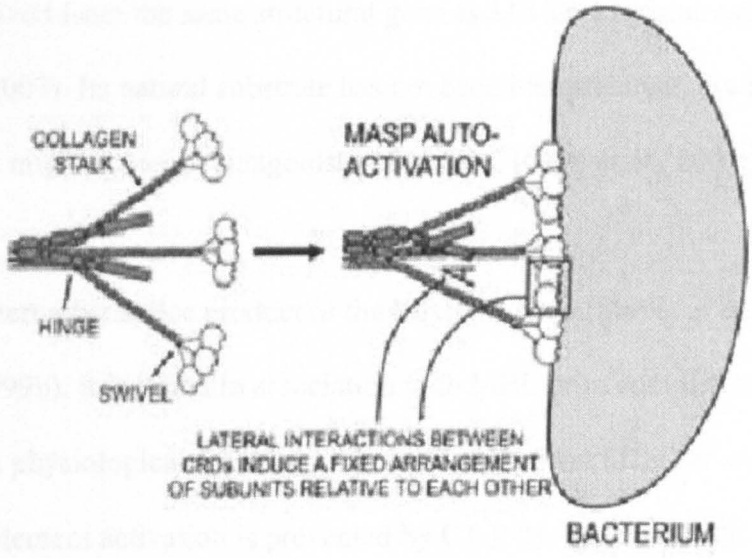

Fig. 1.4: Proposed mechanism of complement activation by MBL.
Lateral interactions between CRDs are stabilised by CRD/carbohydrate interactions when MBL binds to the surface of a microorganism. Binding imparts a fixed geometry as a result of locking the conformation at the hinge and swivel regions of MBL. These changes in turn induce a conformational change in MASP-2 that leads to autoactivation (Wallis, 2002).

The exact role of MASP-1 is still far from being clear at the moment. Initially it has been reported to directly cleave C3 (Matsushita *et al.*, 2000b), but other studies have cast doubt on this (Wong *et al.*, 1999; Hajela *et al.*, 2002; Ambrus *et al.*, 2003). However, it has been shown that MASP-1 seems to cleave C2 (Ambrus *et al.*, 2003), and a role as enhancer of complement activation was suggested (Chen and Wallis, 2004). It also demonstrates a thrombin-like activity by cleaving fibrinogen, thus releasing fibrinopeptide B (a chemotactic factor), and by activating plasma transglutaminase (Factor XIII) (Hajela *et al.*, 2002). Interestingly, MASP-1 is absent in birds (Lynch *et al.*, 2005).

MASP-3 is derived from the same structural gene as MASP-1 by alternative splicing (Stover *et al.*, 2003). Its natural substrate has not been identified yet, but it was proposed that it might act as an antagonist of MASP-2 (Dahl *et al.*, 2001; Zundel *et al.*, 2004).

MAp19 is an alternative splice product of the MASP-2 gene (Stover *et al.*, 1999a; Stover *et al.*, 1999b). It is found in association with MBL or in complex with MASP-1 in serum, but its physiological role has not been identified yet (Thiel *et al.*, 2000).

Excessive complement activation is prevented by C1-INH, which binds to both MASP-2 and MASP-1 (Matsushita *et al.*, 2000b; Petersen *et al.*, 2000; Ambrus *et al.*, 2003). MASP-1 is also inhibited by α_2-macroglobulin (Ambrus *et al.*, 2003). Heparin has been shown to inhibit both MASP-1 and MASP-2 (Presanis *et al.*, 2004).

Furthermore MBL can act as an opsonin via CR1 (Ghiran *et al.*, 2000) or calreticulin/CD91 (Stuart *et al.*, 1997). It opsonises bacteria (Neth *et al.*, 2002), apoptotic cells (Ogden *et al.*, 2001) and necrotic cells (Nauta *et al.*, 2003).

MBL binds to monosaccharides such as N-acetyl-D-glucosamine, mannose, N-acetyl-D-mannosamine, L-fucose and glucose (Hansen *et al.*, 2000). Ligand binding to one single CRD, however, is very weak, and multiple contacts are necessary for activation.

These repetitive carbohydrate structures are found on a wide range of microorgansims, including bacteria, viruses and fungi (Jack *et al.*, 2001; Townsend *et al.*, 2001; Jack and Turner, 2003), but not on mammalian cells, because of the prevalent termination of self-glycoproteins with sialic acid or galactose (Ezekowitz, 1998; Petersen *et al.*, 2001b; Wallis, 2002). Some bacteria protect themselves from MBL-mediated complement attack by sialylating their surface structures (Jack *et al.*, 2001; Gadjeva *et al.*, 2004a).

1.3.2. MBL-deficiency

MBL is mainly produced in the liver. Although a modest acute phase reaction has been reported (Sastry *et al.*, 1991; Thiel *et al.*, 1992; Liu *et al.*, 2001), serum levels are genetically determined and relatively stable (Nielsen *et al.*, 1995; Naito *et al.*, 1999; Siassi *et al.*, 2003).

MBL-deficiency is one of the most common immunodeficiencies. Three different variant alleles encoding for structurally abnormal proteins were identified with single nucleotide polymorphisms in codons 52, 54 and 57 of exon 1 of the *mbl2* gene located on chromosome 10. The wild type allele is designated A. A point mutation at codon 54 leads to a substitution of aspartic acid for glycine at position 34 in the mature protein (allele B). Another point mutation at codon 57 results in a change of glycine to glutamic acid at position 37 (allele C). These exchanges disrupt the Gly-X-Y repeats in the collagenous domain and alter the disulfide bonding pattern, thus disrupting the MASP-binding site (Petersen *et al.*, 2001b). The mutation at codon 52 (allele D) changes the arginine at position 32 to cysteine. This disrupts the oligomer formation by generation of aberrant disulfide bonds (Wallis *et al.*, 2004). These mutated proteins do not bind their ligands, are defective in MASP activation and are unable to fix complement (Garred *et al.*, 2003a; Larsen *et al.*, 2004).

Serum levels are further modified by different promoter polymorphisms. A nucleotide substitution (G to C) at position –550 and –221 gives rise to the H/L and Y/X variant, respectively. The haplotypes HY, LY and LX are associated with high, medium and low serum levels, respectively. Another substitution in the 5' untranslated region of exon 1 at position +4 gives rise to the P/Q variant. Because of a linkage disequilibrium only seven MBL haplotypes (HYPA, LYQA, LYPA, LXPA, LYPB, LYQC and HYPD) were established. Linkage describes the proximity of two or more markers on a chromosome. The closer together the markers are, the lower the probability that they will be separated during DNA replication processes, and hence the greater the probability that they will be inherited together.

But even when all these variants are taken into account, there is still considerable variation in serum MBL levels between individuals with the same genotype (Petersen *et al.*, 2001b). The B allele is found in many Eurasian populations at frequencies ranging from 0.11 – 0.50, whereas the codon 57 mutation is more frequently found in Sub-Saharan Africans (0.23 – 0.29) (Turner and Hamvas, 2000). The frequency of the D allele is 0.05 or less in both Africans and Non-Africans (Turner, 1996). Promoter-polymorphism-frequencies also differ between populations (Turner and Hamvas, 2000).

The gene or allele frequency measures the frequency of a particular gene in the population relative to other genes at its locus. It is expressed as a proportion or percentage. While the gene frequency can be calculated from the genotype frequency, the opposite is not true, so it is not possible to calculate how many people would be deficient in MBL from the data mentioned above. However, it has been reported that 5 to 10% of the population carry polymorphisms resulting in low MBL levels (Dahl *et al.*, 2004; Kilpatrick *et al.*, 2005).

Recently, a point mutation with a frequency of 0.055 was identified in exon 3 of the MASP-2 gene. This mutation results in an exchange of glycine for aspartic acid in the

CUB1 domain, which prevents the mutant protein from binding to MBL (Stengaard-Pedersen *et al.*, 2003).

1.3.3. Disease association

The fact that serum from MBL-deficient individuals is unable to opsonise bakers' yeast presented the first indication that deficiency for MBL might be of clinical relevance (Super *et al.*, 1989). MBL binds to a wide range of pathogenic bacteria, e.g. *Staphylococcus*, *Neisseria*, *Salmonella* and *Escherichia coli* (Jack *et al.*, 2001; Jack and Turner, 2003), and MBL-deficiency is associated with an increased risk of infections in children (Super *et al.*, 1989; Summerfield *et al.*, 1997; Cedzynski *et al.*, 2004) and adults (Summerfield *et al.*, 1995). In children with recurrent infections also low L-ficolin concentrations have been found (Atkinson *et al.*, 2004).

However, the fact that most MBL-deficient individuals are healthy (Dahl *et al.*, 2004), has given rise to the theory that a second immune defect needs to be present for a higher susceptibility to infection. This view is supported by a number of studies: Patients deficient for MBL have an increased risk of severe infections after operations (Siassi *et al.*, 2003), chemotherapy (Peterslund *et al.*, 2001; Kilpatrick *et al.*, 2003) or stem cell transplantation (Mullighan *et al.*, 2002). Patients admitted to intensive care units suffering from systemic inflammatory response syndrome are more likely to develop severe sepsis or septic shock when they have low MBL serum levels (Garred *et al.*, 2003b). MBL-deficiency has also been associated with unexplained, recurrent miscarriage (Christiansen *et al.*, 1999; Kilpatrick *et al.*, 1999). MBL-deficient cystic fibrosis patients showed a decreased lung function and a shorter survival time (Garred *et al.*, 1999b). Another study failed to confirm this association, but showed that MBL-deficiency is detrimental for cystic fibrosis patients colonized with *Staphylococcus aureus* (Carlsson *et al.*, 2005).

Furthermore, MBL plays a role in viral infections. MBL-deficient individuals have an increased risk of HIV infection either directly or indirectly due to an increased susceptibility to coinfections (Garred *et al.*, 1997a; Garred *et al.*, 1997b). MBL binds to the virus protein gp120, inhibits infection of $CD4^+$ cells by HIV and is able to recognise HIV-infected cells (Turner and Hamvas, 2000; Thielens *et al.*, 2002).

MBL also protects from Influenza A infection (Thielens *et al.*, 2002).

The data regarding Herpes simplex virus 2 and hepatitis B virus are conflicting. A study carried out in The Gambia showed no association between hepatitis B and MBL-deficiency (Bellamy *et al.*, 1998). This was also true for asymptomatic carriers of hepatitis B or C in Asia. However, an increased frequency of the B variant allele was found in those patients responding poorly to interferon therapy or in patients with spontaneous bacterial peritonitis or hepatitis B cirrhosis (Turner and Hamvas, 2000). MBL has been shown to enhance herpes simplex virus 2-infection in a mouse model (Fischer *et al.*, 1994). In contrast, the frequency of MBL-deficiency was higher in people with recurrent HSV-2 infections (Gadjeva *et al.*, 2004b). Mice deficient for MBL display defective virus clearance from the liver and compromised liver function (Gadjeva *et al.*, 2004b).

MBL is also involved in parasitic infections. MBL binds to *Trichinella spiralis* (Gruden-Movsesijan *et al.*, 2003) and *Schistosoma mansoni* (Klabunde *et al.*, 2000) and recognises parasite-derived proteins on the surface of cells infected with *Plasmodium falciparum* (Klabunde *et al.*, 2002). In Gabonese children with severe *Plasmodium falciparum* malaria a higher frequency of MBL-deficiency was observed (Luty *et al.*, 1998), whereas no association with MBL-deficiency was found in asymptomatic carriers (Mombo *et al.*, 2003). Mice deficient for MBL-A showed no altered resistance to infection with *Plasmodium yoelii* (Lee *et al.*, 2002).

The high prevalence of variant alleles has led to the assumption that MBL-deficiency might be beneficial for the host in some cases, e.g. in infections with intracellular pathogens. High serum levels of MBL may be involved in the pathogenesis of tuberculosis (Garred *et al.*, 1997b; Bellamy *et al.*, 1998). MBL also enhances the susceptibility to visceral Leishmaniasis (Santos *et al.*, 2001).

MBL is also able to bind to altered self-structures, thus decreasing the likelihood of chronic inflammation and autoimmune triggering (Saevarsdottir *et al.*, 2004). It has been documented previously that MBL is able to bind to apoptotic (Ogden *et al.*, 2001) and necrotic (Nauta *et al.*, 2003) cells. Also some cancer cell lines are recognised by MBL (Gadjeva *et al.*, 2004a; Saevarsdottir *et al.*, 2004).

Modification of self-surfaces after ischemia-reperfusion injury leads to tissue damage, which is partly mediated by complement (Yasuda *et al.*, 1990; Collard *et al.*, 1999). Recognition of ischemic cells by MBL via cytokeratin 1 contributes to this damage (Collard *et al.*, 2001). MBL binds to hypoxic and reoxygenated human umbilical vein endothelial cells (HUVECs) (Collard *et al.*, 1999) and is deposited in heart (Collard *et al.*, 2000) and kidney (de Vries *et al.*, 2004) in an ischemia-reperfusion model. Administration of an anti-MBL-A antibody reduced postischemic myocardial reperfusion injury (Jordan *et al.*, 2001). Mice deficient for MBL, but not C1q-deficient mice, were protected in a myocardial and a kidney ischemia/reperfusion model (Gadjeva *et al.*, 2004a).

MBL has also been associated with autoimmune diseases like systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). MBL-deficiency is a minor risk factor for acquiring SLE and increases the risk of complicating infections (Garred *et al.*, 1999a). In RA the frequency of variant alleles is higher in patients with early onset of disease (Garred *et al.*, 2000) and fast progression of radiographic joint destruction (Graudal *et al.*, 2000; Saevarsdottir *et al.*, 2001). In cases of late onset and advanced

disease, however, high serum MBL levels could contribute to additional inflammation (Garred *et al.*, 2000), underlining the notion of MBL as a modifier in chronic diseases. Another report showed that in rheumatic heart disease, a streptococcal-induced autoimmune disease, carriers of MBL variant alleles were more frequently found in the healthy control group than in patients (Schafranski *et al.*, 2004). This supports the hypothesis that high MBL levels are not always beneficial.

When considering the importance of MBL in health and disease, it has to be taken into account that a large proportion of the population carry variant alleles, most of which are healthy. In the diseases mentioned above, being deficient for MBL increases the risk of acquiring the disease, but it is by no means the only, causative predisposing factor. A large population-based study carried out in Denmark showed no increased morbidity and mortality in MBL-deficient people (Dahl *et al.*, 2004), indicating that in most adults other parts of the immune system are able to compensate for MBL-deficiency. Lack of MBL may only become important when the immune system is compromised, e.g. in children or after chemotherapy. Studies associating MBL-deficiency with a higher risk for severe infections were almost solely hospital-based (Eisen and Minchinton, 2003). Thus, it appears that MBL-deficiency may only increase risk of infection in certain contexts or subgroups of patients, or when additional impairments of the immune system are present (Dahl *et al.*, 2004).

1.3.4. MBL replacement therapy

The fact that low serum MBL levels and their cognate haplotypes are associated with a wide variety of infectious diseases hints at a clinical potential of MBL-replacement therapy. This might be beneficial in MBL-deficient patients to reduce susceptibility to, or enhance recovery from, bacterial infection (Summerfield, 2003). Addition of purified MBL to serum of MBL-deficient patients restores its ability to opsonise bakers' yeast in

vitro (Super *et al.*, 1989). This has also been shown in vivo, when MBL purified from human plasma was infused into two MBL-deficient individuals (Valdimarsson *et al.*, 1998). MBL replacement seems to be safe, and no anti-MBL antibodies have been detected in the recipients (Valdimarsson *et al.*, 1998; Valdimarsson *et al.*, 2004). This is because also people who are considered MBL-deficient produce low amounts of MBL, which, however, is non-functional. Therefore administered MBL is not recognised as foreign by the recipient's immune system.

MBL-infusion-treatment of a cystic fibrosis patient colonized with *Pseudomonas aeruginosa* stabilised the clinical condition; lung function deteriorated again when MBL infusions ceased (Garred *et al.*, 2002). It was shown that in allogenic stem cell transplantation also the genotype of the donor influences the risk of infection (Mullighan *et al.*, 2002). The authors suggested that lymphocytes, macrophages or the haemopoietic progenitor cells might synthesize MBL. Another study, however, demonstrated that haemopoietic stem cell transplantation does not correct MBL-deficiency (Kilpatrick *et al.*, 2005). Thus, infusion of recombinant or purified MBL seems to be the only possible way to correct MBL-deficiency in high-risk patients.

1.4. Previous work and aims

MBL plays a role in a large number of diseases. MBL-deficiency has been associated with severe infections, but also with autoimmune diseases like SLE or RA and ischemia/reperfusion injury (Petersen *et al.*, 2001b). The possibility of MBL replacement therapy calls for a more thorough understanding of the mechanisms of action of MBL in different diseases in order to identify the patients who are most likely to benefit from this therapy.

The aim of this work is to further elucidate the role of the lectin pathway, and especially MBL, in mouse models of septic and anaphylactic shock.

Recently, it has been shown in a different model of septic peritonitis that complement activation is necessary for survival (Celik *et al.*, 2001). The model of cecal ligation and puncture (CLP), which is used in this work, is considered as a more clinic-related model of septic peritonitis with bacteraemia (Urbaschek *et al.*, 1984). By using mouse strains deficient for C1q and factors B and C2 in this model, the relative importance of the classical and alternative pathway for host defence can be determined. To see whether and to which extent the lectin pathway is involved, serum MBL levels will be measured.

Systemic anaphylaxis is the most severe form of immediate hypersensitivity reaction. The classic anaphylactic reaction is mediated by allergen binding to IgE on mast cells, resulting in the activation of Fcε-receptors and subsequent mast cell degranulation (Serafin and Austen, 1987). In addition, the complement system is activated in anaphylactic shock. Common allergens like house dust and *Aspergillus fumigatus* lead to the generation of the anaphylatoxins C3a, C4a, and C5a and to aggregation of polymorphonuclear leukocytes when incubated with human serum (Nagata and Glovsky, 1987). Elevated levels of the anaphylatoxin C3a have been found in a cardiac anaphylaxis model in the guinea pig (del Balzo *et al.*, 1988) and in the blood of patients undergoing immediate hypersensitivity reactions (van der Linden *et al.*, 1990). It is

believed that this activation occurs via the classical pathway, as serum C1q levels are diminished after anaphylactic shock (Tannenbaum *et al.*, 1975; van der Linden *et al.*, 1990). To see whether the lectin pathway is also involved, serum MBL levels will be measured in a mouse model of anaphylactic shock. To determine the pathophysiological importance of the complement system and the relative contribution of different mechanisms of complement activation, mouse strains with selective deficiencies in complement components as well as mast cell-deficient mice will be used.

2. Materials and Methods

2.1. Animal Experiments

2.1.1. Mice

C1q a$^{-/-}$ (Botto *et al.*, 1998) and *H2-Bf/C2*$^{-/-}$ (Taylor *et al.*, 1998) breeding pairs were obtained from M. Botto (Imperial College School of Medicine, London). Both strains were on a 129/SVJ genetic background. *C1q a*$^{-/-}$ mice are selectively deficient in the antibody-mediated classical pathway of complement activation (Botto *et al.*, 1998), as they lack one of the three peptide chains that make up C1q and therefore are unable to produce a functional recognition molecule. The lectin and alternative pathways are functionally active in these mice. *H2-Bf/C2*$^{-/-}$ mice are deficient in both the classical and alternative routes of complement activation (Taylor *et al.*, 1998). Recently, it has been demonstrated that these mice also lack complement activation via the lectin pathway (Celik *et al.*, 2001). They still possess the recognition molecules of both the classical and the lectin pathway, which, upon binding a target, can cleave C4. However, since C2, the next molecule in the cascade is missing, further downstream complement activation cannot occur, and lack of factor B makes formation of the alternative C3 convertase impossible. Therefore, they exhibit complete deficiency of all three complement activation pathways. These mice as well as control 129/SVJ mice were reared, bred, and kept under conventional conditions in the animal facilities of the University of Regensburg. All genetically modified mice used in the experiments were genotyped.

Female NMRI mice and DBA/2 mice were purchased from Charles River (Sulzfeld, Germany). NMRI is an outbred mouse strain; DBA/2 mice are deficient in C5 (van den Berg *et al.*, 1991). These mice are unable to form the membrane attack complex or produce the anaphylatoxin C5a.

Mice with a double deficiency for MBL-A and MBL-C ($MBL^{-/-}$) were kindly provided by A. Ezekowitz (Laboratory of Developmental Immunology, Massachusetts General Hospital, Boston, MA). These mice lack lectin pathway activation via MBL but not via ficolins, while the classical and alternative pathways are fully functional (Shi *et al.*, 2004).

Mice genetically deficient for the C5a receptor ($C5aR^{-/-}$) (Hopken *et al.*, 1996) were kindly provided by J. E. Gessner (Dept. of Clinical Immunology, Medical School Hannover, Germany). The C5aR is found on neutrophils, monocytes, macrophages and mast cells. Its activation leads to chemotaxis and activation of leukocytes, including increased expression of adherence proteins and the generation of oxygen and nitrogen radicals, as well as mast cell degranulation.

Mast cell-sufficient $WBB6F_1$-+/+ ($kit^{+/+}$) and their mast cell-deficient littermates, $WBB6F_1$-$kit^{W}/kit^{W\text{-}V}$ (W/W^{V}) were kindly provided by L. Hueltner (Institute of Clinical Molecular Biology and Tumor Genetics, GSF, Neuherberg, Germany). W/W^{V} mice have a double dose of mutant alleles at the *W* locus, which encodes the c-kit tyrosine kinase growth factor receptor (Chabot *et al.*, 1988). Lack of this receptor results in the inability of mast cell precursors to develop into mature mast cells (Galli and Kitamura, 1987).

All groups were age matched. The study was performed in accordance with German federal regulations of animal experimentation.

2.1.2. Immunisation

Mice were immunised on day 0 and day 10 intraperitoneally with 10 μg antigen (OVA or BSA, both from Sigma, Taufkirchen, Germany) in 200 μl PBS with 35% (v/v) Alum (Perbio Science, Bonn, Germany). From day 21 onwards mice were bled and the antibody titre (the reciprocal of a serum dilution whose fluorescence intensity was 50% of the maximum level) was determined using an antigen-specific ELISA as described in section 2.5.5.

2.1.3. Induction of anaphylactic shock

For active anaphylactic shock mice were immunised as described above and challenged on day 21 with 500 μg antigen in 200 μl PBS intravenously.

For passive anaphylaxis mice received 200 μl of antiserum with a titre of at least 3200 intraperitoneally and were challenged 24 hours later with 500 μg antigen in 200 μl PBS intravenously. Body temperature was monitored with a rectal thermometer (Greisinger electronic, Regenstauf, Germany) for mice.

2.1.4. Cecal Ligation and Puncture (CLP)

CLP was performed as described earlier (Echtenacher *et al.*, 1990). Briefly, mice were anaesthetised and the cecum was exteriorised. The distal 30% of the cecum were ligated and punctured once with a needle with a 0.4 mm diameter opening. The cecum was then replaced and the skin closed with clamps.

2.1.5. Bacterial counts

Mice were sacrificed 24 hours after CLP and livers and spleens were removed. The organs were homogenised in an Ultra-Turrax (IKA-Labortechnik, Staufen, Germany) at 8000 rpm in 2 ml of Standard 1 Nutrient Broth (VWR, Darmstadt, Germany) for 5 seconds. Homogenates were diluted serially in 0.85 % NaCl and incubated on Mueller-Hinton plates at 36°C for 24 hours in aerobic atmosphere. The resulting bacterial colonies were counted and expressed as bacteria per organ.

2.2. Molecular Biology

2.2.1. Genotyping of *C1q* $a^{-/-}$ and *H2-Bf/C2* $^{-/-}$ mice

2.2.1.1. Isolation of genomic DNA from mouse tails

Mouse tails (approximately 0.6 cm) were digested overnight at 56°C in 500 µl lysis buffer with 10 µl Proteinase K (Sigma, Taufkirchen, Germany) and 25 µl Pronase E (Sigma, Taufkirchen, Germany). The Pronase E needs to be preactivated for 1 hour at 37°C before it is added to the mixture. After the tail had been completely digested, samples were centrifuged at 9,300*g* for 15 minutes. To the supernatant, containing the DNA, 500 µl phenol-chloroform were added. The mixture was vortexed and centrifuged at 13,400*g* for 5 minutes. The upper phase was carefully removed, mixed with 500 µl chloroform and centrifuged again. The DNA in the upper phase was then precipitated with ethanol: 2x volume 96% ethanol and 1/10 volume 3 M CH_3COONa (pH 4.8) were added and samples incubated at –20°C for at least 20 minutes. After centrifuging the tubes at 13,400*g* for 15 minutes the supernatant was decanted and the pellet washed with 1 ml 70% ethanol. The pellet was then air-dried and resuspended in 50 µl DNAse free water.

2.2.1.2. Genotyping with PCR

Genomic DNA from mouse tails was diluted 1:50, denatured at 75°C for 15 minutes and put on ice immediately. For each PCR reaction 0.5 µl dNTP-mix (10mM), 2.5 µl 10x PCR reaction buffer (Roche, Mannheim, Germany), 0.5 U Taq-polymerase (Roche, Mannheim, Germany), 0.5 µM of each primer and 5 µl DNA were used. The final reaction volume was 25 µl.

Primers used for *C1q a*$^{-/-}$: Forward: 5′-GGG GCC TGT GAT CCA GAC AG-3′

Reverse 1: 5′-ACC AAT CGC TTC TCA GGA CC-3′

Reverse 2: 5′-GGG GAT CGG CAA TAA AAA GAC-3′

Primers used for *H2-Bf/C2*$^{-/-}$: Forward: 5′-ACT GGA CTC TCT GGT GCT TTC-3′

Reverse 1: 5′-TCT GCA TTC CGC CTT CTG GAC-3′

Reverse 2: 5′-TTC TGA AGG AAA GTC CTT GGG-3′

For amplification of the template a touchdown-PCR program was performed as follows: After a first denaturation step at 94°C for 1 minute and 30 seconds, further denaturation and elongation steps were done for 30 seconds. The initial annealing temperature was 70°C and this was lowered in the following cycles by 0.5°C per cycle to a final annealing temperature of 58°C. At this temperature further 30 cycles were performed, followed by a final elongation for 5 minutes at 72°C. PCR products were separated on a 2% agarose gel. The wild type gene gives rise to a 380 base pair band for *C1q a* and a 400 bp band for *H2-Bf/C2*. When homologous recombination has taken place the band has a size of 180 bp (*C1q a*$^{-/-}$) or 118 bp (*H2-Bf/C2*$^{-/-}$). When the mouse is homozygous only one fragment is amplified, in heterozygous mice both bands are seen.

2.2.2. mRNA expression studies

2.2.2.1. RNA isolation

A piece of mouse liver was passed through a sterile sieve and flushed with 5 ml PBS into a sterile falcon tube. After centrifuging for 3 minutes at 200*g* the PBS was discarded, the pellet thoroughly dissolved in 1 ml Trizol (Invitrogen, Karlsruhe, Germany), transferred to a 1.5 ml reaction tube and incubated at room temperature for 10 minutes. Then 100 µl chloroform were added, the mixture was shaken gently and incubated for further 8 minutes. The cup was then centrifuged at 13,400*g* for 15 minutes at 4°C. The upper phase was transferred into a new tube, mixed 1:1 with ice-cold isopropanol, left at –20°C for at least 30 minutes and centrifuged again at 13,400*g* for 15 minutes at 4°C. The pellet was washed with 500 µl ice-cold 70% ethanol (diluted with DEPC-water) and centrifuged again at 13,400*g* for 5 minutes at 4°C. The pellet was air dried and resuspended in 100 µl RNAse-free water. RNA concentration was estimated by measuring the absorbance in a UV-meter.

2.2.2.2. RT-PCR

1 µg RNA was transcribed into cDNA using oligo-dT primers from the Reverse Transcription System (Promega, Mannheim, Germany) according to the manufacturer's instructions. The cDNA was used for PCR or to generate a probe for Northern blot.

2.2.2.3. PCR for MBL

5 µl of cDNA were amplified using the following primers:

MBL-A: Forward: 5′-CCA AAG GGG AGA AGG GAG AAC-3′

Reverse: 5′-GCC TCG TCC GTG ATG CCT AG-3′

MBL-C: Forward: 5′- GAC GTG ACG GTG CCA AGG G-3′

Reverse: 5′- CTT TCT GGA TGG CCG AGT TTT C-3′

β-actin: Forward: 5′- TGA CGG GGT CAC CCA CAC TGT-3′

Reverse: 5′- CTA GAA GCA TTT GCG GTG GAC-3′

For each PCR reaction 1 µl dNTP-mix (10mM), 5 µl 10x PCR reaction buffer (Roche, Mannheim, Germany), 2.5 U Taq-polymerase (Roche, Mannheim, Germany), 50 pmol of each primer and 5 µl cDNA were used. The final reaction volume was 50 µl.

For amplification of the template the following PCR program was performed: After an initial denaturation step of 7 minutes at 95°C further denaturation and elongation steps (72°C) lasted 30 seconds. Annealing was done at 56°C for 45 seconds. For MBL-A 25 cycles and for MBL-C 30 cycles were performed. For β-actin 35 cycles were done with each step lasting 1 minute. The annealing temperature for β-actin was set at 60°C. The final elongation was done for 10 minutes at 72°C.

2.2.2.4. Preparation of probes for Northern Blot

Total RNA was isolated from mouse liver as described in section 2.2.2.1, and transcribed into cDNA using oligo-dT primers from the Reverse Transcription System (Promega, Mannheim, Germany) according to the manufacturer's instructions. Full-length cDNA for MBL-A and MBL-C was amplified using the following primers:

MBL-A: Forward: 5′- CCC TAG TAA GGA CCA TGC TTC TGC-3′

Reverse: 5′-GGC ACT CGT TTC CTC AGG CTG GG-3′

MBL-C: Forward: 5′-CCT GCA CGT GAG GAG CAT GTC CC-3′

Reverse: 5′- GAG AAA CAA GCA CCC TCA GTC AGA G-3′

For each PCR reaction 1 µl dNTP-mix (10mM), 5 µl 10x PCR reaction buffer (Roche, Mannheim, Germany), 10 U Taq-polymerase (Roche, Mannheim, Germany), 100 pM of each primer and 5 µl cDNA were used. The final reaction volume was 50 µl.

For amplification of the template the following PCR program was performed: After an initial denaturation step of 5 minutes at 95°C further denaturation and elongation steps (72°C) lasted 1 minute. Annealing was done at 60°C for 1 minute. 35 cycles were performed. The final elongation was done for 10 minutes at 72°C. cDNA for β-actin was amplified as described in section 2.2.2.3. PCR products were separated on a 1% agarose gel. Bands (size approximately 700 bp) were cut; DNA was extracted with the Quiaex II Gel Extraction Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions and extracted with phenol-chloroform (see section 2.2.1.1). The DNAs were cloned into Topo Vector (Topo TA Cloning Kit, Invitrogen, Karlsruhe, Germany). 2 µl of the ligation mixture were added to 200 µl competent Top 10 *E. coli*. After incubation on ice for 30 minutes the bacteria were heat-shocked for 30 seconds at 42°C, added to 250 µl SOC medium and incubated for 1 hour at 37°C with gentle shaking. The transformed bacteria were spread on LB plates containing 100 µg/ml ampicillin with 40 µl X-gal solution and 4 µl of 1M IPTG and incubated at 37°C overnight. White, positive clones were inoculated into 3 ml LB medium containing 100 µg/ml ampicillin and incubated again at 37°C overnight with gentle shaking. Plasmid DNA was extracted from the overnight culture using the Wizard Plus Minipreps DNA purification system (Promega, Mannheim, Germany). Positivity of clones was confirmed by PCR and by restriction enzyme digest using EcoRI for MBL-A and BamHI and EcoRV for MBL-C (all from Roche, Mannheim, Germany). Two positive clones were selected for further amplification in 200 ml LB medium containing 100 µg/ml ampicillin. Plasmid DNA was extracted using the Plasmid Maxi Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. Probes were cut from the vector using EcoRI for MBL-A and for MBL-C BamHI and EcoRV, separated on a

1% agarose gel and extracted from the gel with the Quiaex II Gel Extraction Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions.

Probes were labeled with α^{32}P dCTP using the random prime labeling kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions.

2.2.2.5. Northern Blot

RNA was resolved on a 1.5% agarose gel containing 2.2 M formaldehyde. 20 µg RNA were diluted 1:2 with 2x loading dye solution (Fermentas GmbH, St. Leon-Rot, Germany), denatured at 70°C for 10 minutes and loaded immediately onto the gel. Prior to loading the sample, the gel was run for 5 minutes in 1x MOPS electrophoresis buffer at 5 V/cm. Then the samples were loaded and the gel was run for 3 hours. Afterwards the gel was soaked for 10 minutes in DEPC-water to remove formaldehyde and then for further 15 minutes in 20x SSC. RNA was blotted overnight in 20x SSC onto an uncharged nylon membrane (Osmonics, Minnetonka, MN, USA). The membrane was then crosslinked with UV light (254 nm, for 1 minute 45 seconds at 1.5 J/cm^2) and hybridised with probes for either MBL-A or MBL-C using Ultrahyb Hybridisation buffer (Ambion, Huntingdon, UK) overnight at 45°C. The membrane was then rinsed with 2x SSC containing 0.1% SDS and washed as follows: 20 minutes with 2x SSC/0.1% SDS at 45°C, 20 minutes with 0.1x SSC/0.1% SDS at 45°C and 20 minutes with 0.1x SSC/0.1% SDS at 65°C. The blot was wrapped in plastic, placed in a radiography cassette and exposed to a special autoradiography film (Kodak BioMax Film).

2.3. Protein Methods

2.3.1. C4-preparation

C4 preparation from human blood was performed as described earlier (Dodds, 1993). Briefly, 50 ml of fresh EDTA-plasma were centrifuged at 805*g* for 10 minutes at 4°C. To the supernatant (25 ml) 10 mM EDTA, 2.5 mM Pefabloc (serine protease inhibitor, Roche Applied Science, Mannheim, Germany), 3 ml buffer B and 7.5 ml buffer A were added and the mixture centrifuged for 10 minutes at 3,220*g* at 4°C. The supernatant was then loaded at a flow rate of 1 ml/min onto a 10 ml Q-sepharose column (Pharmacia/GE Healthcare Bio-Sciences, Freiburg, Germany), which had been equilibrated with buffer A. The column was then washed with buffer A and bound proteins eluted with a linear gradient from 0 to 500 mM NaCl. 2.5 ml fractions were collected and analysed on a 12.5% SDS-PAGE gel, which was Coomassie stained. Fractions containing three bands for the C4 γchain (33kDa), C4 βchain (75 kDa) and C4 αchain (97 kDa) were pooled, diluted with 1.5 volumes of buffer A and loaded at a flow rate of 5 ml/min onto a 1 ml Poros 20 HQ column (Applied Biosystems, Foster City, CA, USA), equilibrated with 100 mM NaCl. To avoid overloading the column the sample was split and two runs were performed. The column was washed with 100 mM NaCl and bound proteins eluted with a linear gradient from 100 mM to 500 mM NaCl. 1 ml fractions were collected and analysed on a 12.5 % SDS-PAGE gel. C4 containing fractions were pooled, aliquoted and stored at –80°C. Functional integrity was tested in a C4 cleavage assay as described in section 2.5.1. As C4 is not stable at room temperature, samples were kept on ice whenever possible.

2.3.2. SDS-PAGE gel electrophoresis

The samples (30 µl) were denatured by heating them for 5 minutes at 95°C in 10 µl 4x loading buffer and loaded onto a 12,5% SDS-Gel. The gel was run in 1x SDS-Gel running buffer with a constant current of 30mA in the stacking gel and 40mA in the resolving gel. The sizes of the fragments were compared with a broad range molecular weight standard (Biorad, Munich, Germany).

2.3.3. Coomassie stain

In order to visualise bands on the SDS-PAGE gel or the Western Blot membrane, they were stained with Coomassie. For this, membranes or gels were soaked in 0.2% Coomassie Brilliant Blue R250 for a few seconds and destained with 10% acetic acid and 40% methanol until bands could be distinguished. Before drying the membranes or gels were washed intensively with water to get rid of the acetic acid.

2.3.4. Western Blot

Proteins were transferred from the gel to a PVDF membrane by a semi dry blotting system. The stacking gel was cut off and the resolving gel was immersed in blot buffer B for 15 minutes. 3MM chromatography paper was cut to the same size as the gel and three sheets each were immersed in either blot buffer A, B, or C. A piece of PVDF-membrane Immobilon P (Millipore, Schwalbach, Germany) was cut the same size as the gel and wetted in methanol, then washed in H_2O and placed for 5 minutes into blot buffer B. The blotting apparatus was assembled as follows: On the anode the chromatography paper immersed in blot buffer A and B was placed. The membrane, the gel and the chromatography paper immersed in blot buffer C were placed on top. Proteins were blotted onto the membrane with 0.8 mA/cm^2 for 1 hour. The membrane was then blocked with 1% powdered milk in TBS/Tw overnight at 4°C. After three 10-

minute washing steps with TBS/Tw the membrane was incubated for 1 hour at room temperature with peroxidase-conjugated goat anti-mouse IgG antibodies (Sigma, Taufkirchen, Germany) at a dilution of 1/2,000 in the blocking buffer. After further washing steps the membrane was incubated with ECL-solution (Nowa Solution A and B, MoBiTec, Göttingen, Germany), placed between two foils in a radiography cassette and exposed to an autoradiography film (Hyperfilm from Amersham/GE-Healthcare, Freiburg, Germany).

2.3.5. Antibody purification on BSA-coupled sepharose

2.3.5.1. Coupling of BSA to sepharose beads

0.5 g Cn-Br-activated Sepharose 4B (Amersham Pharmacia, Little Chalfont, UK) was washed with 100 ml 1 M HCl on a sintered glass filter. 14 mg BSA were dissolved in 2.5 ml coupling buffer, added to the beads and rotated gently for 1 hour at room temperature. The mixture was then centrifuged at 3,220*g* for 5 minutes and the pellet was washed with 9 ml coupling buffer. After another centrifugation step residual binding sites were blocked with 5 ml of 0.1 M Tris-HCl pH 8 for 2 hours at room temperature. The sepharose beads were then washed with 3 cycles of alternating pH. Each cycle consists of a wash with 9 ml wash buffer 1 and 9 ml wash buffer 2. After the final centrifugation step the beads were kept in PBS containing 0.02% (w/v) NaN_3 until use.

2.3.5.2. Ammonium sulfate precipitation

To 8.5 ml anti-BSA antiserum ammonium sulfate was slowly added until a final concentration of 50% (w/v) was reached. The mixture was stirred overnight at 4°C and centrifuged at 12,100*g* for 10 minutes at 4°C. The pellet was resuspended in PBS and thoroughly dialysed against PBS.

2.3.5.3. Purification of anti-BSA antibodies

The antiserum was added to the BSA-coupled sepharose beads, mixed thoroughly by gentle rotation and the mixture was centrifuged at 3,200*g* for 5 minutes. The supernatant was kept for a second elution step. The beads were then washed three times with 5 ml PBS and bound antibodies were eluted with 1 ml of 0.1 M glycine, pH 2.5. The eluate was neutralised immediately with 1 M Tris-HCl, pH 11. The beads were regenerated with 3 washes with 0.1 M Tris-HCl, pH 8.5 and the elution repeated. Eluates and supernatants were dialysed overnight against PBS and analysed in a 12.5% SDS-PAGE gel (described in section 2.3.2), which was stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, Leiden, The Netherlands) and on Western Blot as described in section 2.3.4. Protein content was estimated using the DC Protein assay (Bio Rad Laboratories, Munich, Germany) and integrity of eluted antibodies was tested in an antigen specific ELISA as described in section 2.5.5.

2.4. Immunhistochemistry

Tissue was snap-frozen in liquid nitrogen. Cryostat sections (5µm) were mounted on poly-lysine-coated glass slides (Super Frost Plus from Menzel GmbH, Braunschweig, Germany) and fixated in 4% PBS-buffered formaldehyde for 15 minutes at room

temperature, washed three times in PBS for 5 minutes each and endogenous peroxidase activity was blocked with 2% H_2O_2 in methanol for 20 minutes at 4°C. After another wash with PBS sections were blocked with 20% BSA (Sigma, Taufkirchen, Germany) in PBS for 30 minutes at room temperature. Primary antibody was diluted in 1% BSA in PBS and left to bind overnight at 4°C. Antibodies used were rat anti-mouse MBL-A (clone 8G6), rat anti-mouse MBL-C (clone 16A8), both at a concentration of 2 µg/ml (both kindly provided by J. Jensenius, Department of Microbiology, University of Aarhus, Aarhus, Denmark), and rat anti-mouse C3 (HyCult Biotechnology, Uden, Netherlands, clone 11H9) at a concentration of 5 µg/ml. Negative controls received rat IgG_{2a} (Cymbus Biotechnology, Dianova, Hamburg, Germany) at a concentration of 5 µg/ml or just buffer. Sections were then washed again and incubated with biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Burlingame, CA, USA, concentration 10 µg/ml in 0.2% normal rat serum in PBS) for 45 minutes at room temperature. After washing in PBS sections were incubated with the mixture of biotin and avidin DH (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 45 minutes at room temperature, washed again and stained with Peroxidase/DAB (ChemMate Detection Kit, Dako Cytomation, Glostrup, Denmark). The sections were then counterstained with haematoxylin and cover slipped.

Sections for haematoxylin-eosin staining and controls for endogenous peroxidase activity were put aside after the fixation step.

In tissues where endogenous biotin gave high background staining (liver, kidney and small intestine) the method was slightly modified. Fresh cryostat sections were left to air-dry, fixed in acetone (10 minutes at room temperature) and air dried again. Since the peroxide-blocking step severely damaged the tissue and controls showed no endogenous peroxidase activity, this step was omitted. As secondary antibody a directly peroxidase-

labelled goat anti-rat IgG antibody (Jackson Immunoresearch, Soham, UK) was used at a concentration of 8 μg/ml. The antibody was diluted in 1% BSA and 5 % normal mouse serum in PBS and preincubated for 30 minutes at room temperature in the dark before adding it to the sections to avoid cross-reaction of antibodies with mouse tissue.

2.5. ELISA techniques

2.5.1. C4 cleavage assay

A C4 cleavage assay was performed as described earlier (Petersen *et al.*, 2001a). Briefly, microtiter wells (Maxisorp, Nunc, Kamstrup, Denmark) were coated overnight at room temperature with 1 μg mannan (Sigma, Taufkirchen, Germany) in 100 μl of coating buffer. Residual protein binding sites were blocked with TBS/HSA for 1 hour. After washing with TBS/Tw/Ca^{++} wells were filled with 100 μl of serum dilutions (starting at 1/100) in MBL-binding buffer. Plates were incubated overnight at 4°C and washed thrice with TBS/Tw/Ca^{++}. Wells then received 1 μg human C4 (prepared as described in section 2.3.1) in 100 μl TBS^{++}. After incubation for 90 minutes at 37°C and another wash in TBS/Tw/Ca^{++} deposited C4b was detected by adding alkaline phosphatase conjugated chicken anti-human C4 antibody (100 μl of 1:1,000 dilution in TBS/Tw/Ca^{++}; Immunsystem AB, Uppsala, Sweden). Alkaline phosphatase activity was determined by adding 100 μl of p-nitrophenyl phosphate substrate solution (Sigma, Taufkirchen, Germany). Plates were read at 405 nm. The optical density induced by mouse serum samples was determined in duplicates and normalised to an internal standard (human serum) assigned the arbitrary activity of 1 unit per ml.

2.5.2. MBL TRIFMA

Plates were coated and blocked as described in section 2.5.1. Samples and standard serum were diluted in MBL-binding buffer. After incubation overnight at 4°C plates were washed and biotinylated anti-mouse MBL-A (clone 13H6) or MBL-C antibody (clone 14D12, both kindly provided by J. Jensenius, Department of Microbiology, University of Aarhus, Aarhus, Denmark) diluted in TBS/Tw/Ca^{++} was added. Anti-mouse MBL-A was used at a dilution of 0.4 µg/ml, anti-mouse MBL-C at 0.025 µg/ml. Plates were incubated for 2 hours at room temperature and washed, followed by 90 minutes incubation with Eu^{3+}-conjugated streptavidin (Wallac, Turku, Finland) at a dilution of 1:1,000 in TBS/Tw with 25 µM EDTA. After another wash Enhancement solution (Wallac, Turku, Finland) was added. The fluorescent chelate was estimated by time-resolved immunofluorometry using a DELFIA fluorometer (Wallac, Turku, Finland).

2.5.3. MBL-antigen ELISA

Microtiter plates (Maxisorb, Nunc, Kamstrup, Denmark) were coated with 10 µg/ml OVA or BSA (both from Sigma, Taufkirchen, Germany) in coating buffer overnight at room temperature. Plates were then blocked with 0.5% (w/v) powdered milk in TBS for 1 hour and washed with TBS/Tw/Ca^{++}. Serial dilutions of serum in MBL binding buffer, starting from 1:10, were added and plates were incubated at 4°C overnight. To show specificity of binding, serum was also diluted in MBL binding buffer containing 10 mM EDTA instead of $CaCl_2$ or MBL binding buffer also containing 1 µg/ml mannan, 50 mg/ml Ovalbumin or 1 mg/ml BSA (all from Sigma, Taufkirchen, Germany). As positive control wells were coated with 10 µg/ml mannan. After another wash rat anti-mouse MBL-A (clone 8G6, 0.5 µg/ml in TBS/Tw/Ca^{++}) or rat anti-mouse

MBL-C (clone 16A8, 0.05 µg/ml in TBS/Tw/Ca^{++}, both kindly provided by J. Jensenius, Department of Microbiology, University of Aarhus, Aarhus, Denmark) was added and plates incubated for 90 minutes at room temperature. As secondary antibody alkaline phosphatase- conjugated goat anti-rat IgG (Sigma, Taufkirchen, Germany) was used at a dilution of 1: 1,000 in TBS/Tw/Ca^{++}. Alkaline phosphatase activity was determined by adding p-nitrophenyl phosphate substrate solution (100 µl; Sigma, Taufkirchen, Germany). Plates were read at 405 nm.

To see whether MBL binds to immune complexes, BSA coated wells were incubated for 1 hour with anti-BSA-Ig, prepared as described in section 2.3.5, at 1 µg/ml in TBS after blocking. Further steps were performed as described above.

2.5.4. Proteoglycan-Inhibition

Normal mouse serum was diluted 1:400 in MBL-binding buffer, mixed with decreasing amounts of either heparin (low molecular weight heparin sodium salt from porcine intestinal mucosa/Sigma, Taufkirchen, Germany) or chondroitin sulfate (Chondroitin 4-sulfate sodium salt from bovine trachea/Fluka, Buchs, Switzerland) and incubated for 45 minutes at room temperature. The mixture was added to mannan-coated ELISA-plates and incubated overnight at 4°C to allow the remaining free MBL to bind to the plate. Then either a C4 cleavage assay was performed as described in section 2.5.1, or bound MBL was detected directly with monoclonal rat anti-mouse MBL-A or MBL-C antibodies as described in section 2.5.3.

2.5.5. Antigen-ELISA

96-well plates (Falcon/BD Becton Dickinson Biosciences, Heidelberg, Germany) were coated with 10 µg/ml OVA or BSA in coating buffer overnight at room temperature and blocked with TBS containing 1% powdered milk for 2 hours at room temperature. Plates were then washed three times with TBS/Tw and serum samples, diluted in TBS, were added. After incubating the plates for 2 hours at room temperature and washing with TBS/Tw bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, Taufkirchen, Germany), diluted 1:1,000 in TBS. Alkaline phosphatase activity was determined by adding p-nitrophenyl phosphate substrate solution (100 µl; Sigma, Taufkirchen, Germany). Plates were read at 405 nm.

2.5.6. Sandwich-ELISA

Microtiter plates (Maxisorb, Nunc, Kamstrup, Denmark) were coated with monoclonal rat anti-mouse MBL-A (clone 8G6) or rat anti-mouse MBL-C (clone 16A8, both kindly provided by J. Jensenius, Department of Microbiology, University of Aarhus, Aarhus, Denmark) at a concentration of 1 µg/ml in coating buffer overnight at room temperature. Plates were blocked with TBS/HSA for 2 hours at room temperature and washed three times with TBS/Tw/Ca^{++}. Serum samples and standard serum were diluted in MBL-binding buffer. After incubation overnight at 4°C plates were washed and biotinylated anti-mouse MBL-A (clone 13H6) or MBL-C antibody (clone 14D12, both kindly provided by J. Jensenius, Department of Microbiology, University of Aarhus, Aarhus, Denmark) diluted in TBS/Tw/Ca^{++} was added. Anti-mouse MBL-A was used at a dilution of 0.4 µg/ml, anti-mouse MBL-C at 0.025 µg/ml. Plates were incubated for 2 hours at room temperature and washed, followed by 90 minutes incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch, Soham, UK) at a

dilution of 1:1,000 in TBS/Tw with 25 µM EDTA. Alkaline phosphatase activity was determined by adding p-nitrophenyl phosphate substrate solution (100 µl; Sigma, Taufkirchen, Germany). Plates were read at 405 nm.

2.6. Statistical evaluation

All experiments were performed at least three times with similar results and one representative experiment is shown. Descriptive statistics were expressed as means ± standard deviations. Statistical significance was determined using the Student's T-test for lectin pathway activity and the Man-Whitney U-Test for bacterial counts. Time courses of survival were analysed by Kaplan-Meier survival curve calculations and the log-rank test. P values of < 0.05 were defined as significant for discrimination. Data were analysed using SPSS software.

2.7. Buffers and Solutions

2.7.1. DNA and RNA methods

Lysis buffer for mouse tails: 0.1 M TrisHCl
0.2 M NaCl
5 mM EDTA
1% (w/v) SDS
pH 8

Proteinase K stock solution: 10 mg/ml

Pronase E stock solution: 10 mg/ml
10 µl of 1 M TrisHCl (pH 8.0)
2 µl of 5 M NaCl

X-gal solution: 20 mg/ml in dimethylformamide
protect from light and store at –20°C

DEPC-H_2O: 0.1% (v/v) DEPC, stir overnight and autoclave

10x MOPS buffer: 0.2 M MOPS
20 mM CH_3COONa
10 mM EDTA
pH 11

20x SSC: 3 M NaCl
0.3 M $C_6H_5Na_3O_7$
pH 7.2

LB agar: LB medium with 1.5% agar agar

LB medium: 10 g/l tryptone
5g/l yeast extract
10 g/l NaCl
pH 7.0, autoclaved

SOC medium: 20 g/l tryptone
5 g/l yeast extract
8.6 mM NaCl
2.5 mM KCl
2 mM MgCl
pH 7.0, autoclave and
add glucose to 20 mM final concentration right before use

2.7.2. Antibody purification on BSA-sepharose

Coupling buffer: 0.1 M $NaHCO_3$
0.5 M NaCl
pH 8.3

Wash buffer 1: 0.1 M CH_3COONa
0.5 M NaCl
pH 4

Wash buffer 2: 0.1 M Tris-HCl
0.5 M NaCl
pH 8

2.7.3. C4 preparation

Buffer A: 20 mM TrisHCl
5 mM EDTA
50 mM EACA
0.02 % (w/v) NaN_3
pH 7.5
Add 500 µM Pefabloc (serine protease inhibitor, Roche, Mannheim, Germany) just before use and filtrate.

Buffer B: Buffer A with 1 M NaCl

2.7.4. SDS-PAGE and Western Blot

stacking gel: 0.85 ml 30% acrylamide
1.5 ml 0.5 M Tris-HCl pH 6.8
3.75 ml H_2O
60 µl 10% SDS
5 µl TEMED
50 µl 10% APS

resolving gel: 6.25 ml 30% acrylamide
3.75 ml 1.5 M Tris-HCl pH 8.8
5 ml H_2O
150 µl 10% SDS
10 µl TEMED
100 µl 10% APS

4x Laemmli loading buffer: 250 mM Tris-HCl
4% SDS
40% glycerol
4% β-Mercaptoethanol
pH 6.8

5x SDS-PAGE running buffer: 120 mM Tris-HCl
950 mM glycine
0.5% SDS

Western blot buffer A: 300 mM Tris-HCl
10% methanol
pH 10.4

Western blot buffer B: 25 mM Tris-HCl
10% methanol
pH 10.4

Western blot buffer C: 25 mM Tris-HCl
40 mM 6-amino-n-capronic acid
10% methanol
pH 9.4

Coomassie staining: 10% glacial acetic acid
40% methanol
0.2% Coomassie brillant blue R250
filter before use

Coomassie destaining: 10% glacial acetic acid
40% methanol

2.7.5. ELISA

Coating buffer:	15 mM Na_2CO_3 35 mM $NaHCO_3$ pH 9.6
TBS:	10 mM Tris-HCl 140 mM NaCl 1.5 mM NaN_3 pH 7.4
TBS/Tw:	TBS with 0.05% Tween 20 (v/v) (Fluka, Buchs, Switzerland)
TBS/Tw/Ca^{++}:	TBS with 0.05% Tween 20 (v/v) (Fluka, Buchs, Switzerland) and 5 mM $CaCl_2$
TBS^{++}:	TBS with 2 mM $CaCl_2$ and 1 mM $MgCl_2$
TBS/HSA:	TBS with 0.1% (w/v) HSA (Statens Serum Institute, Kopenhagen, Denmark)
MBL-binding buffer:	20 mM Tris-HCl 10 mM $CaCl_2$ 1 M NaCl 0.05% (v/v) Triton X-100 (Serva Feinbiochemica, Heidelberg, Germany) 0.1% (w/v) HSA (Statens Serum Institute, Kopenhagen, Denmark) pH 7.4

MBL-BB with EDTA:
20 mM Tris-HCl
1 M NaCl
10 mM EDTA
0.05% Triton X-100
pH 7.4

PBS:
137 mM NaCl
2.7 mM KCl
6.5 mM Na_2HPO_4
1.5 mM KH_2PO_4
pH 7.4

3. Part I: Septic Shock

3.1. Introduction: The CLP model

As an experimental model of polymicrobial septic infection we have chosen cecal ligation and puncture (CLP). This is considered as a clinically relevant model of septic peritonitis with bacteraemia (Urbaschek *et al.*, 1984).

The cecum, also called blind gut, is a blind-ended pouch forming the first part of the large intestine. Its size and function varies between species. In carnivores it is rather small, the main function here being absorption of salts and water. In herbivores it is often quite large, as it contains a large number of symbiotic bacteria, whose function it is to digest cellulose. Since the large intestine is not able to absorb nutrients, these animals practice coprophagy (i.e. consumption of their own feces) to utilise the cellulose breakdown products.

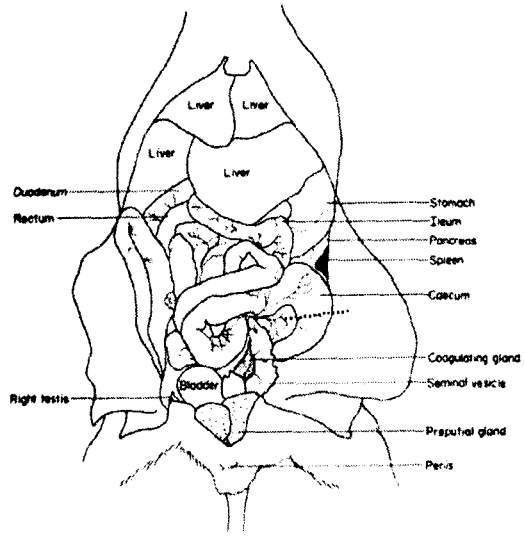

Fig. 3.1: Schematic drawing of the abdominal organs in the mouse.
The cecum is located on the right hand side. The dotted line indicates the approximate position of the ligature in CLP. (Adapted from Cook, 1965).

In the CLP model, as the name implies, part of the cecum is ligated and punctured. This leads to leakage of intestinal bacteria into the peritoneal cavity. Through lymph drainage these bacteria reach the bloodstream and are distributed to other organs (Urbaschek *et al.*, 1984). Ligating a larger part and/or puncturing twice can increase mortality of immune-competent wild type mice. Mice can be saved by antibiotics from CLP-induced mortality, demonstrating that the animals die as a consequence of the bacterial infection (Echtenacher *et al.*, 2001). The ligated part of the cecum becomes necrotic. After a short time further spreading of bacteria is prevented by the generation of fibrous adhesions and later abscess formation. For this and for the recruitment of neutrophils TNF stored in mast cells has been shown to be important (Echtenacher *et al.*, 2001). It has also been indicated that activation of the classical pathway by binding of natural antibodies to microbial antigens is important (Brown *et al.*, 2002).

Apart from CLP, there are a number of other models that can be used to study the effects of bacterial infection.

One of them is LPS-injection. LPS is a component of the cell wall of gram-negative bacteria. Intravenous injection of LPS leads to shock-like symptoms, but the pathophysiological changes are different from those in septic patients, owing to the fact that release of LPS is only one of many factors contributing to the sepsis syndrome.

Another model is the infusion of live bacteria. This can be used to study the kinetics of bacterial clearance and leukocyte response, but does not resemble the clinical situation, as the host is suddenly overwhelmed with a large number of bacteria. Patients, on the other hand, usually harbour a septic focus that is persistently showering the body with a lower number of bacteria, and that can also be walled off by the host defence mechanisms (Wichterman *et al.*, 1980).

Intraperitoneal injection of human feces into mice can be used as a model of polymicrobial peritonitis and sepsis, as would occur e.g. in the case of anastomotic

leakage. The severity of the symptoms can be determined by titrating the injected amount (Celik et al., 2001). When interpreting the results, however, it has to be taken into account that the pathogenicity of bacteria can differ from species to species and that the results gathered in the mouse model might not be transferable to humans.

Another model of polymicrobial septic peritonitis is the colon ascendens stent peritonitis (CASP). In this model a stent is implanted in the colon ascendens, a part of the large intestine, which leads to a continuous leakage of bacteria into the abdominal cavity, causing severe peritonitis and sepsis (Maier *et al.*, 2004). However, the host is not able to contain the septic focus as would normally happen after anastomotic leakage.

Peritoneal bacterial infection and sepsis induced by CLP is the model that resembles the clinical situation most closely, because an early hyperinflammatory phase is followed by a subsequent hypoinflammatory phase during which the animals are extremely sensitive to bacterial super-infections (Wichterman *et al.*, 1980; Echtenacher *et al.*, 2003). This parallels the clinical observation of immunoparalysis developing in patients during the course of sepsis (Volk *et al.*, 1996), and can therefore serve as a clinically relevant model for severe abdominal infection to study the impact of abdominal trauma with bacterial infection on the innate and adaptive immune system.

3.2. Results

3.2.1. Preparatory Work

3.2.1.1. Genotyping of *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice

Breeding pairs of *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice were genotyped to confirm their selective complement deficiency. For this, DNA was isolated from mouse tails as described in Materials and Methods and PCR was performed. In the PCR reaction two reverse primers were used. One primer is located in the inserted cassette that disrupts the wild type gene, the other primer is located outside this cassette. When no homologous recombination has taken place, the forward and second reverse primer will generate a "wild type" fragment with a size of 380 bp and 400 bp for *C1q a* and *H2-Bf/C2*, respectively. If the gene is interrupted by the cassette, the fragment that would be generated by these two primers is too big to be amplified in PCR under these conditions, but the reverse primer located in the cassette will give rise to a fragment with a size of 180 bp and 118 bp for *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$, respectively. When the mouse is homozygous only one fragment is amplified, in heterozygous mice both bands are seen. As some of the fragments are quite small, a 2% agarose gel was used to separate the PCR products.

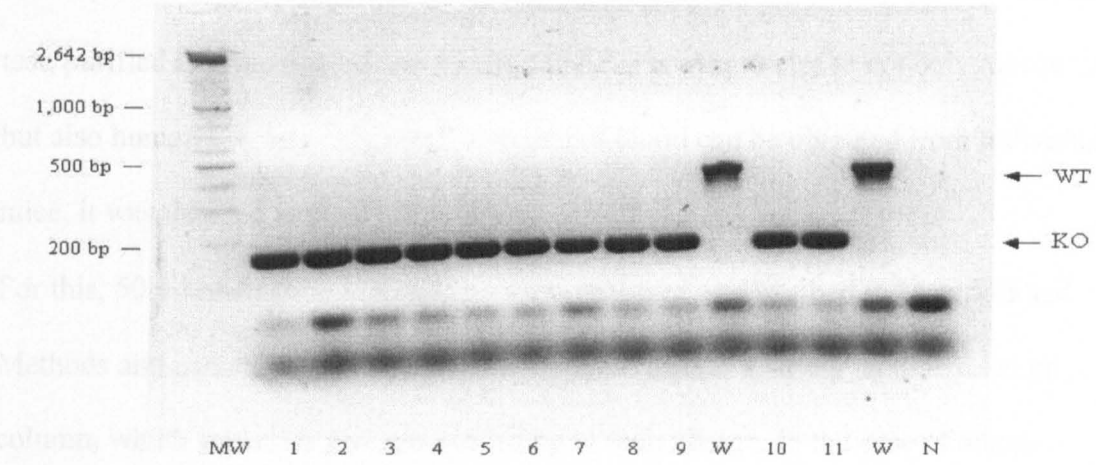

Fig. 3.2: Genotyping of *C1q a*$^{-/-}$ mice.

PCR products were separated on a 2% agarose gel and visualised by ethidium bromide staining. MW is the molecular weight standard; numbers indicate different mice that were tested. W are wild type 129/SVJ mice, N denotes the no template control. Arrows on the right indicate the bands for the wild type fragment (WT, size approximately 380 bp) and the fragment in C1q-deficient animals (KO, size approximately 180 bp). The bands below that are primer oligomers.

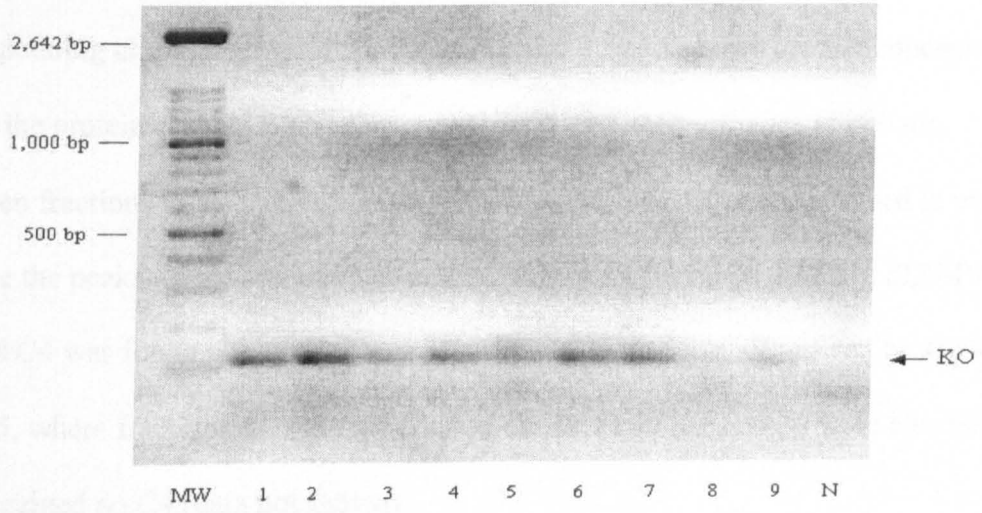

Fig. 3.3: Genotyping of *H2-Bf/C2*$^{-/-}$ mice.

PCR products were separated on a 2% agarose gel and visualised by ethidium bromide staining. MW is the molecular weight standard; numbers indicate different mice that were tested. N denotes the no template control. The arrow on the right indicates the bands for the fragment in animals deficient in factors B and C2 (KO, size approximately 118 bp).

3.2.1.2. C4 Preparation

It was planned to use a C4 cleavage assay to measure lectin pathway activity. For this test, purified C4 was needed. As mouse MASP-2 is able to cleave not only mouse C4, but also human C4, and only small amounts of blood can be obtained from individual mice, it was decided to purify human C4.

For this, 50 ml of fresh EDTA-plasma were prepared as described in Materials and Methods and passed over a Q-Sepharose column. This is a strong anion-exchange column, which separates proteins according to their charge. In the case of anion-exchange chromatography, the column matrix is positively charged and allows for binding of negatively charged ions or proteins. Once the proteins are bound, they can be eluted by increasing the ionic strength of the buffer.

The column was equilibrated with a buffer containing 0 mM NaCl, the sample was loaded onto the column, which was then washed with the no-salt buffer until all unbound proteins were removed. Bound protein was eluted with a linear salt gradient ranging from 0 mM to 500 mM NaCl and 2.5 ml fractions were collected. The corresponding chromatogram is shown in Fig. 3.4. Line A shows the salt concentration; line B the protein coming from the column, measured as absorbance at 280 nm. Between fractions 22 and 26 the sensitivity of the photometer was decreased in order to still see the peaks on the chromatogram. Fractions were tested on a 12.5% SDS-PAGE gel and C4 was found to elute in the final peak (fractions 25 to 29), as can be seen in Fig. 3.5, where fractions 21 to 30 are shown. Earlier elution fractions were also tested, but contained no C4 (data not shown).

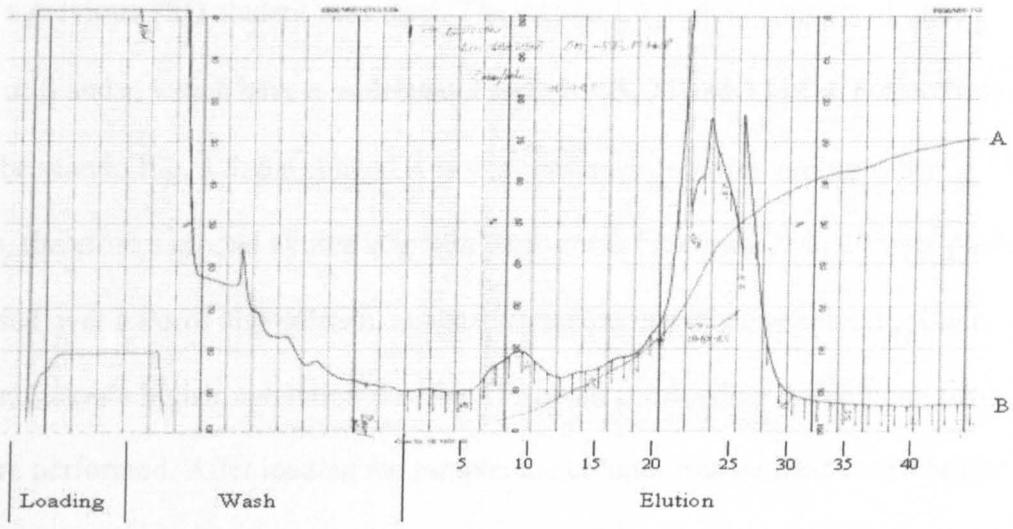

Fig. 3.4: Elution profile of C4 on a Q-Sepharose column.
Line A shows the salt concentration of the elution buffer, line B gives the amount of protein coming from the column. Elution fractions are marked on line B, but for greater clarity are also indicated at the bottom of the chromatogram. C4 was found to elute in the final peak (between fractions 25 to 29).

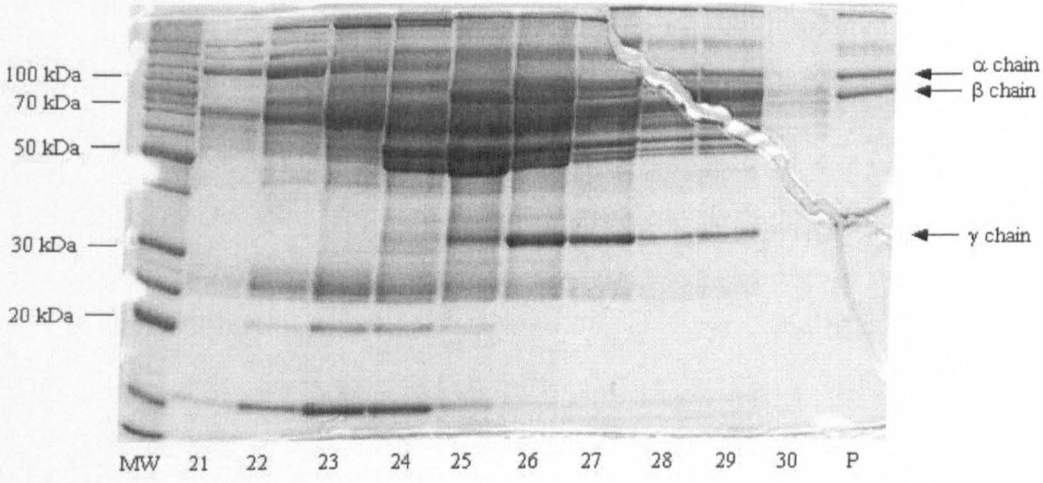

Fig. 3.5: Elution fractions after ion-exchange chromatography on a Q-Sepharose column.
Samples from the elution fractions were separated on a reducing 12.5% SDS-PAGE gel and visualised by Coomassie staining. Fraction numbers are indicated at the bottom of the gel. MW denotes the molecular weight standard, P is the positive control.

As positive control on the SDS-PAGE gel a sample of C4 that was purified by Sotiria Tzima, a previous PhD student, was used. The mature C4 protein consists of three chains, α, β and γ, which have a molecular weight of 95, 75 and 33 kDa, respectively. As can be seen in Fig. 3.5, the eluted C4 is still contaminated with various other proteins, therefore a second elution step was performed. Fractions 26 to 29 were pooled and passed over a Poros HQ-column, another strong anion exchange column, which, however, makes a higher resolution possible. To avoid overloading the column, two runs were performed. After loading the sample, the column was washed with a buffer containing 100 mM NaCl, bound protein was eluted with a linear salt gradient ranging from 100 to 500 mM NaCl and 1 ml fractions were collected. The corresponding elution profile of the first run is shown in Fig. 3.6. As another peak emerged at the end of the gradient elution, elution was continued with 500 mM NaCl for a couple of minutes. Representative elution fractions were analysed on a reducing 12.5% SDS-PAGE gel (Fig. 3.7). The big peak was found to contain C4.

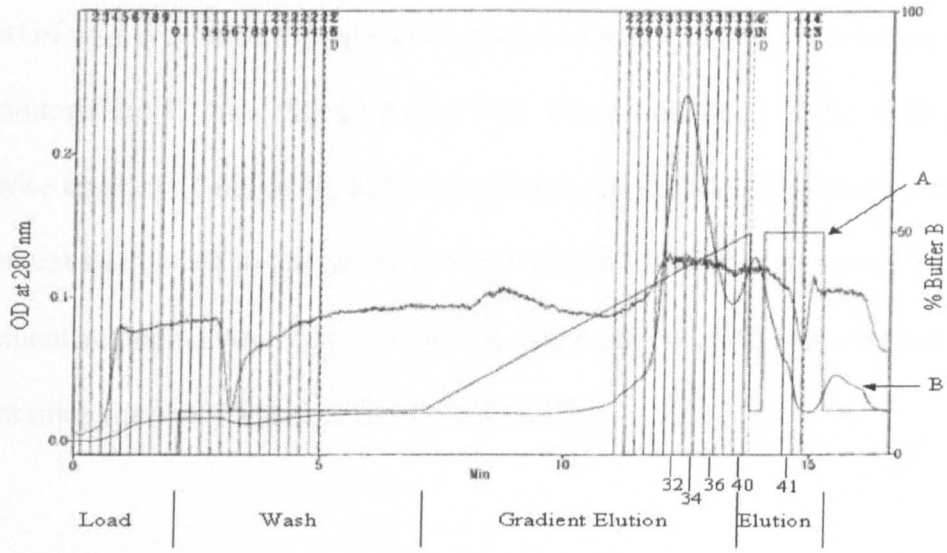

Fig. 3.6: Elution profile of C4 on a Poros HQ column.
Line A shows the salt concentration of the elution buffer, shown as % buffer B on the right hand y-axis. Line B gives the amount of protein coming from the column, measured as absorbance at 280 nm (left hand y-axis). The parallel vertical lines indicate collected fractions. Fractions shown in the SDS-PAGE gel are indicated at the bottom of the chromatogram.

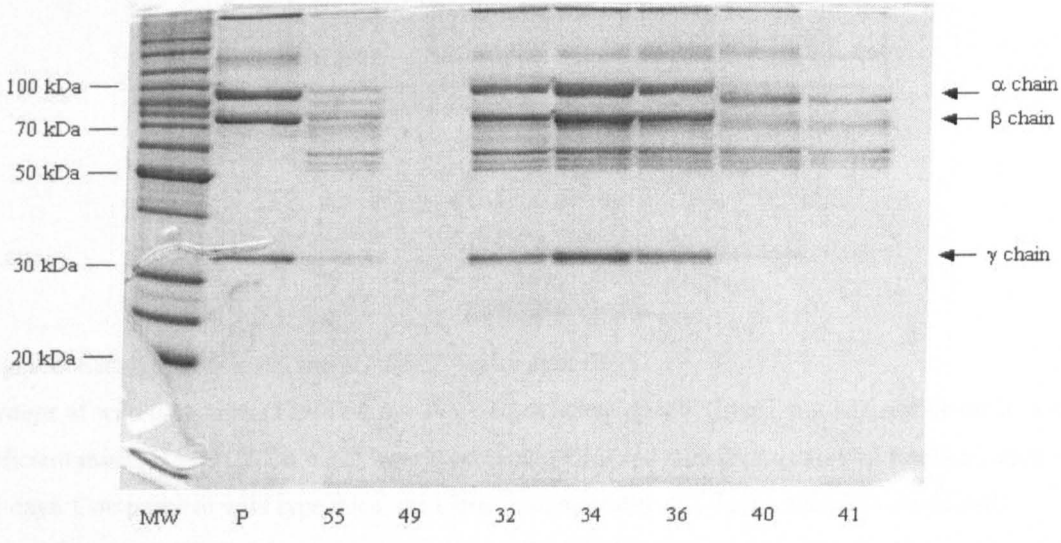

Fig. 3.7: Elution fractions after ion-exchange chromatography on a Poros HQ column.
Samples from the elution fractions were separated on a reducing 12.5% SDS-PAGE gel and visualised by Coomassie staining. Fraction numbers are indicated at the bottom of the gel. Fractions 49 and 55 represent the flow-through of the second run. MW denotes the molecular weight standard, P is the positive control.

3.2.2. Survival of complement-deficient mice after CLP

Survival of wild type mice, C1q-deficient mice, and factor B- and C2-deficient mice was monitored for 12 days after CLP (Fig. 3.8). While 3 out of 11 (27%) wild type mice died, twice as many (6 out of 10, 60%) mice lacking the classical activation pathway of complement succumbed to the infection. Survival of mice lacking all three pathways of complement activation was severely impaired. Only one out of 12 factor B- and C2-deficient mice survived the infection (92% mortality).

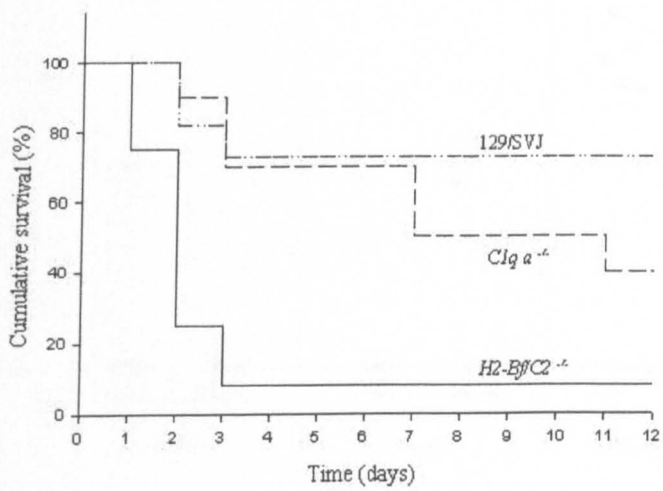

Fig. 3.8: Survival of *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice after CLP.
Groups of wild type mice (129/SVJ, n = 11), C1q-deficient mice (*C1q a*$^{-/-}$, n = 10), and factor B- and C2-deficient mice (*H2-Bf/C2*$^{-/-}$, n = 12) were subjected to CLP and cumulative survival was monitored over 12 days. Compared to wild type mice, the increase in mortality of *C1q a*$^{-/-}$ mice was statistically insignificant ($p = 0.219$) whereas the mortality of *H2-Bf/C2*$^{-/-}$ mice was significantly higher ($p = 0.001$).

3.2.3. Bacterial loads after CLP

In accordance to their increased susceptibility to infection, the ability to clear bacteria from liver and spleen was also impaired in mice lacking the classical, the alternative, and the lectin pathway of complement activation. Bacteria were detected in livers ($3.1x10^4 \pm 3.3x10^4$ CFU/organ) and spleens ($8.3x10^3 \pm 6.8x10^3$ CFU/organ) from all tested mice deficient for factor B and C2 one day after CLP (Fig. 3.9), while bacteria were found only in very few control or C1q-deficient mice.

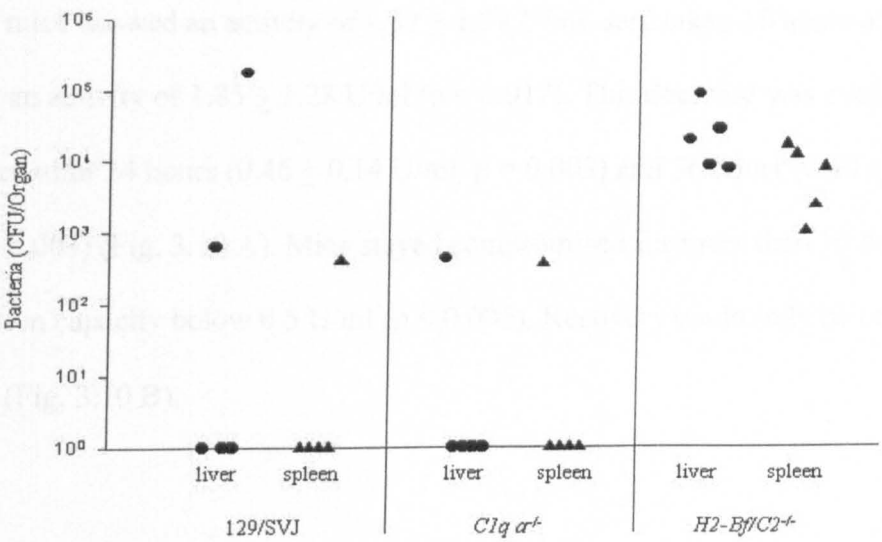

Fig. 3.9: Bacterial loads after CLP.

Bacterial counts were determined in spleen or liver cultures of groups (n = 5) of control 129/SV mice (left), *C1q a*$^{-/-}$ mice (middle), and *H2-Bf/C2*$^{-/-}$ mice (right) 24 hours after CLP. Bacterial counts are given as CFU per organ. Dots denote bacterial counts in liver, triangles symbolise bacterial counts in spleen. A statistical significant enhancement in bacterial counts from liver ($p = 0.007$) and spleen ($p = 0.007$) of *H2-Bf/C2*$^{-/-}$ mice compared to *C1q a*$^{-/-}$ was determined.

3.2.4. Lectin pathway activity after CLP

To further investigate whether the lectin pathway is involved in host defence in the CLP model of peritonitis, a C4 cleavage assay was performed. Plasma samples of a complement-sufficient strain (NMRI) were assayed in a mannan-dependent C4 cleavage test by trapping plasma MBL-MASP complexes on mannan-coated plates and measuring MASP-2-mediated C4 cleavage. This test specifically measures lectin pathway activation via MBL without interference from the classical pathway. Already 10 hours after CLP the MBL-dependent C4 cleavage capacity in the mouse sera was significantly diminished compared to control serum (Fig. 3.10 A). Whereas the serum of untreated mice showed an activity of 4.37 ± 1.38 U/ml, sera taken 10 hours after CLP displayed an activity of 1.85 ± 1.28 U/ml ($p = 0.017$). This decrease was even more pronounced after 24 hours (0.46 ± 0.14 U/ml, $p = 0.003$) and 36 hours (0.40 ± 0.14 U/ml, $p = 0.003$) (Fig. 3.10 A). Mice stayed compromised for more than 10 days with a C4 activation capacity below 0.5 U/ml ($p < 0.005$). Recovery could only be observed 14 days later (Fig. 3.10 B).

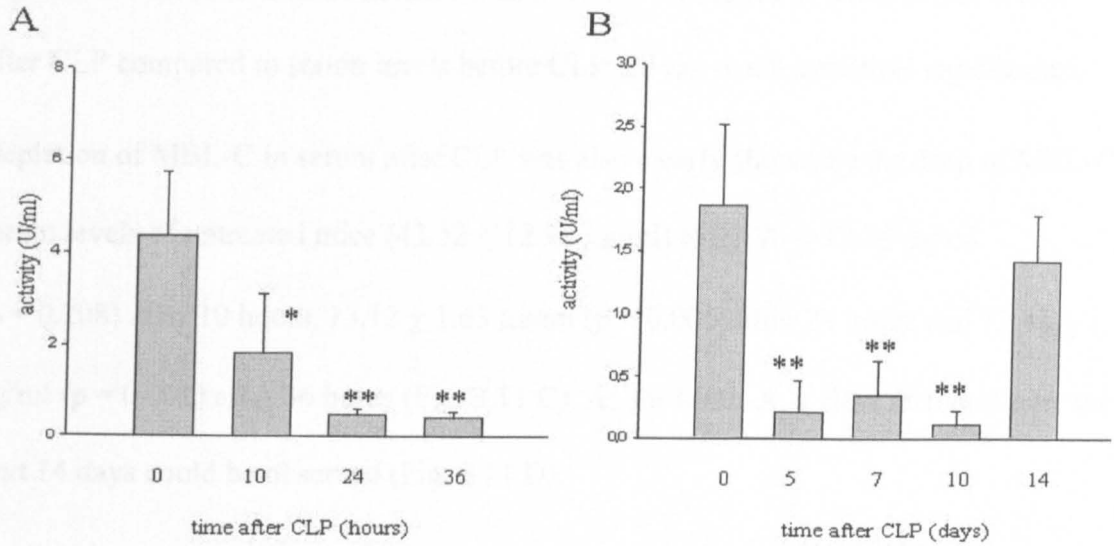

Fig. 3.10: C4 activation after CLP.

Serum samples (NMRI, n = 5) were taken at different time points after CLP and MBL-dependent C4 cleavage activity was compared to samples from untreated control mice (timepoint 0). A) Statistical significant reduced C4-activation in sera was determined at 10, 24 and 36 hours after CLP. * $p < 0.05$ ** $p < 0.005$. B) MBL-dependent C4 cleavage activity 5 (n = 4), 7 (n = 4), 10 (n = 3) and 14 days (n = 3) after CLP. ** $p < 0.005$

3.2.5. Serum MBL levels after CLP

MBL-A and MBL-C serum levels were determined to directly test whether the observed decrease in lectin pathway-dependent C4 activation was due to a lack of MBL or consumption of MASP-2. Already 10 hours after CLP the serum MBL-A levels dropped in mice that had undergone CLP (4.76 $\pm$ 2.49 µg/ml) when compared to serum levels of untreated mice (8.26 $\pm$ 1.15 µg/ml) ($p = 0.021$) (Fig. 3.11 A). Depletion of MBL-A from the sera was even more pronounced after 24 hours (3.29 $\pm$ 2.87 µg/ml, $p = 0.015$) and 36 hours (2.62 $\pm$ 1.96 µg/ml, $p = 0.001$) with two mice below the detection range of 2.5 ng/ml after 24 and 36 hours, respectively. The MBL-A serum levels rose gradually

over the next week, reaching normal levels again after 14 days (Fig. 3.11 B). Due to the high variation of MBL titres in individual mice the difference of MBL serum levels after CLP compared to serum levels before CLP did not reach statistical significance.

Depletion of MBL-C in serum after CLP was also clearly shown by the drop of MBL-C serum levels of untreated mice (43.52 $\pm$ 12.92 µg/ml) to 28.96 $\pm$ 19.36 µg/ml ($p = 0.208$) after 10 hours, 13.12 $\pm$ 1.63 µg/ml ($p = 0.006$) after 24 hours and 12.46 $\pm$ 1 µg/ml ($p = 0.001$) after 36 hours (Fig. 3.11 C). As for MBL-A, a slow recovery over the next 14 days could be observed (Fig. 3.11 D).

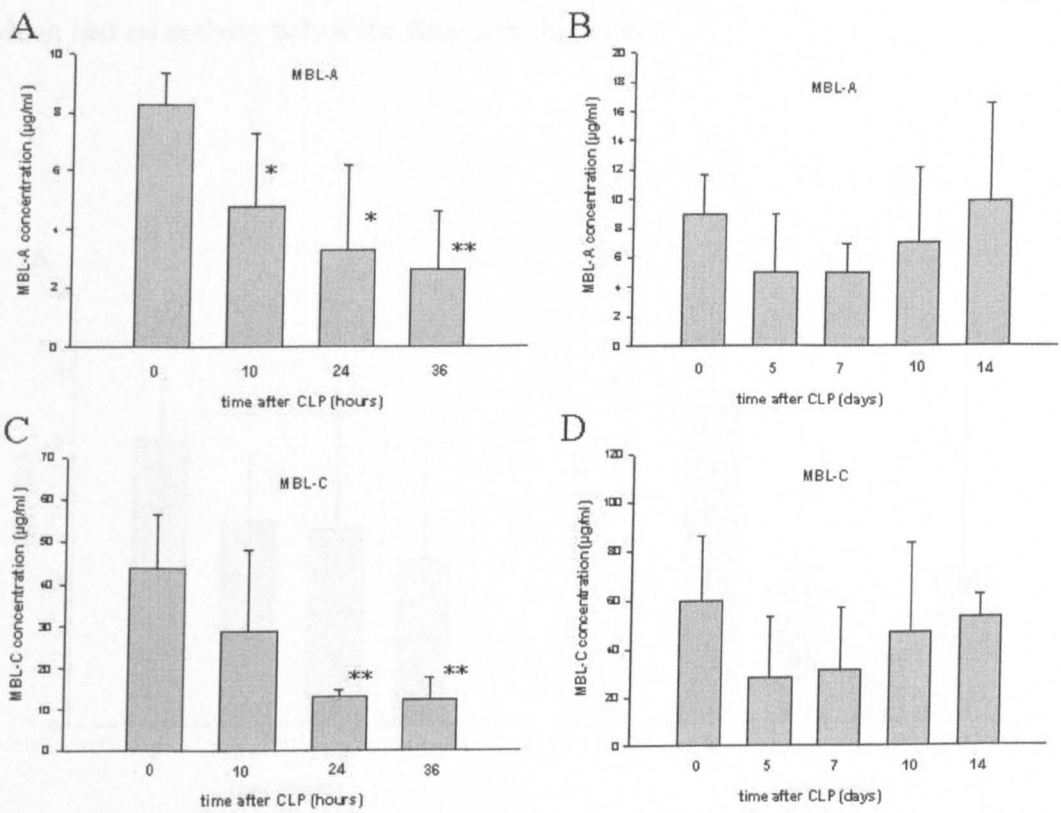

Fig. 3.11: Serum MBL levels after CLP.

Levels of MBL-A and MBL-C were determined in mouse serum samples (NMRI, n = 5) at different time points after CLP and compared to the serum levels of untreated control mice (timepoint 0). A) MBL-A levels in untreated mice and 10, 24, and 36 hours after CLP. * $p < 0.05$, ** $p < 0.005$ B) MBL-A levels 5, 7, 10, and 14 days after CLP. C) MBL-C levels in untreated mice and 10, 24, and 36 hours after CLP. ** $p < 0.005$ D) MBL-C levels 5, 7, 10, and 14 days after CLP.

3.2.6. Lectin pathway activity in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice

Activation of the lectin pathway after CLP was also determined in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice. In *C1q a*$^{-/-}$ mice the C4 cleavage capacity of serum samples (4.03 ± 0.83 U/ml in samples from naive mice) was only slightly reduced at 10 hours (2.86 ± 0.94 U/ml), 24 hours (2.74 ± 2.32 U/ml), and 36 hours (2.33 ± 1.57 U/ml) after CLP (Fig. 3.12 A). In *H2-Bf/C2*$^{-/-}$ mice, however, this decrease was much more pronounced. Untreated control animals showed an activity of 3.71 ± 0.45 U/ml. The activity dropped to 1.59 ± 0.45 U/ml ($p = 0.001$) at the 10-hour time point, and to 0.58 ± 0.31 U/ml ($p < 0.001$) after 24 hours (Fig. 3.12 B). After 36 hours only 2 mice were still alive, one of which had an activity below the detection threshold.

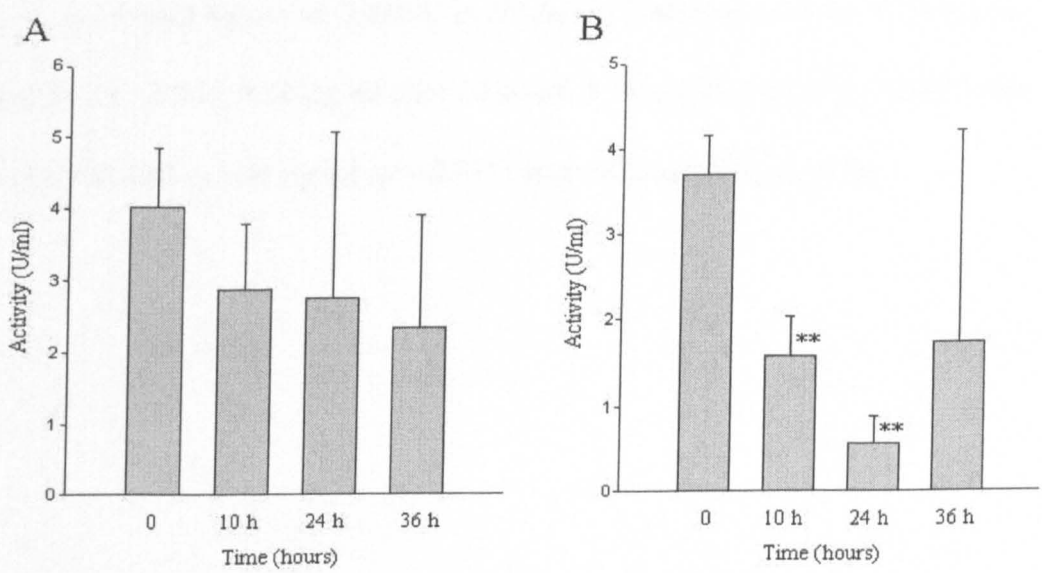

Fig. 3.12: C4 activation after CLP in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice.

Serum samples were taken at different time points after CLP and MBL-dependent C4 cleavage activity was compared to samples from untreated control mice. A) C4 cleavage activity of serum samples (n = 4) from untreated *C1q a*$^{-/-}$ mice and from samples taken 10, 24, and 36 hours after CLP. B) C4 cleavage activity of serum samples from untreated *H2-Bf/C2*$^{-/-}$ mice (n = 3) and from samples taken 10 (n = 5), 24 (n = 5), and 36 hours (n = 2) after CLP. ** $p < 0.005$

Serum MBL levels were significantly reduced in both mouse strains. Samples from untreated *C1q a*$^{-/-}$ mice had an MBL-A concentration of 18.01 ± 8.14 μg/ml. This dropped to 6.92 ± 1.76 μg/ml ($p = 0.037$) after 10 hours, 9.21 ± 4.10 μg/ml ($p = 0.063$) after 24 hours and 4.37 ± 3.02 μg/ml ($p = 0.016$) after 36 hours (Fig. 3.13 A). The serum MBL-C concentration in naive *C1q a*$^{-/-}$ mice was 23.32 ± 2.96 μg/ml. At the 10-hour time point this concentration was more than halved (9.46 ± 2.53 μg/ml, $p < 0.001$) and dropped even further after 24 hours (1.33 ± 1.90 μg/ml, $p < 0.001$) and 36 hours (0.93 ± 1.07 μg/ml, $p < 0.001$) (Fig. 3.13 B).

Untreated *H2-Bf/C2*$^{-/-}$ mice had an MBL-A serum concentration of 17.09 ± 2.53 μg/ml. This was markedly reduced 10 hours (7.28 ± 3.33 μg/ml, $p = 0.001$), 24 hours (3.94 ± 3.48 μg/ml, $p < 0.001$), and 36 hours after CLP (1.59 ± 2.24 μg/ml, $p = 0.001$) (Fig. 3.13 C). Normal values for MBL-C in *H2-Bf/C2*$^{-/-}$ mice were 21.28 ± 7.69 μg/ml. They dropped to 14.96 ± 9.24 μg/ml after 10 hours, 3.38 ± 3.27 μg/ml ($p = 0.001$) after 24 hours, and 0.95 ± 1.34 μg/ml ($p = 0.017$) after 36 hours (Fig. 3.13 D).

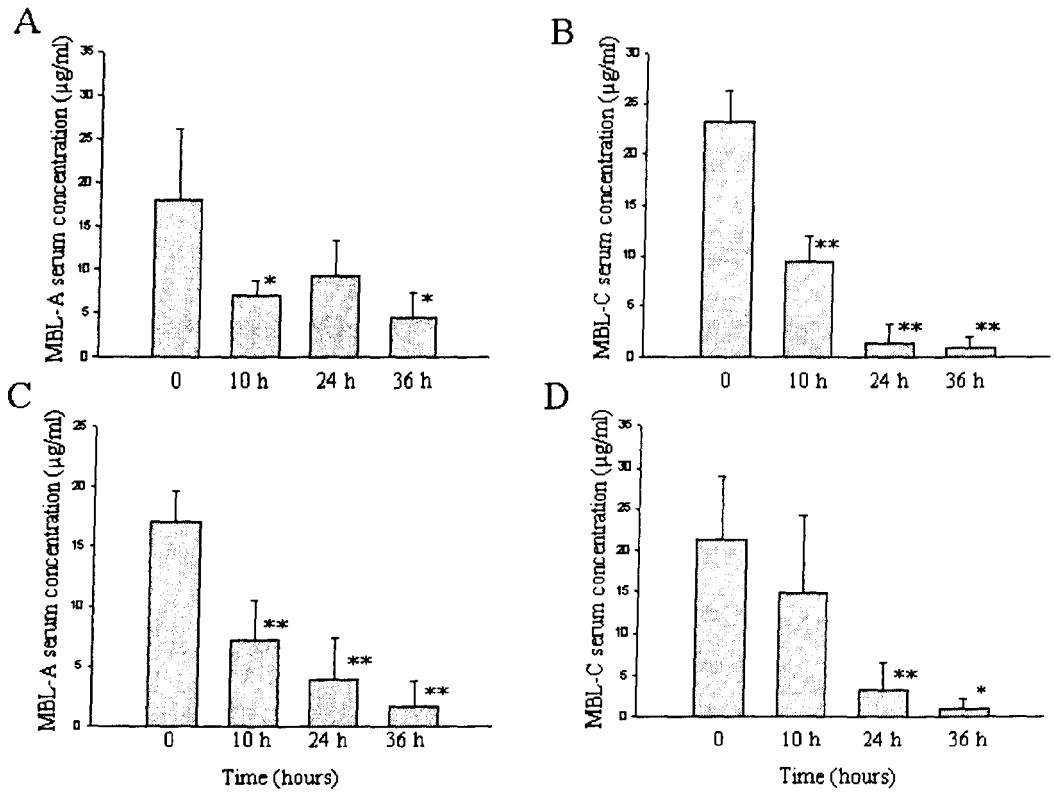

Fig. 3.13: Serum MBL levels after CLP in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice.

Serum samples (n = 5) were taken from untreated mice (timepoint 0) and 10, 24, and 36 hours (n = 4 for *C1q a*$^{-/-}$ and n = 2 for *H2-Bf/C2*$^{-/-}$ mice) after CLP. MBL levels were determined by TRIFMA. A) Serum MBL-A levels of *C1q a*$^{-/-}$ mice. B) Serum MBL-C levels of *C1q a*$^{-/-}$ mice. C) Serum MBL-A levels of *H2-Bf/C2*$^{-/-}$ mice. D) Serum MBL-C levels of *H2-Bf/C2*$^{-/-}$ mice. * $p < 0.05$; ** $p < 0.005$

3.2.7. mRNA expression after CLP

To establish whether the decreased MBL serum levels were due to consumption or decreased protein expression MBL-A and MBL-C mRNA expression from livers of mice that had undergone CLP was determined by Northern blot analysis. Groups of three mice each were sacrificed 5 and 10 hours as well as 5 and 10 days after CLP. Bands were normalised to β-actin. The results demonstrated that transcription of both MBL-A and MBL-C was not significantly changed by CLP over the whole period of 10 days after CLP (Fig 3.7).

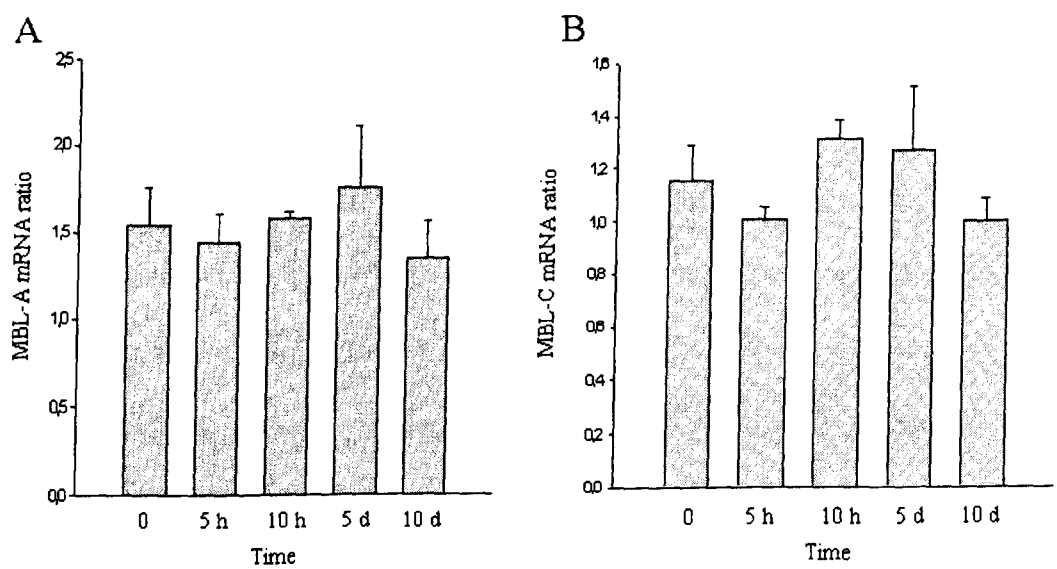

Fig. 3.14: mRNA expression after CLP.

Hybridisation bands on the Northern Blot were quantified using ImageQuant Software (Molecular Dynamics/GE Healthcare, Freiburg, Germany) and normalised to β-actin. A) mRNA ratio for MBL-A in untreated control mice (timepoint 0) and 5 and 10 hours as well as 5 and 10 days after CLP. B) mRNA ratio for MBL-C in untreated control mice (timepoint 0) and 5 and 10 hours as well as 5 and 10 days after CLP.

3.2.8. MBL deposition on abscess

It is reasonable to assume that MBL binding to microbial antigens - after they are released from the intestinal lumen - accounts for the rapid decrease in serum levels after CLP. However, it is unlikely that sufficient numbers of bacteria are present over a prolonged period of time to explain the long lasting depletion. Therefore it can be assumed that MBL might also be deposited elsewhere in the tissue. To test this hypothesis, an abscess was removed 10 days after CLP and cryostat sections of its wall were stained for MBL-A and MBL-C. Sections were also stained for C3 to monitor C3 deposition as an indicator for complement activation.

Positive staining for both MBL-A and MBL-C was observed in necrotic parts of the tissue, whereas newly formed granulation tissue was not reactive. The demarcation zone separating healthy from necrotic tissue showed strong staining. This area is rich in fibrin and highly vascularised with leaky blood vessels, which enable cells and plasma proteins to extravasate, thereby facilitating the healing process. The staining pattern of C3 was similar to staining for MBL, indicating that complement is activated. Controls, which were either stained without use of the primary antibodies (not shown) or using an irrelevant rat IgG_{2a} antibody (isotype control, Fig. 3.15 A) showed no reactivity, thereby confirming specificity.

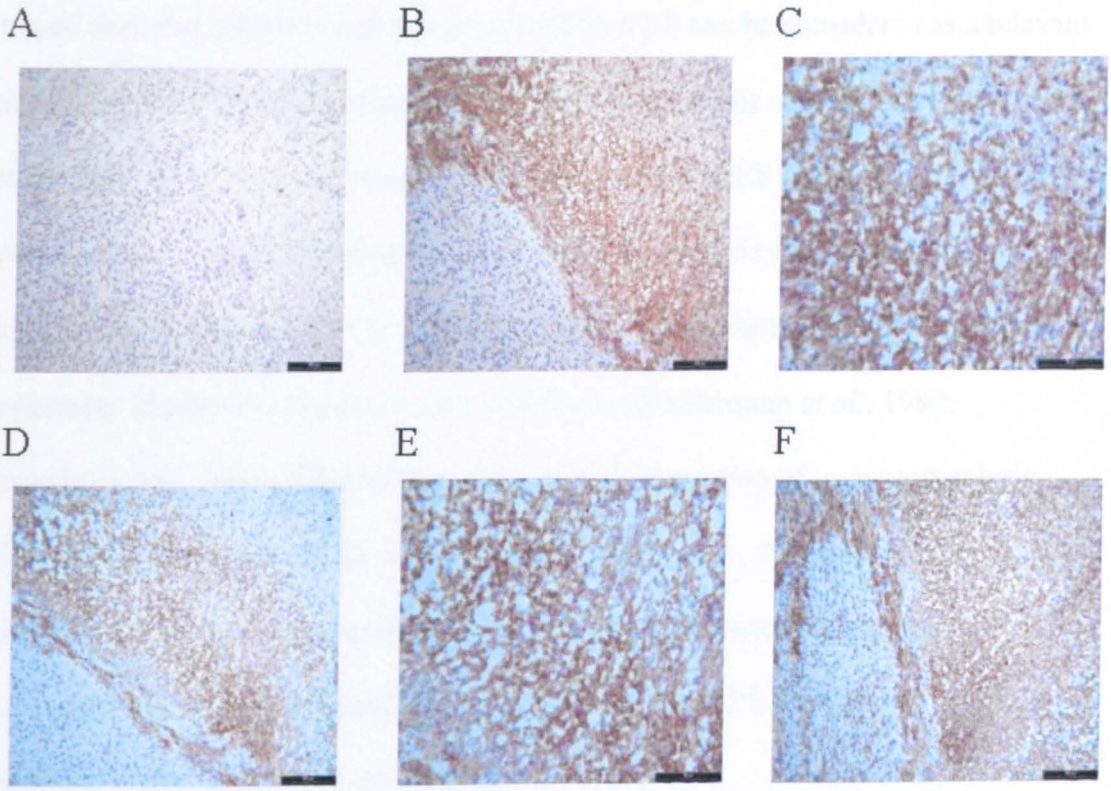

Fig. 3.15: Immunhistochemical staining of abscess.
An abscess was removed 10 days after CLP and cryostat sections of its wall were stained for MBL-A (B and C), MBL-C (D and E) and C3 (F). A) Isotype control. Granulation tissue can be seen in the bottom left hand corner of A, B, D, and F (magnification x160). The bars correspond to 100 μm. C) Higher magnification of B (x400). E) Higher magnification of D (x400). The bars in C and E correspond to 50 μm.

3.3. Discussion

Peritoneal bacterial infection and sepsis induced by CLP can be considered as a relevant animal model to study the importance of different mechanisms of host innate and adaptive immune responses to combat bacterial infections. CLP serves as a clinically relevant model for severe abdominal infection because an early hyperinflammatory phase is followed by a subsequent hypoinflammatory phase during which the animals are extremely sensitive to bacterial super-infections (Wichterman *et al.*, 1980; Echtenacher *et al.*, 2003). This parallels the clinical observation of immunoparalysis developing in patients during the course of sepsis (Volk *et al.*, 1996), and is therefore better suited to study the impact of abdominal trauma with bacterial infection on the innate and adaptive immune system than other models like LPS-injection or infection with a single bacterial strain (Wichterman *et al.*, 1980).

The results from this study clearly demonstrate the importance of an intact complement system for survival after CLP. Mice deficient in factors B and C2 were extremely sensitive to CLP (Fig. 3.8). 90% of these mice died within the first three days after CLP. Also mice lacking only the classical pathway were more susceptible to CLP than the wild type mice, with a survival rate of 40% after 12 days. In the first few days after CLP, however, their mortality was not much different from that of wild type mice, possibly because an intact lectin pathway was sufficient to give them protection. Only after 7 days, at a time when the first specific antibodies against the invading microorganisms would appear, did their mortality increase.

All mice were housed under conventional conditions. Untreated mice with complement deficiencies living in the same surroundings do not show a higher mortality rate than complement-sufficient mice, showing that without infection, complement deficiency does not influence general well-being to a great extent. Another possibility to show that

the deficiencies do not have an effect without infection would be to use sham-operated mice. In this case a laparatomy is performed without ligating or puncturing the cecum. This usually does not influence mortality, but it does give an inflammatory stimulus, which needs to be taken into account when interpreting the results of subsequent measurements. Because of this and because age- and sex-matched groups were not big enough due to a limited number of breeding cages, sham-operated controls were not used in this work.

As already shown recently in a different model of experimentally induced polymicrobial peritonitis, both the classical pathway as well as the lectin pathway of complement activation provide essential support in combating bacterial infection (Celik *et al.*, 2001). A dominant role of the classical pathway for innate immunity to bacterial pathogens by binding of natural antibodies to microbial antigens has previously been indicated (Brown *et al.*, 2002). Recognition of microorganism-specific carbohydrate patterns by serum defence molecules such as MBL may constitute another important mechanism by which the innate immune system is activated, thus providing critical residual protection for C1q-deficient mice as also supported by the results of this work. Mouse MBL-A, which is associated with MASP-2 under physiological conditions, initiates the lectin pathway of complement activation after binding to specific oligosaccharides (Matsushita and Fujita, 1992; Thiel *et al.*, 1997; Vorup-Jensen *et al.*, 2000). Mouse MBL-C has been detected in sera of different mouse strains in about 6-fold higher concentration than MBL-A and was also shown to activate the lectin pathway via MASP-2 (Liu *et al.*, 2001). MBL-A and MBL-C have different avidities for carbohydrate structures, which may broaden the spectrum of microorganisms they recognise (Hansen and Holmskov, 1998).

The two molecules may also have other effector functions besides serving as recognition molecules of the lectin pathway of complement activation. Both MBL and C1q have been shown to bind to CR1/CD35 (Ghiran *et al.*, 2000) and calreticulin/CD91 (Ogden *et al.*, 2001; Vandivier *et al.*, 2002), thereby facilitating phagocytosis. However, this mechanism alone does not seem to be able to protect mice from bacterial infection, as *H2-Bf/C2*$^{-/-}$ mice, which still possess both molecules but lack all complement activation downstream of C4, were significantly more susceptible to CLP than wild type or C1q-deficient mice (Fig. 3.8). Moreover, these mice showed an impaired bacterial clearance from organs, indicating a clear need for further complement activation to successfully combat infections (Fig. 3.9).

However, also mice deficient in C1q, which did not show increased bacterial counts (Fig. 3.9), were much more sensitive to CLP than the wild type controls (Fig. 3.8). There are a number of possible explanations for this discrepancy. To remove organs for bacterial counts mice were killed 24 hours after CLP, at a time when the mortality of *C1q a*$^{-/-}$ mice did not differ from that of wild type controls. At this time mice might have enough protection through an intact lectin pathway, whereas a functional classical pathway becomes important at later stages of the infection. A more pronounced increase of mortality in *C1q a*$^{-/-}$ mice occurred after seven days, at a time when the first specific antibodies against the invading organisms would have been produced. Also, mice were killed especially to remove organs for bacterial counts. Their bacterial situation might not reflect the situation of a mouse that dies from CLP. Furthermore only aerobic bacteria growing on Mueller-Hinton agar were counted. This is a universal agar that allows for growth of all aerobic strains. However, anaerobic bacteria were not looked at but might also contribute to the different mortality rates. Nevertheless it can be assumed that bacterial infection and its consequences are major contributors to mortality after

CLP, as mice can be saved by antibiotic treatment (Echtenacher et al., 2001), helped by an intact complement system.

To further elucidate the role of the lectin pathway of complement activation after CLP, serum samples were taken and analysed. At each time point examined a number of mice were bled and killed afterwards. Sequential bleeding of the same mouse would reflect the actual changes in MBL levels after CLP in an individual more closely. On the other hand, the repeated manipulation of serially bleeding the same mouse might also have an influence on the observed changes.

Lectin pathway activity involvement was first tested by measuring C4 cleavage activity on a mannan-coated plate. This test determines whether MBL oligomers are present in serum, able to bind their ligand and whether they are associated with functionally active MASP-2. The concentration of native C4 in mouse serum does not influence the results, as a standardised amount of purified human C4, which can be cleaved by mouse MASP-2, is added to every sample. A decrease in C4 cleavage activity was observed within hours after CLP, which lasted for more than 10 days (Fig. 3.10).

To find out whether this was caused by a lower number of MBL complexes or by their inability to cleave C4, mannan-bound MBL was measured with monoclonal rat anti-mouse MBL-A and MBL-C antibodies. In this test the serum concentration of MBL complexes that can bind their targets is determined. Also in these experiments a rapid and long lasting decrease in MBL serum levels was seen (Fig. 3.11), which mirrors the decrease in C4-activation, indicating that a lower number of functional MBL oligomers is the main reason for the decreased C4 cleavage capacity. However, there is not always a linear correlation between decrease in MBL levels and decrease in C4 cleavage activity, suggesting that the latter might be influenced by factors other than number of MBL oligomers as well, e.g. altered MASP-2 or inhibitor activity.

To find out whether the decrease in MBL serum levels was due to consumption or decreased production, RNA was isolated from livers and Northern Blot was performed. This showed no significant changes in MBL-A or MBL-C mRNA expression at the examined time points after CLP (Fig. 3.14). However, it might be possible that due to posttranscriptional modification not all of that mRNA is translated into protein. Also, protein oligomerisation might be affected. MBL monomers have a very weak affinity for their ligands (Jack *et al.*, 2001) and might not bind sufficiently to a mannan-coated plate to be detected. To circumvent this problem, MBL serum levels could also be determined in a Sandwich-ELISA. This was done in the second part of this work, to see whether MBL loses its ability to bind to carbohydrates after anaphylactic shock (Fig. 4.7). All anti-MBL antibodies utilised in this assay can be used in a mannan-based ELISA, so it is likely that their epitopes are different from the carbohydrate binding site. Like in the mannan-based ELISA, MBL serum levels were decreased after challenge. However, the situation after anaphylactic shock might not be comparable to CLP. Also, antibodies were raised against recombinant protein and might not be able to detect monomers. As in a non-reducing SDS-PAGE gel the quaternary structure of proteins is not disrupted, this might give a hint as to whether the oligomerisation state of MBL in serum has changed, but the large number of different proteins present in serum may make identification of the bands difficult. This problem could be solved by Western Blot.

This work shows for the first time that the lectin pathway is actively engaged in combating bacterial infection to such an extent that a rapid depletion of MBL from serum occurs. Most interestingly, this depletion lasted for a period of more than 10 days, even though transcription of both MBL-A and MBL-C was still going on. Although surviving mice seem to have overcome the infection and give a healthy impression after about one week, resolution of peritoneal adhesions and abscesses that

are formed after CLP and which are crucial for survival (Echtenacher *et al.*, 2001), takes up to four weeks, since they are quite big with a diameter of up to 2 cm (unpublished observation). Continuous consumption of MBL during this process could explain the long lasting depletion.

To test whether this could be possible, immunhistochemistry was done on cryostat sections of the abscess wall. Positive staining for both MBL-A and MBL-C was observed in areas with fragmented and destroyed nuclei and indistinct cell borders, whereas granulation tissue with healthy fibroblasts was not reactive (Fig. 3.15). Therefore the staining can be considered specific, especially as the isotype control showed no reaction. Areas rich in fibrin also stained for MBL. This has to be interpreted with caution, as fibrin has a tendency to adsorb antibodies unspecifically, but again the isotype control showed no staining in the same areas. C3 staining could be observed in the same areas, indicating that MBL-binding leads to complement activation in necrotic tissues.

Since these abscesses are structures that are formed especially as a reaction to the gut injury, there is no corresponding healthy tissue that could serve as negative control. Sections of other tissues from healthy mice showed staining for MBL-A and MBL-C of hepatocytes, which are the main producers of MBL, as well as of glomerular cells of the kidney and of endothelial cells in the small intestine (MBL-C only), where extrahepatical MBL expression has been reported (Uemura *et al.*, 2002; Wagner *et al.*, 2003; de Vries *et al.*, 2004). Other healthy cells are not reactive (see section 4.2.6. for examples). C3 staining in healthy tissue can be observed on endothelial cells (Ueki *et al.*, 1987), epithelial cells of the lung (Strunk *et al.*, 1988) and intestine (Andoh *et al.*, 1993) as well as along the tubular basement membrane, the Bowman's capsule and on

mesangial cells in the kidney (Thurman *et al.*, 2003). Again, other healthy cells show no staining.

Therefore it can be argued that MBL binds specifically to dead tissue and may lead to complement activation in those areas, as the staining pattern for C3 was identical. This is in accordance with previous reports showing that MBL is able to bind to apoptotic (Ogden *et al.*, 2001) and necrotic (Nauta *et al.*, 2003) cells. Complement activation via the lectin pathway after tissue injury was also suggested by a report demonstrating binding of MBL to endothelial cells after oxidative stress (Collard *et al.*, 2000), indicating a role for MBL in maintaining tissue integrity. The finding that MBL binds to necrotic parts of the abscess wall supports this.

A rise in MBL-A levels after CLP could not be detected, even though MBL-A has been reported to behave like an acute phase protein as elevated levels were induced by injection of azocasein, thioglycolate medium, or lipopolysaccharide (Sastry *et al.*, 1991; Liu *et al.*, 2001). A modest increase in MBL levels was also reported in patients undergoing major surgery (Thiel *et al.*, 1992). Another study, however, showed no significant difference in serum MBL between pre- and post-operative samples (Siassi *et al.*, 2003). In addition, promoter studies have cast doubt on the notion of MBL being an acute phase protein, as transcriptional activity was not increased upon stimulation with proinflammatory cytokines. Dexamethasone even downregulated MBL expression (Naito *et al.*, 1999). The observation that MBL-A and MBL-C mRNA levels were not significantly increased at any measured time point after CLP does not support the viewpoint of MBL being an acute phase protein, either. It could be argued, however, that quantitating bands in Northern Blot is not the most accurate of methods and that quantitative real-time PCR would be a better method to detect an acute phase reaction, but because MBL expression varies considerably even in healthy mice (Liu *et al.*,

2001), a modest acute phase reaction might be masked by these inter-individual differences.

A recent report shows that MBL-A deficiency enhanced survival in the same mouse model of peritonitis, but using mice with the C57/BL6 genetic background (Takahashi *et al.*, 2002). As a possible explanation the authors discuss their unexpected finding by the significant decrease in the TNF response in MBL-A-deficient mice 48 hours after CLP. In contrast, other reports showed that TNF neutralisation up to 8 hours after CLP increased lethality (Echtenacher *et al.*, 1990). Also, TNF-deficient mice as well as mice deficient in the TNF receptor type 1 were reported to be significantly more susceptible to CLP compared to their wild type controls (Maurer *et al.*, 1998; Echtenacher and Mannel, 2002). Thus, the relative importance of either MBL-A or MBL-C for survival of CLP-induced septic peritonitis is far from being clear at the moment, especially as the recently generated mice with a double deficiency in MBL-A and MBL-C have not been tested in this model yet. However, they were significantly more susceptible to infection with Staphylococcus aureus (Shi *et al.*, 2004).

When comparing the human system to the mouse model it has to be taken into account that humans express only one form of MBL, which is more closely related to the rodent MBL-C form (Ezekowitz *et al.*, 1988; Holmskov *et al.*, 2003). However, there is good evidence that also in man MBL plays an important role in protection from bacterial infection (Super *et al.*, 1989; Madsen *et al.*, 1995). A recent study in patients admitted to hospital with systemic inflammatory response syndrome underlines the importance of MBL during sepsis, showing that patients carrying MBL variant alleles had a higher risk of developing severe sepsis and septic shock and were more likely to have positive cultures for microbial species (Garred *et al.*, 2003b).

In conclusion, these results show that the lectin pathway of complement activation plays an important role in combating bacterial peritonitis, and that CLP leads to a long lasting depletion of MBL from serum, with little or no effect on MBL biosynthesis. Furthermore, MBL binding could be demonstrated on necrotic parts of the abscess wall, explaining some of the MBL depletion. However, as the decrease of MBL serum levels is so pronounced and long lasting, it is conceivable that also other, as yet unidentified mechanisms contribute to this effect.

4. Part II: Anaphylactic Shock

4.1. Introduction

Anaphylactic shock is an immediate, systemic and life threatening hypersensitivity reaction. The classic anaphylactic reaction is mediated by allergen binding to IgE on mast cells (Serafin and Austen, 1987). This results in activation of Fcε receptors, degranulation and release of mast cell mediators, e.g. histamine, tryptase, heparin, platelet-activating factor (PAF) or leukotriene B4, thus causing some or all of the following symptoms: lethargy, pruritus, urticaria, bronchospasm, hypotension and increased vasopermeability (Lei *et al.*, 1996). The cause of death in the mouse is hypotension, resulting in cardiac failure (Munoz and Bergman, 1965). In mice, the decrease in respiratory function and blood pressure is accompanied by a decrease in body temperature (Makabe-Kobayashi *et al.*, 2002), which can therefore be used as an indicator of the severity of shock.

However, IgE dependent mast cell activation is not the only mechanism involved, since anaphylaxis can also be induced in IgE-deficient mice (Oettgen *et al.*, 1994) and mast-cell-deficient mice (Ha *et al.*, 1986; Choi *et al.*, 1998; Okunuki *et al.*, 2000) as well as mast cell-deficient rats (Guo *et al.*, 2001). In these cases a role for IgG1 and macrophages (Strait *et al.*, 2002), granulocytes (Kimura *et al.*, 1997) or basophils (Takeda *et al.*, 1997; Choi *et al.*, 1998) has been suggested. Also platelets (Cara *et al.*, 2004) and PAF (Choi *et al.*, 1998; Okunuki *et al.*, 2000) seem to be involved.

In addition, the complement system is activated in anaphylactic shock. Common allergens like house dust and *Aspergillus fumigatus* lead to generation of the anaphylatoxins C3a, C4a, and C5a and to aggregation of polymorphonuclear leukocytes when incubated with human serum (Nagata and Glovsky, 1987). Elevated levels of the anaphylatoxin C3a have been found in a cardiac anaphylaxis model in the guinea pig (del Balzo *et al.*, 1988) and in the blood of patients undergoing immediate

hypersensitivity reactions (van der Linden *et al.*, 1990). Pretreatment with soluble complement receptor 1 (sCR1), which binds C3b and C4b and inhibits further C3a generation, ameliorated the symptoms (Regal *et al.*, 1993), whereas inhibition of C3a inactivation prolonged the anaphylactic reaction (del Balzo *et al.*, 1988).

Anaphylatoxins are small cleavage products of complement components, which are generated when the complement system is activated via one of its pathways. C5a, a glycopeptide consisting of 74 amino acid residues, is the most potent of the anaphylatoxins generated during complement activation. It binds to C5aR (CD88) on granulocytes and monocytes/macrophages, acting as a chemotactic factor. It also causes vascular permeability, elicits the generation of oxygen and nitrogen radicals and stimulates smooth muscle contraction. Both C3a and C5a have been shown to directly stimulate mast cell degranulation (Johnson *et al.*, 1975; Schulman et al., 1988; el Lati *et al.*, 1994). Complement activation also plays an important role in immune-complex induced Arthus reaction (Hopken *et al.*, 1997) and can cause severe lung inflammatory reactions (Mulligan *et al.*, 1996).

Although the main function of the recently discovered lectin pathway of complement activation seems to be host defence against bacteria (Celik *et al.*, 2001; Windbichler *et al.*, 2004), its role in other immunological reactions, such as anaphylactic shock, remains to be elucidated.

4.2. Results

4.2.1. Preparatory Work: Purification of anti-BSA antibodies

During this work it was investigated whether MBL is able to bind to immune complexes. To do this, it was necessary to purify anti-BSA antibodies, which were then added to BSA-coated ELISA plates in order to generate plate-bound immune complexes.

Anti-BSA antibodies were enriched from mouse immune serum by ammonium sulfate precipitation as described in Materials and Methods. The precipitate was dissolved in PBS and thoroughly dialysed against PBS to remove the ammonium sulfate. Antibodies were then affinity-purified on BSA-coupled sepharose (prepared as described in Materials and Methods) in a batch system, i.e. without the use of columns. The sample was first passed over uncoupled sepharose to remove proteins, which would unspecifically interact with the sepharose, and then over BSA-coupled sepharose. The supernatant from this step was used again in a second purification step. The sepharose was washed thrice and bound protein was eluted with 0.1 M glycine, pH 2.5. After the elution the sepharose was washed with 0.1 M Tris, pH 8.5. All supernatants and eluates were dialysed against PBS and used for further analysis.

SDS-PAGE gel analysis showed that the supernatants from the washing and binding steps contained a large amount of protein and also immunoglobulins (Fig. 4.1). This is not surprising, as only a small amount of antibodies would be specific for BSA. In the lanes showing the eluates, however, hardly any bands can be seen. The IgG heavy chain would be expected to run at a height of 50 kDa, the light chain has a size of 25 kDa.

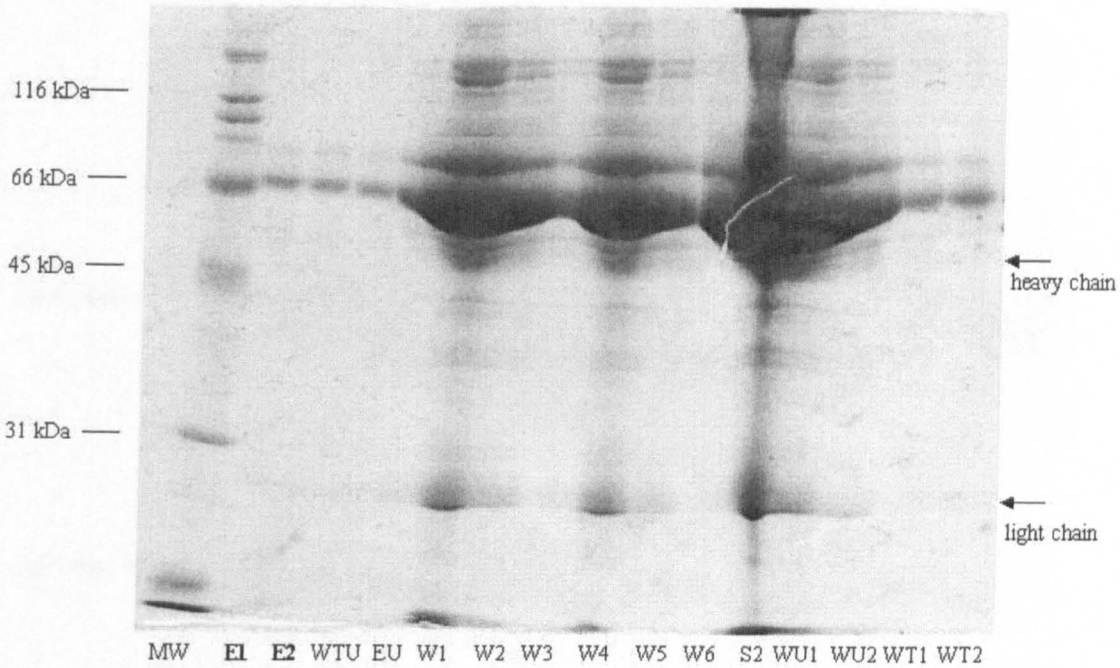

Fig. 4.1: Affinity-purification of anti-BSA antibodies on BSA-coupled sepharose.

Eluates and supernatants were separated on a reducing 12.5% SDS-PAGE gel and visualised by Coomassie staining. MW denotes the molecular weight standard, E1 and E2, printed in bold, are the eluates from the first and second elution step, respectively. WTU is the final washing step of the uncoupled sepharose, EU shows the eluate from uncoupled sepharose. Washing steps of the BSA-coupled sepharose before elution are denoted W, those after elution are denoted WT. WU are washing steps of the uncoupled sepharose, S2 is the supernatant of the second purification step. The arrows on the right indicate the approximate positions of the IgG heavy and light chains at 50 and 25 kDa, respectively.

To see whether the eluates from the BSA-coupled sepharose do contain antibodies, the more sensitive method of Western Blot was employed, using a peroxidase-conjugated anti-mouse IgG antibody for detection. Again, as in the SDS-PAGE gel, supernatants from the washing steps were found to contain such a large amount of mouse immunoglobulins that the signal is very strong and therefore indistinct, but also in the eluates a small signal could be obtained (Fig. 4.2), indicating that antibodies specific for BSA could be purified.

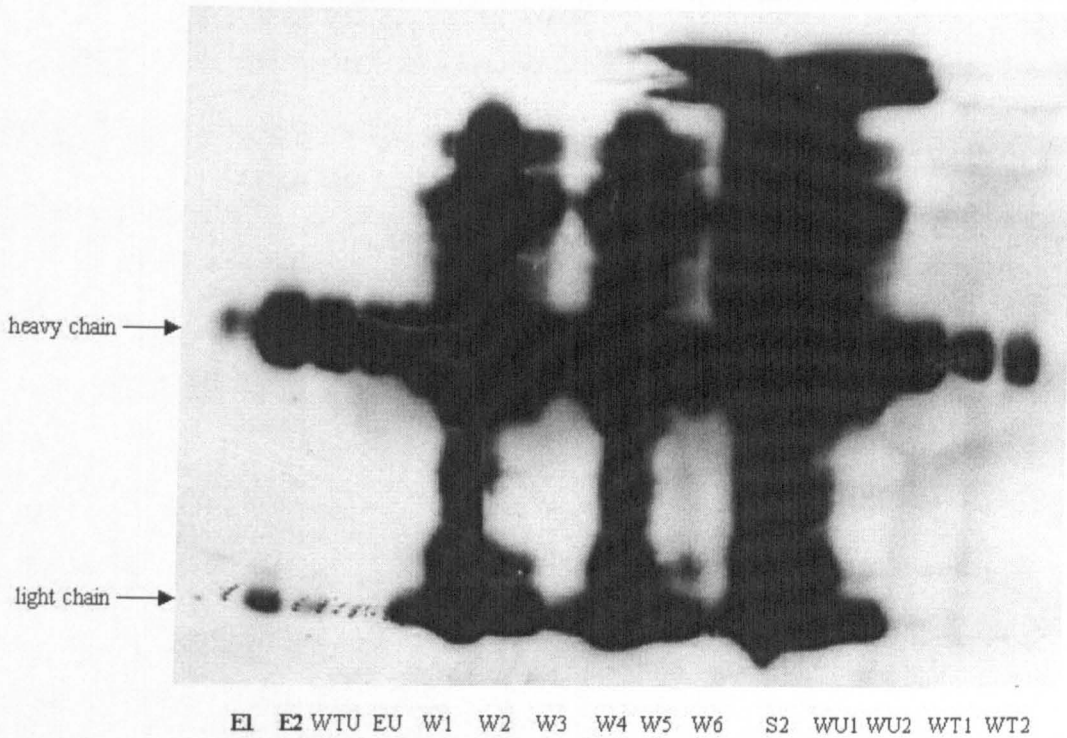

Fig. 4.2: Detection of mouse antibodies in eluates from BSA-coupled sepharose.

Eluates and supernatants from washing steps were separated on a 12.5% SDS-PAGE gel, blotted onto a membrane which was then incubated with a peroxidase-labelled goat anti-mouse IgG antibody. Lanes containing the first and second eluate are indicated in bold at the bottom of the blot. Use of the other abbreviations is identical to Fig. 4.1. The arrows denote the signals for the IgG heavy and light chains.

The titer of anti-BSA antibodies and total protein content of eluates and supernatants were determined as described in Materials and Methods. Results are shown in Table 4.1. The first eluate from the BSA-coupled sepharose was found to contain antibodies specific for BSA with a concentration of 180 μg/ml. Also in the second eluate an antibody concentration of 150 μg/ml was found. The supernatants from the washing steps have a high protein concentration. This is not surprising, as these fractions would contain contaminating proteins and antibodies unspecific for BSA, which do not bind to the BSA-coupled sepharose. But they have a low anti-BSA titer, so not a lot of specific antibodies are lost during these steps. As the starting material had an anti-BSA titer of

6400, this purification was not very efficient. Most of the antibodies were lost during the passage over the uncoupled sepharose. This step could perhaps be omitted in the future to increase the yield. But since the amount of purified mouse antibodies specific for BSA was enough for subsequent experiments, the method was not optimised further.

Fraction	Protein conc. mg/ml	α-BSA titer
EU	0,31	12
E1	**0,18**	**24**
W1	> 1,5	6
W2	2,14	6
W3	0,21	6
E2	**0,15**	**16**
W4	> 1,5	4
W5	0,63	3
W6	0,2	3
S2	>> 1,5	6
WT	0,08	8
WT 2	0,05	8
WTU	0,11	6
WU 1	> 1,5	> 48
WU 2	0,6	32

Table 4.1: Protein concentration and titer of anti-BSA antibodies after antibody purification on BSA-coupled sepharose.

Protein concentration was estimated using the DC Protein assay; the antibody titer was determined with ELISA. E1 and E2, printed in bold, are the eluates from the first and second elution step, respectively. WTU is the final washing step of the uncoupled sepharose, EU shows the eluate from uncoupled sepharose. Washing steps of the BSA-coupled sepharose before elution are denoted W, those after elution are denoted WT. WU are washing steps of the uncoupled sepharose, S2 is the supernatant of the second purification step.

4.2.2. Anaphylactic shock induced by Ovalbumin

4.2.2.1. Characterisation of OVA-induced anaphylactic shock

Female NMRI mice were immunized with albumin from chicken egg white (Ovalbumin, OVA). When challenged with 50 µg OVA intravenously, these mice developed anaphylactic symptoms such as lethargy, tachypnea, and a rapid decrease in body temperature (Fig. 4.3). Naïve mice challenged with the same dose showed no

symptoms. The same reaction could also be induced by passive immunisation with serum from sensitised mice and subsequent challenge (Fig. 4.4 A). Serum from naïve mice (Fig. 4.4 A) or sensitised rats (Fig. 4.4 B) did not cause an anaphylactic reaction.

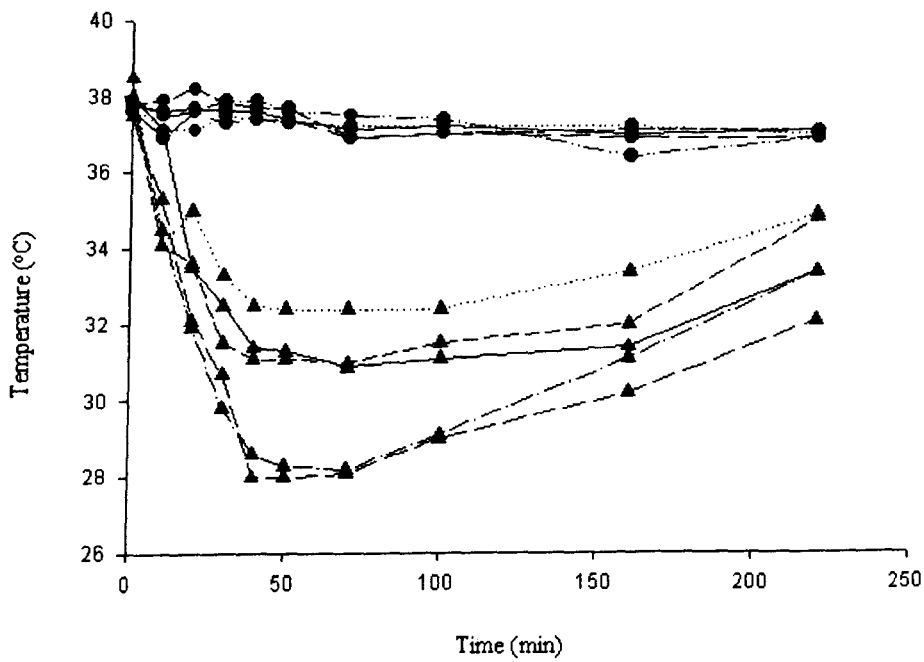

Fig. 4.3: Active OVA-induced anaphylaxis.
NMRI mice (n = 5) were actively immunized with OVA as described in Materials and Methods and challenged on day 21 with 50 μg OVA i.v. (filled triangles). A naïve control group (n = 5) received only the challenging dose (filled circles). Rectal temperature was recorded.

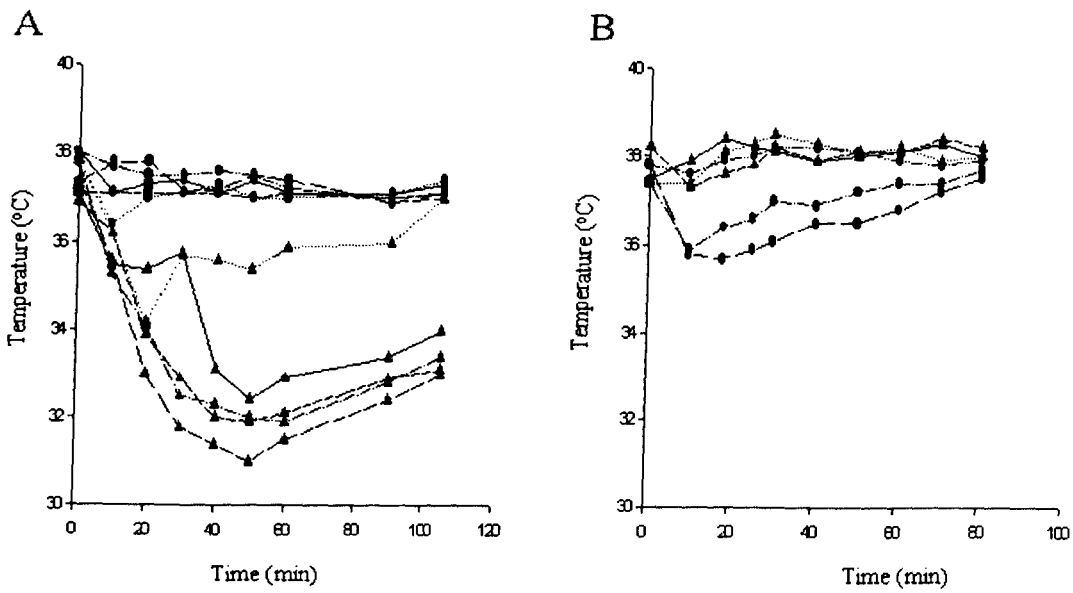

Fig. 4.4: Passive OVA-induced anaphylaxis.

A) Groups of 5 NMRI mice received 200 µl normal mouse serum (filled circles) or serum from OVA-immunised mice (filled triangles). After 24 hours they were challenged with 500 µg OVA and rectal temperature was recorded. B) Groups of 5 NMRI mice received 200 µl normal rat serum (filled circles) or serum from OVA-immunised Wistar rats (filled triangles). 24 hours later they were challenged with 500 µg OVA and rectal temperature was recorded.

4.2.2.2. Lectin pathway activity after OVA-induced anaphylactic shock

To see whether the lectin pathway of complement activation is involved in this model of shock, blood was taken at several time points after antigen challenge and an MBL-dependent C4 cleavage assay was performed. MBL-dependent C4 cleavage capacity in the mouse sera decreased within minutes after challenge. The lowest levels of activity were reached after 30 minutes. Whereas the serum of untreated mice showed an activity of 2.38 $\pm$ 0.55 U/ml, sera taken 30 minutes after challenge displayed an activity of 0.79 $\pm$ 0.26 U/ml ($p = 0.001$). Over the next hours a gradual increase could be observed, with values of 1.3 $\pm$ 0.56 U/ml ($p = 0.015$) after 2 hours and 1.24 $\pm$ 0.28 U/ml ($p = 0.003$)

after 4 hours, respectively. After 8 hours an activity of 1.68 ± 0.30 U/ml (p = 0.037) was reached (Fig. 4.5). After 24 hours the C4 cleavage capacity of the serum samples was back to normal (data not shown).

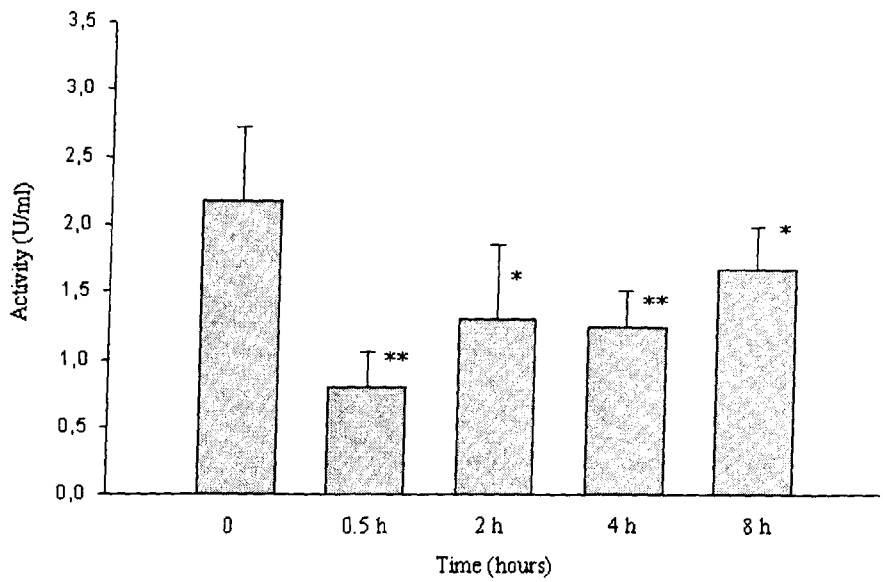

Fig. 4.5: C4 activation after passive anaphylaxis induced by OVA.
Serum samples (n = 5, NMRI) were taken 30 minutes, 2, 4, and 8 hours after antigen challenge and MBL-dependent C4 cleavage activity was compared to samples from untreated control mice (time point 0). * $p < 0.05$; ** $p < 0.005$

4.2.2.3. Serum MBL levels after OVA-induced anaphylactic shock

This decrease in C4-activation capacity was caused by a decrease of both MBL-A and MBL-C serum levels. Already 30 minutes after challenge the serum MBL-A levels dropped considerably (10.00 ± 2.69 µg/ml, p = 0.005) when compared to serum levels of untreated mice (17.59 ± 3.49 µg/ml). Levels stayed low also at 2 hours (13.02 ± 3.25 µg/ml, p = 0.065) and 4 hours (10.80 ± 1.74 µg/ml, p = 0.005) after challenge. After 8 hours normal levels were reached again (20.56 ± 4.90 µg/ml) (Fig. 4.6 A).

Depletion of MBL-C in serum after antigen challenge was also clearly shown by the drop of MBL-C serum levels of untreated mice (108.80 ± 11.78 µg/ml) to 51.46 ± 12.99 µg/ml ($p < 0.001$) after 30 minutes, 62.57 ± 16.82 µg/ml ($p = 0.001$) after 2 hours, 50.94 ± 6.02 µg/ml ($p < 0.001$) after 4 hours and 60.64 ± 11.81 µg/ml ($p < 0.001$) after 8 hours, respectively (Fig. 4.6 B).

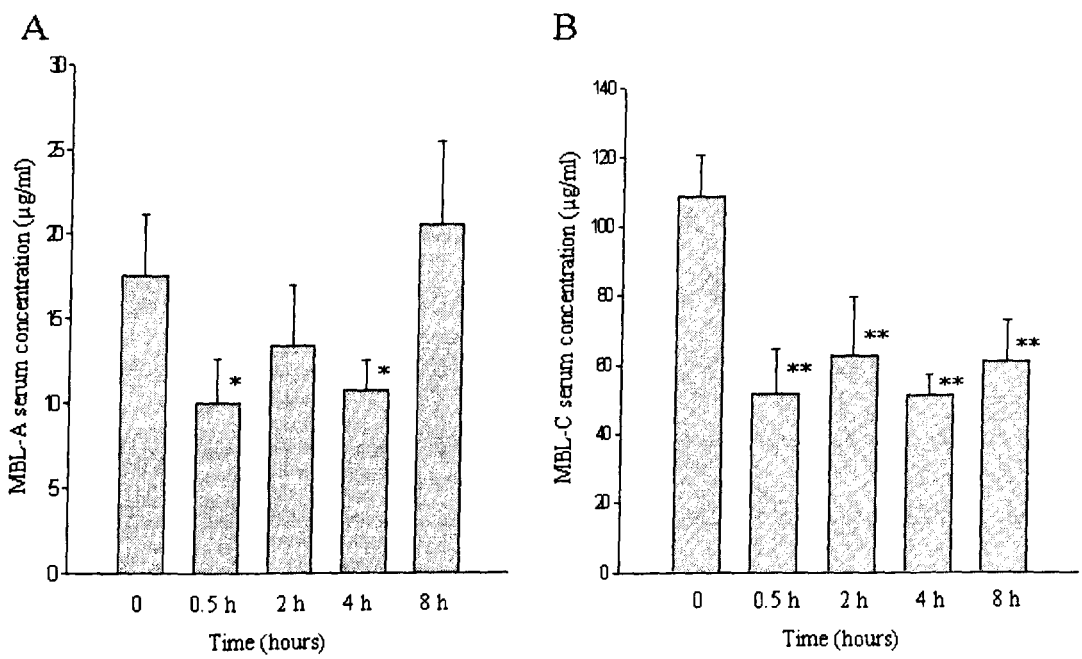

Fig. 4.6: Serum MBL levels after passive anaphylaxis induced by OVA.
Groups of NMRI mice (n = 5) were subjected to passive anaphylaxis and serum MBL levels were determined by TRIFMA. A) Serum MBL-A levels of naïve mice (time point 0) and of samples taken 30 minutes, 2, 4, and 8 hours after antigen challenge. * $p < 0.05$ B) Serum MBL-C levels. ** $p < 0.005$

It has been shown for C1q that increased vasopermeability and loss of proteins into the interstitial space does not explain the decreased serum levels (Tannenbaum *et al.*, 1975; van der Linden *et al.*, 1990). This is also true for MBL, as total serum protein content after anaphylactic shock was unchanged. Total protein content in serum samples from

four untreated mice was 72.83 ± 12.86 mg/ml. Four samples taken 30 minutes after antigen challenge showed a protein content of 84.55 ± 53.13 mg/ml.

To estimate the amount of proteins lost from serum due to unspecific extravasation, total IgG content of sera taken 30 minutes after antigen challenge was measured by Sandwich-ELISA and compared to samples from naïve mice. IgG, considered an inert protein, fell by about 8% during anaphylaxis. MBL levels, on the other hand, dropped by approximately 50%, even though MBL-A and MBL-C are large molecules (850 kDa and 950 kDa, respectively) and would leak less easily through the dilated vessel walls than the much smaller IgG (160 kDa).

To exclude that MBL might have lost its ability to bind to carbohydrate structures and therefore cannot be detected on a mannan-coated plate, the decrease in serum MBL levels was confirmed in a Sandwich-ELISA. MBL was trapped between two monoclonal anti-mouse MBL antibodies, one of which was biotinylated, and detected with alkaline-phosphatase-conjugated streptavidin. Also in this case samples taken after antigen challenge displayed decreased levels of MBL-A (Fig. 4.7 A) and MBL-C (Fig. 4.7 B). Due to the paucity of samples tested this way, statistical evaluation was omitted.

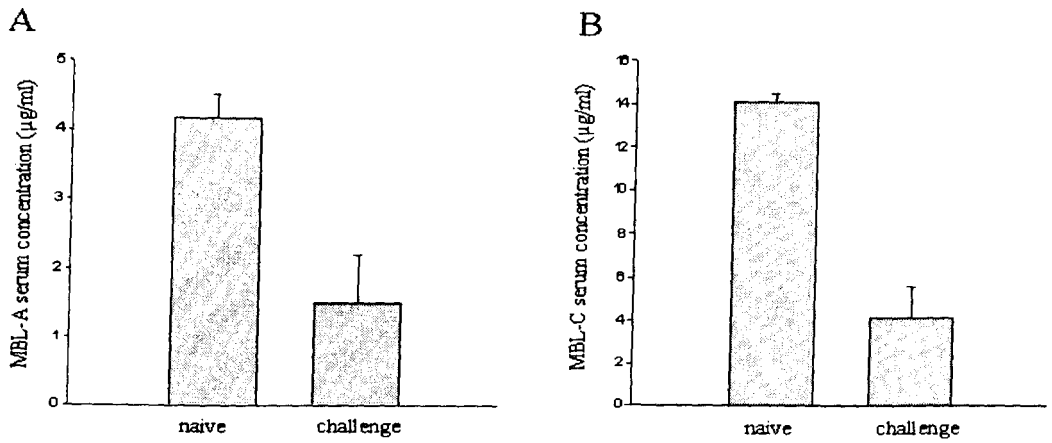

Fig. 4.7: Serum MBL levels determined by Sandwich-ELISA.
NMRI mice were subjected to passive anaphylactic shock induced by OVA. Serum MBL levels from untreated mice (n = 2) and from samples taken 10 minutes after antigen challenge (n = 3) were determined by Sandwich-ELISA as described in section 2.5.6. A) Serum MBL-A levels. B) Serum MBL-C levels.

4.2.3. MBL binding in vitro

The first hypothesis to explain this decrease in serum MBL levels was that MBL might bind to antigen-antibody complexes. To test this, ELISA plates were coated with OVA and normal mouse serum was added in serial dilution in MBL-binding buffer, starting at 1:10. To find out whether MBL-binding was dependent on the carbohydrate recognition domain (CRD), samples were diluted in an EDTA-containing buffer or preincubated with 10 µg/ml mannan or 50 mg/ml OVA. As a positive control wells were coated with mannan. For negative controls serum, the primary or secondary antibody was omitted. Bound MBL was detected with monoclonal rat anti-mouse MBL-A and MBL-C antibodies. Both MBL-A (Fig. 4.8) and MBL-C (Fig. 4.9) bound to solid-phase OVA. This binding is mediated via the carbohydrate-recognition domain. Titration curves (not shown) showed that fluid phase OVA is also able to inhibit MBL binding, but at a

10,000-fold higher concentration than mannan. Since OVA is a glycoprotein with one high mannose group per mole, this binding is not surprising.

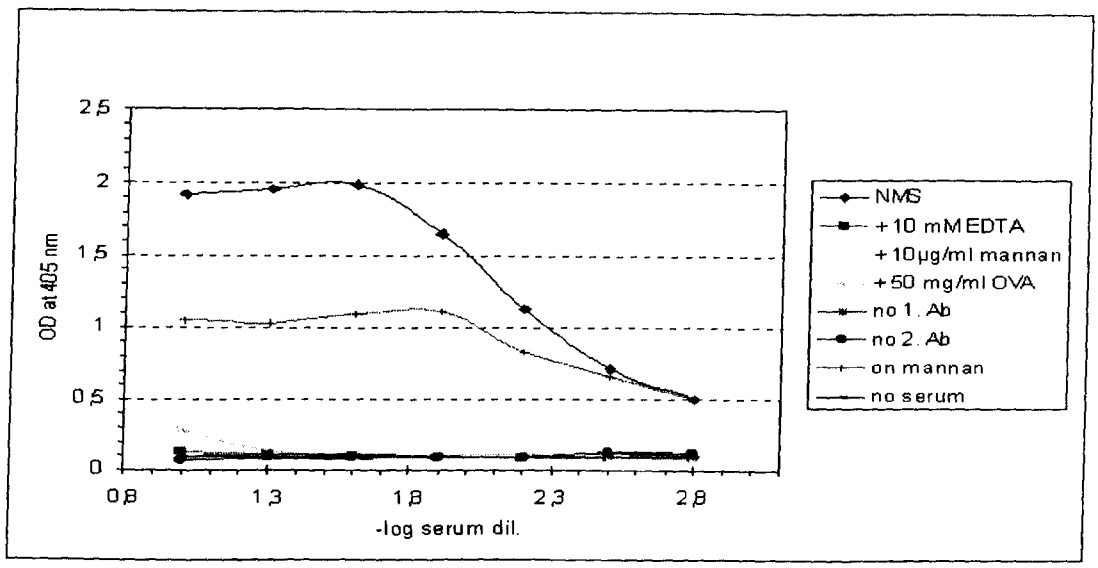

Fig. 4.8: MBL-A binding to OVA.
The x-axis represents the negative logarithm of the serum dilution. The y-axis represents the optical density at 405 nm.

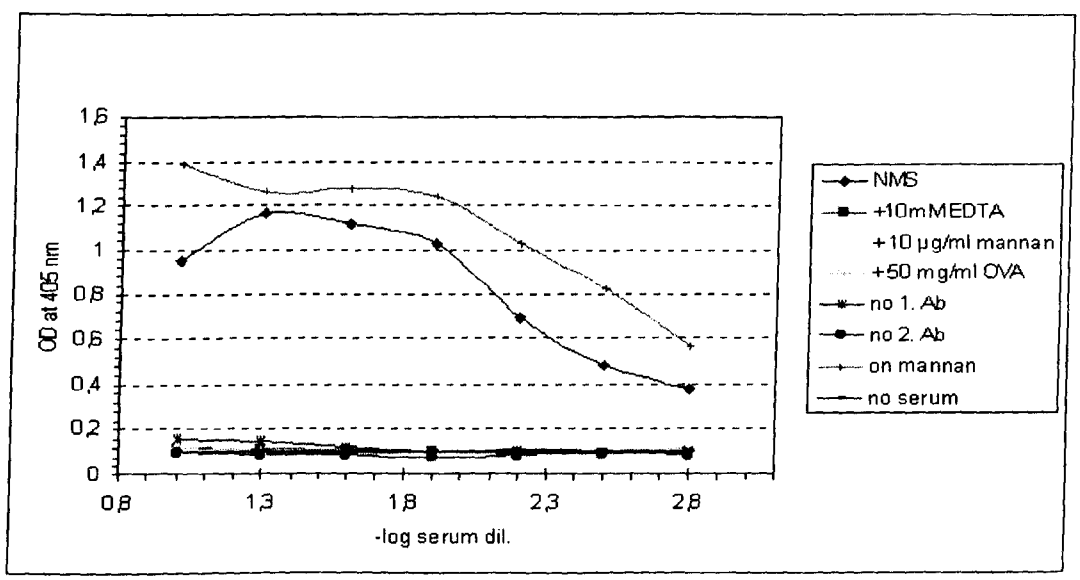

Fig. 4.9: MBL-C binding to OVA.
The x-axis represents the negative logarithm of the serum dilution. The y-axis represents the optical density at 405 nm.

To determine whether the binding of MBL to the antigen is sufficient to account for the decreased MBL serum levels after anaphylactic shock, another protein antigen, which does not bind to MBL, had to be tested. Bovine serum albumin (BSA), which is not glycosylated, seemed a good candidate. An ELISA, performed as described above, showed no MBL binding to BSA-coated plates (diamonds in Fig. 4.10. and 4.11). Since it has been reported that MBL is able to bind to IgG glycoforms (Malhotra *et al.*, 1995) and IgA (Roos *et al.*, 2001), it was also tested whether MBL can bind to the antibody constituent of the immune complex. Anti-BSA antibodies were affinity-purified from serum and added to BSA-coated plates to form solid phase immune complexes. Serial dilutions of mouse serum were added to BSA-coated wells or to wells coated with BSA-anti-BSA immune complexes. As positive control wells were coated with mannan. For negative controls serum, the primary or secondary antibody was omitted. To test for CRD-dependency of binding samples were diluted in an EDTA-containing buffer or preincubated with mannan. No MBL-binding could be observed under these conditions (Fig. 4.10. and 4.11).

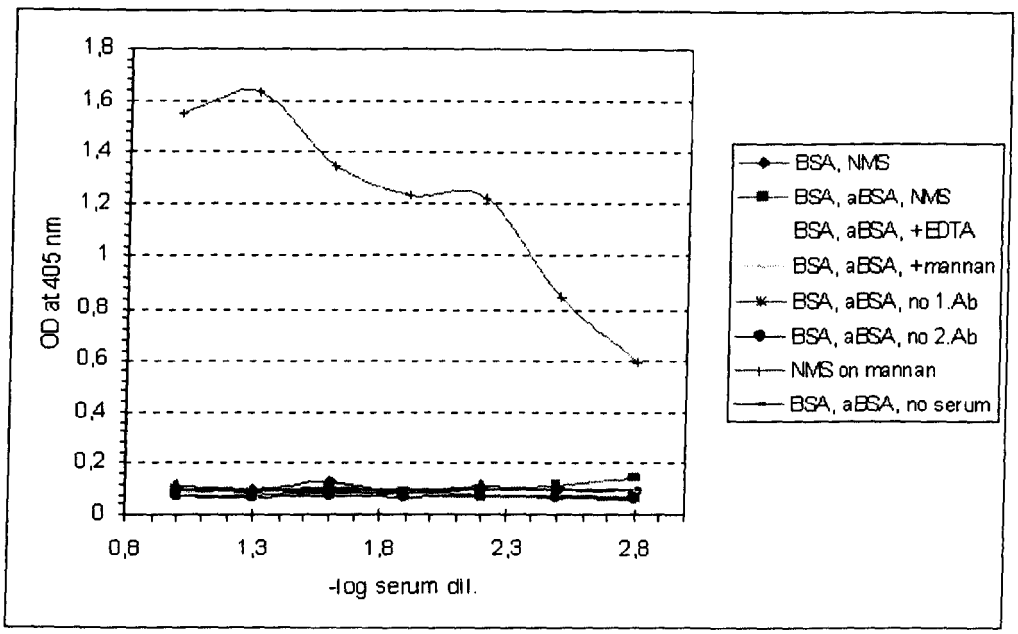

Fig. 4.10: MBL-A binding to solid-phase immune complexes.
The x-axis represents the negative logarithm of the serum dilution. The y-axis represents the optical density at 405 nm.

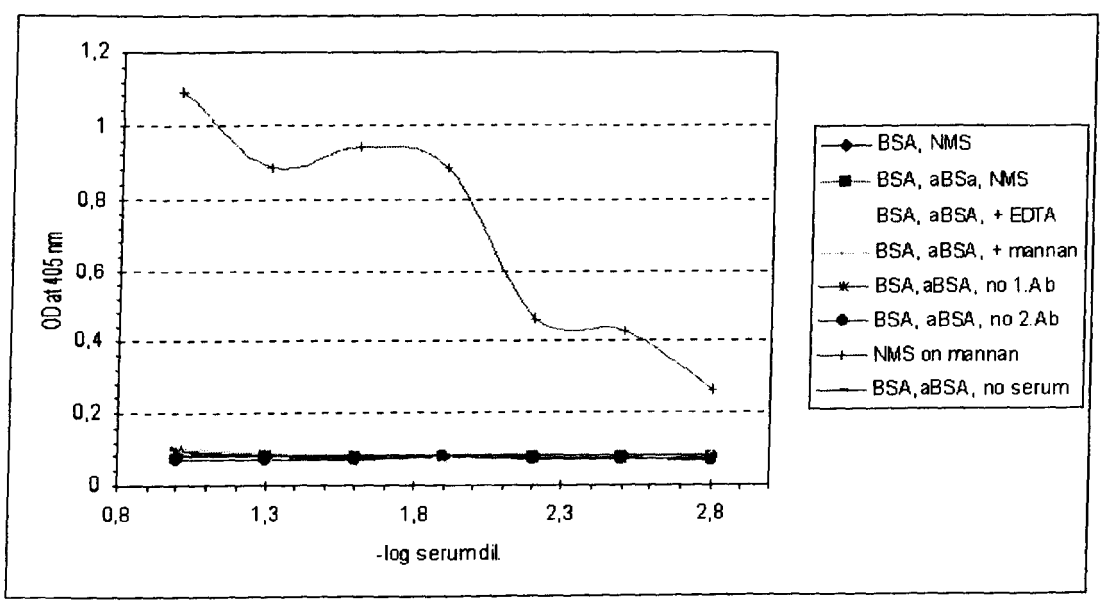

Fig. 4.11: MBL-C binding to solid-phase immune complexes.
The x-axis represents the negative logarithm of the serum dilution. The y-axis represents the optical density at 405 nm.

4.2.4. Anaphylactic shock induced by BSA

4.2.4.1. Characterisation of BSA-induced anaphylactic shock

To test whether MBL-binding to other antigens than OVA also leads to decreased serum MBL levels after antigen challenge, an active and passive model for anaphylactic shock with BSA as antigen was established. Onset of shock, severity, symptoms and decrease in body temperature (Fig. 4.12) were indistinguishable from OVA-induced shock.

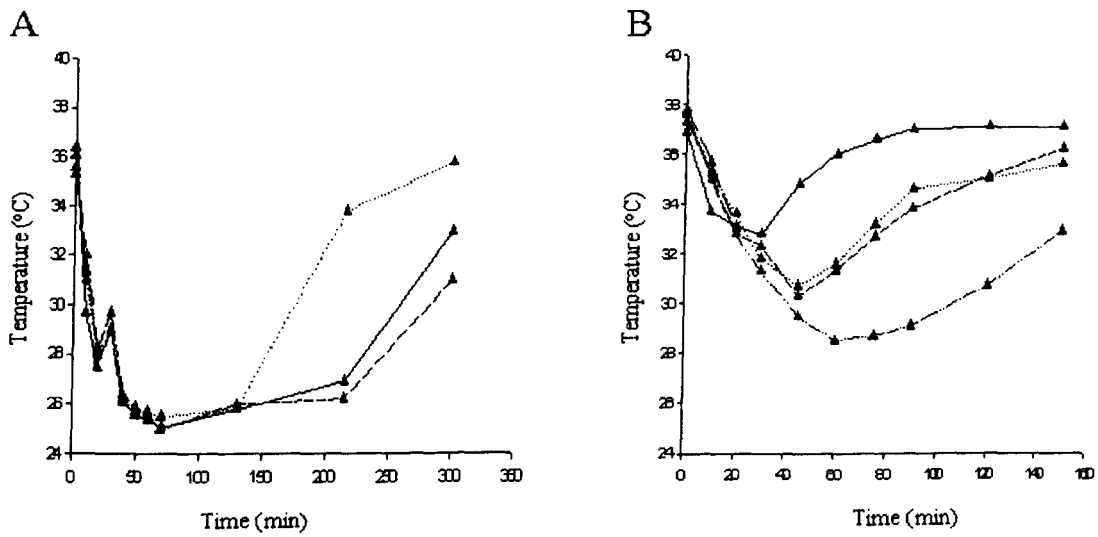

Fig. 4.12: BSA-induced anaphylaxis.

A) NMRI mice (n = 4) were actively immunised with BSA and challenged with 500 µg of antigen on day 21. Rectal temperature was recorded. One mouse died after 20 minutes. B) NMRI mice (n = 5) received 200 µl serum from BSA-sensitised mice and were challenged with 500 µg of antigen 24 hours later. Rectal temperature was recorded. One mouse died after 20 minutes.

4.2.4.2. Lectin pathway activity after BSA-induced anaphylactic shock

Also in BSA-induced shock the capacity of serum samples to cleave C4 decreased after antigen challenge. Whereas untreated serum had an activity of 4.21 ± 2.48 U/ml, 30 minutes after challenge this activity had dropped to 2.23 ± 0.78 U/ml. C4 cleavage capacity was also diminished 2 hours (2.52 ± 1.32 U/ml), 4 hours (2.08 ± 0.87 U/ml) and 8 hours (3.03 ± 1.04 U/ml) after challenge (Fig. 4.13). Due to a high variation of activity in individual serum samples the difference of C4 cleavage capacity of samples after antigen challenge compared to samples from naive mice did not reach statistical significance.

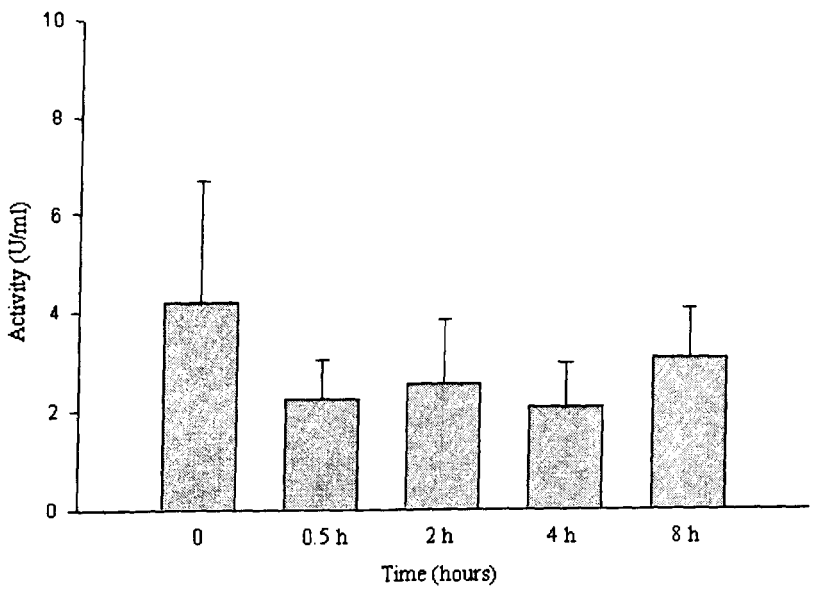

Fig. 4.13: C4 activation after passive anaphylaxis induced by BSA.
Serum samples (n = 5, NMRI) were taken 30 minutes, 2, 4, and 8 hours after antigen challenge and MBL-dependent C4 cleavage activity was compared to samples from untreated control mice (time point 0).

4.2.4.3. Serum MBL levels after BSA-induced anaphylactic shock

Decreased MBL-A and MBL-C serum levels again matched this decreased C4 activation capacity. When compared to untreated mice (20.15 ± 2.37 µg/ml), MBL-A levels were halved 30 minutes after antigen challenge (10.29 ± 2.01 µg/ml, $p < 0.001$) and stayed at this level also at 2 hours (11.98 ± 2.00 µg/ml, $p < 0.001$) and 4 hours (10.80 ± 2,28 µg/ml, $p < 0.001$). After 8 hours a slow recovery could be observed (12.49 ± 2.44 µg/ml, $p = 0.001$) (Fig. 4.14 A).

MBL-C serum levels were also halved at 30 minutes after challenge (50.29 ± 14.24 µg/ml, $p = 0.001$) when compared to untreated mice (105.88 ± 17.08 µg/ml) and stayed low at the 2-hour (70.05 ± 17.48 µg/ml, $p = 0.011$), 4-hour (55.46 ± 23.81 µg/ml, $p = 0.005$) and 8-hour (43.95 ± 35.87 µg/ml, $p = 0.008$) time points (Fig. 4.14 B).

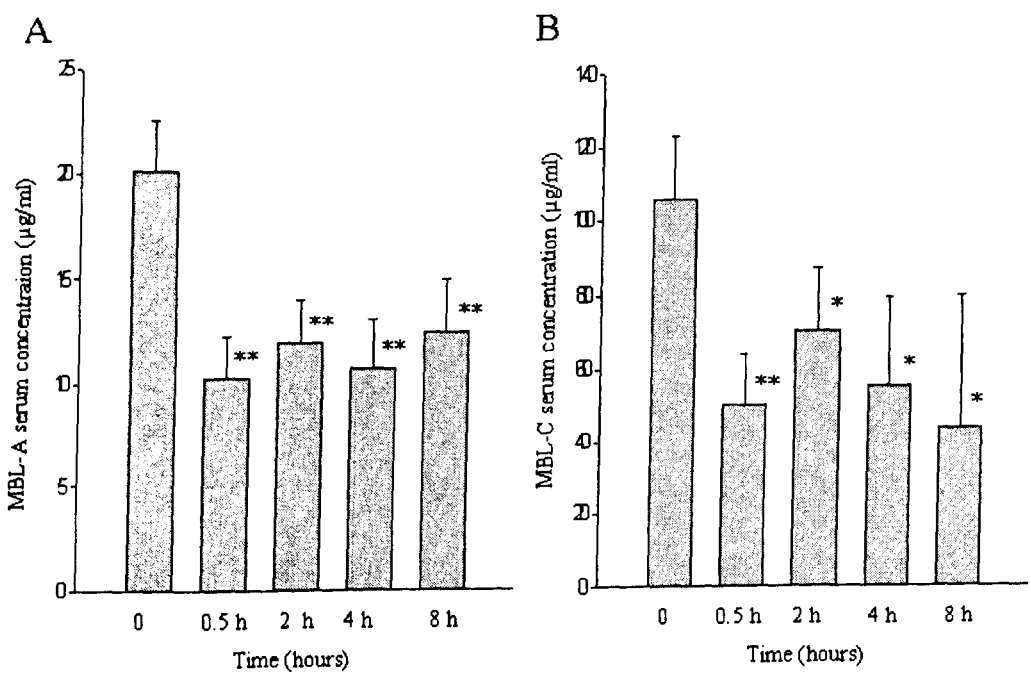

Fig. 4.14: Serum MBL levels after passive anaphylaxis induced by BSA.
Groups of NMRI mice (n = 5) were subjected to passive anaphylaxis and serum MBL levels were determined by TRIFMA. A) Serum MBL-A levels of naïve mice (time point 0) and of samples taken 30 minutes, 2, 4, and 8 hours after antigen challenge. B) Serum MBL-C levels. * $p < 0.05$; ** $p < 0.005$

4.2.4.4. Lectin pathway activity in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice

To test whether the fact that serum MBL levels decrease during anaphylactic shock is restricted to the NMRI mouse strain, *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice were tested as well. Sera from untreated mice showed a C4 cleavage capacity of 1.21 ± 0.25 U/ml in *C1q a*$^{-/-}$ mice and of 2.35 ± 0.26 U/ml in *H2-Bf/C2*$^{-/-}$ mice. This was reduced 30 minutes after antigen challenge, when sera from *C1q a*$^{-/-}$ mice had an activity of 0.74 ± 0.62 U/ml ($p = 0.176$, Fig. 4.15 A) and sera from *H2-Bf/C2*$^{-/-}$ mice an activity of 1.19 ± 0.72 U/ml ($p = 0.009$, Fig. 4.15 B).

Normal serum MBL levels in *C1q a*$^{-/-}$ mice were 19.66 ± 4.07 µg/ml for MBL-A and 13.36 ± 2.15 µg/ml for MBL-C. Both levels dropped slightly, but not significantly, to 11.79 ± 6.90 µg/ml (Fig. 4.16 A) for MBL-A and to 9.41 ± 5.37 µg/ml (Fig. 4.16 B) for MBL-C. In *H2-Bf/C2*$^{-/-}$ mice, this decrease was much more pronounced. Normal values were 25.66 ± 4.99 µg/ml for MBL-A and 22.40 ± 6.01 µg/ml for MBL-C. After antigen challenge both levels dropped to 12.17 ± 5.83 µg/ml for MBL-A ($p = 0.004$, Fig. 4.16 C) and to 10.31 ± 4.83 µg/ml for MBL-C ($p = 0.008$; Fig. 4.16 D).

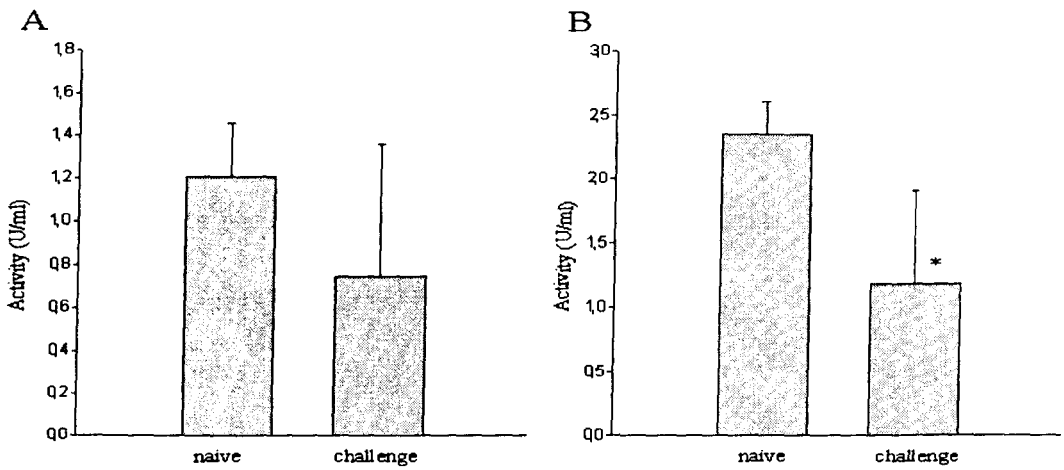

Fig. 4.15: C4 activation after passive anaphylaxis in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice.
Groups of *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice were subjected to passive anaphylactic shock induced by BSA. Serum samples (n = 5) were taken 30 minutes after antigen challenge and MBL-dependent C4 cleavage activity was compared to samples from untreated control mice. A) MBL-dependent C4 cleavage activity of *C1q a*$^{-/-}$ mice. B) MBL-dependent C4 cleavage activity of *H2-Bf/C2*$^{-/-}$ mice. * $p < 0.05$

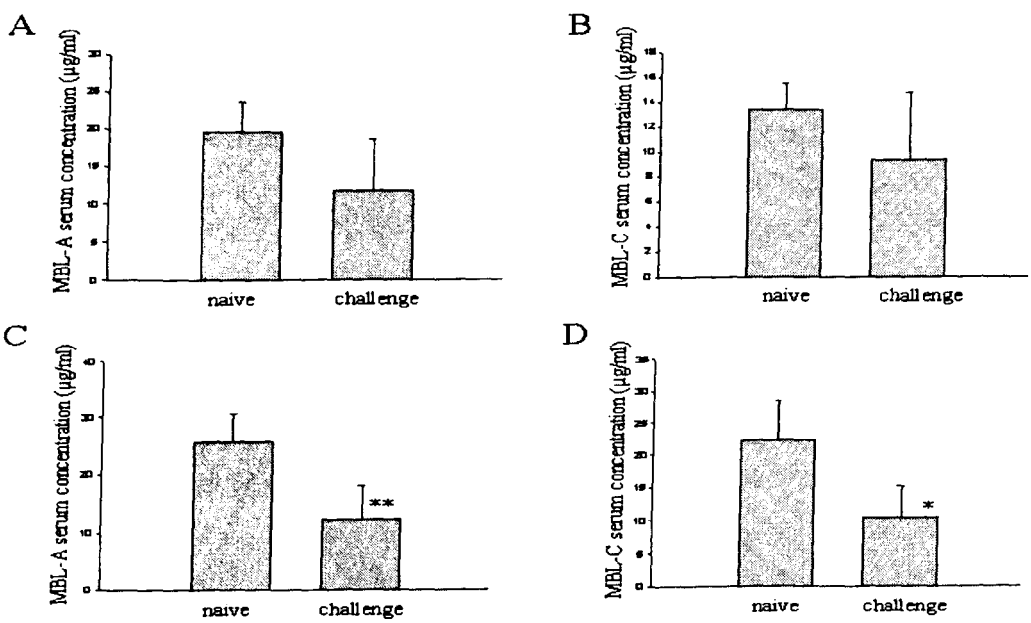

Fig. 4.16: Serum MBL levels after passive anaphylaxis in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice.
Groups of *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice were subjected to passive anaphylactic shock induced by BSA. Sera (n = 5) were taken from naïve mice and 30 minutes after antigen challenge. Serum MBL levels were determined by TRIFMA. A) Serum MBL-A levels of *C1q a*$^{-/-}$ mice. B) Serum MBL-C levels of *C1q a*$^{-/-}$ mice. C) Serum MBL-A levels of *H2-Bf/C2*$^{-/-}$ mice. D) Serum MBL-C levels of *H2-Bf/C2*$^{-/-}$ mice. * $p < 0.05$; ** $p < 0.005$

4.2.5. mRNA expression after anaphylactic shock

To establish whether the decreased MBL serum levels were due to consumption or decreased protein expression, RNA was isolated from livers of mice, transcribed into cDNA and PCR for MBL-A and MBL-C was performed. When the resulting bands were normalised to β-actin, no difference in MBL expression could be observed after antigen challenge when compared to naïve mice (Fig. 4.17).

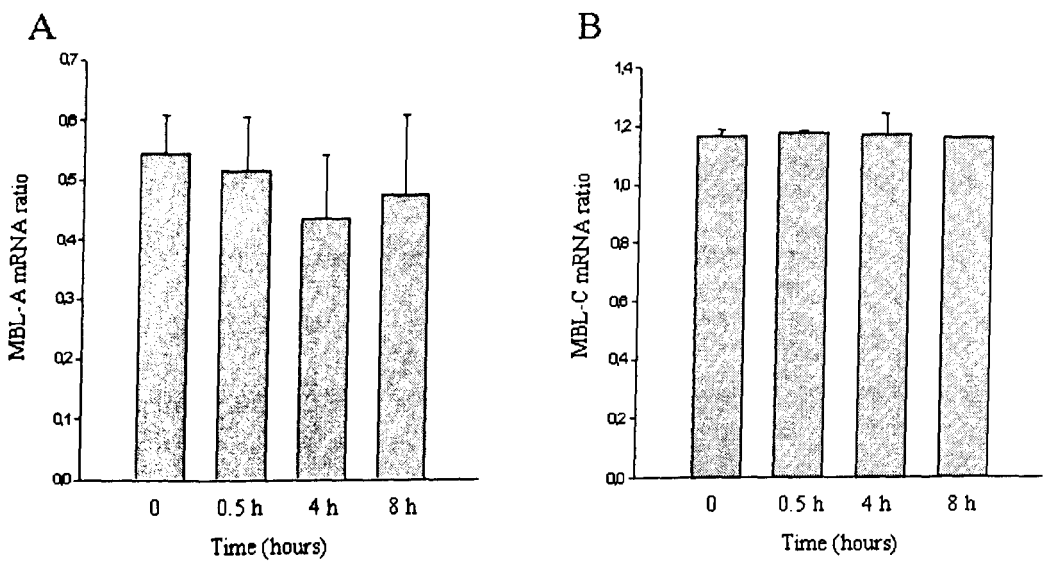

Fig. 4.17: mRNA ratio after anaphylaxis.
Mice were subjected to passive BSA-induced anaphylactic shock and sacrificed 30 minutes, 4 hours, and 8 hours after challenge. RNA was isolated from livers (n = 3), transcribed into cDNA and PCR for MBL-A and MBL-C was performed. Bands were quantified using ImageQuant Software (Molecular Dynamics/GE Healthcare, Freiburg, Germany) and normalised to β-actin. A) MBL-A mRNA ratios for naive mice (time point 0) and at 30 minutes, 4 hours, and 8 hours after challenge. B) MBL-C mRNA ratios for naive mice (time point 0) and at 30 minutes, 4 hours, and 8 hours after challenge.

4.2.6. Immunhistochemistry

Since it has been shown that oxidative stress in ischemia-reperfusion injury leads to the formation of a complement-activating MBL-ligand on human endothelial cells (Collard *et al.*, 2000), it was tested whether this might also be the case in anaphylactic shock. Mice were sacrificed 30 minutes after antigen challenge and cryostat sections of heart, liver, kidney, lung, spleen, thymus, and small intestine were stained for MBL-A, MBL-C, and C3. Previously reported extrahepatical expression of MBL (Uemura *et al.*, 2002; Wagner *et al.*, 2003) and C3 (Ueki *et al.*, 1987; Strunk *et al.*, 1988; Andoh *et al.*, 1993; Thurman *et al.*, 2003) could be confirmed. Negative controls (without primary antibody or with an irrelevant rat IgG_{2a} as isotype control) showed no reactivity (data not shown). However, none of the tissues taken 30 minutes after antigen challenge showed a staining pattern that was different from naïve mouse tissue. As examples, sections from liver (Fig. 4.18), heart (Fig. 4.19) and kidney (Fig. 4.20) are depicted.

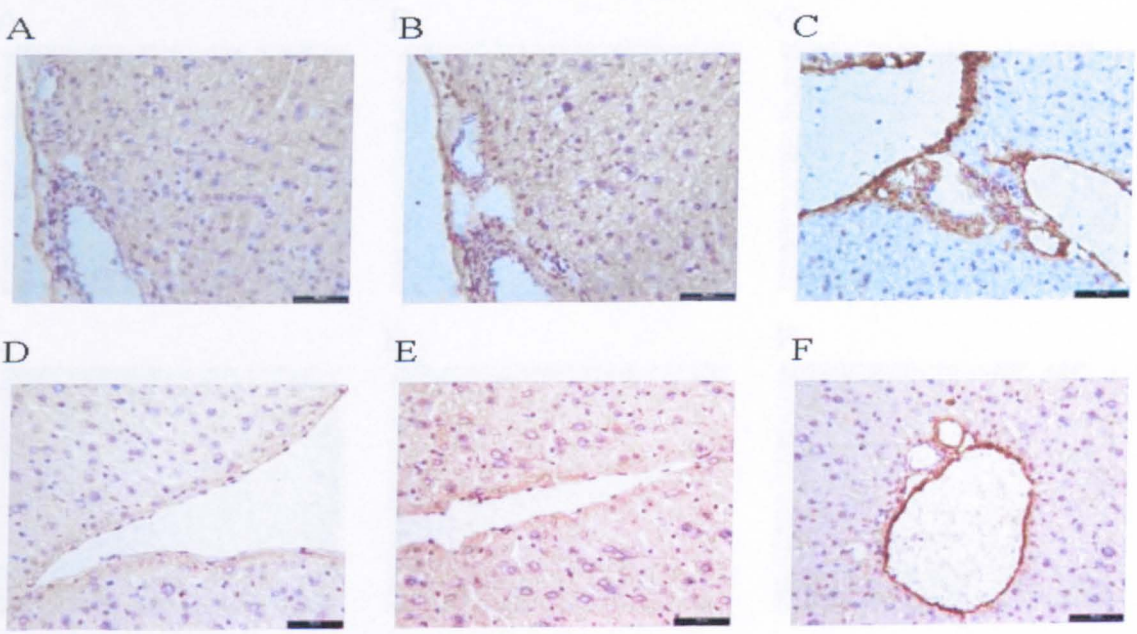

Fig. 4.18: Immunhistochemical staining of liver.
Cryostat sections of liver taken 30 minutes after antigen challenge (bottom row) or from an untreated control mouse (top row) were stained for MBL-A (A and D), MBL-C (B and E) and C3 (C and F). *Original magnification x160, the bars correspond to 100 µm. Hepatocytes stain for MBL-A, MBL-C and* C3. C3-staining can also be seen on endothelial cells.

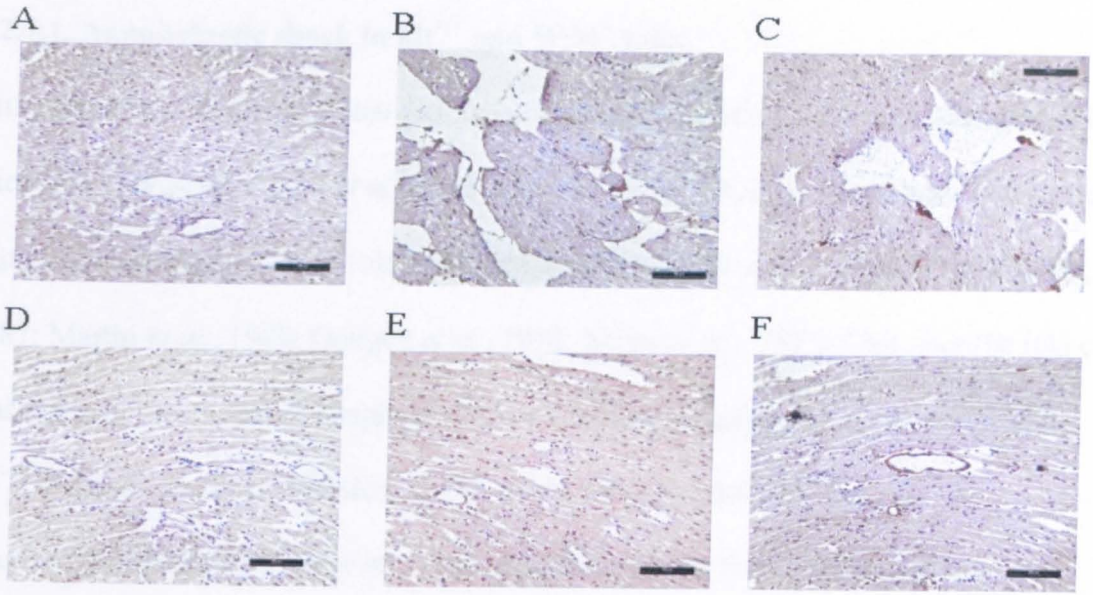

Fig. 4.19: Immunhistochemical staining of heart.
Cryostat sections of heart taken 30 minutes after antigen challenge (bottom row) or from an untreated control mouse (top row) were stained for MBL-A (A and D), MBL-C (B and E) and C3 (C and F). Original magnification x160, the bars correspond to 100 μm. C3-staining can be observed on endothelial cells.

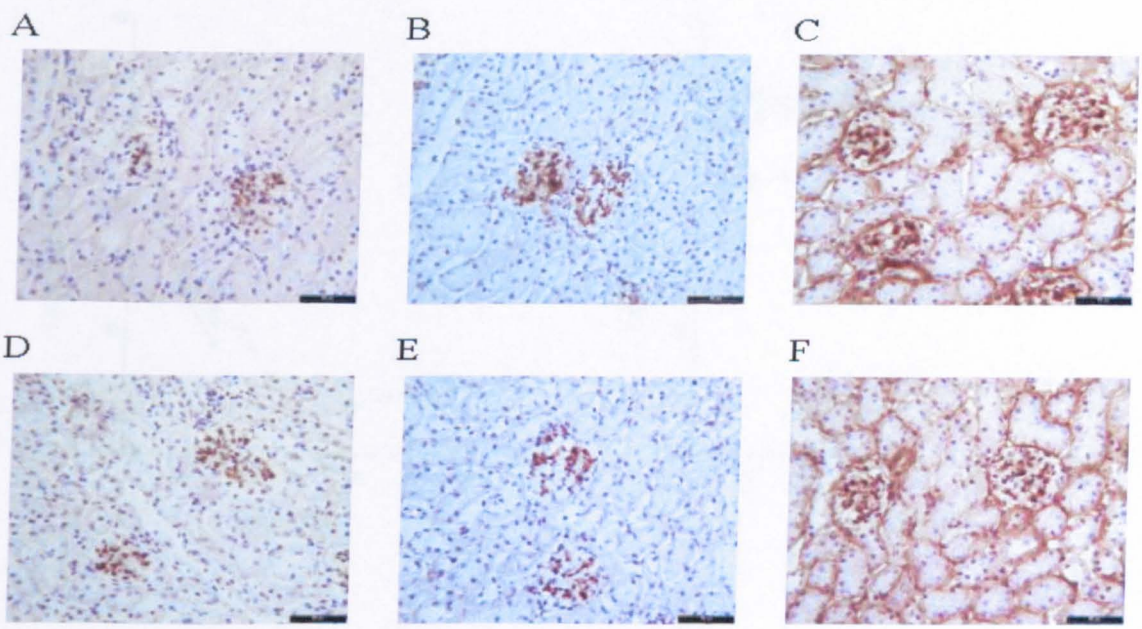

Fig. 4.20: Immunhistochemical staining of kidney.
Cryostat sections of kidney taken 30 minutes after antigen challenge (bottom row) or from an untreated control mouse (top row) were stained for MBL-A (A and D), MBL-C (B and E) and C3 (C and F). Original magnification x160, the bars correspond to 100 μm. Mesangial cells of the glomerula stain for MBL-A and MBL-C, C3 staining can be seen in mesangial cells, along the Bowman's capsule and along the tubular basement membrane.

4.2.7. Role of the mast cell

4.2.7.1. Anaphylactic shock in $kit^{+/+}$ and W/W^v mice

Although it has been shown that anaphylaxis can also be induced in mast cell-deficient mice (Ha *et al.*, 1986; Choi *et al.*, 1998; Okunuki *et al.*, 2000), it is still widely believed that mast cells play a central role in triggering anaphylactic shock (Serafin and Austen, 1987; Martin *et al.*, 1989; Oettgen *et al.*, 1994; Nagai *et al.*, 2000). Therefore the role of mast cells in this model of anaphylactic shock was investigated. Mast cell-sufficient $kit^{+/+}$ and their mast cell-deficient W/W^v littermates were subjected to passive anaphylaxis. While $kit^{+/+}$ mice showed a rapid decrease in body temperature as expected, W/W^v mice were completely protected (Fig. 4.21).

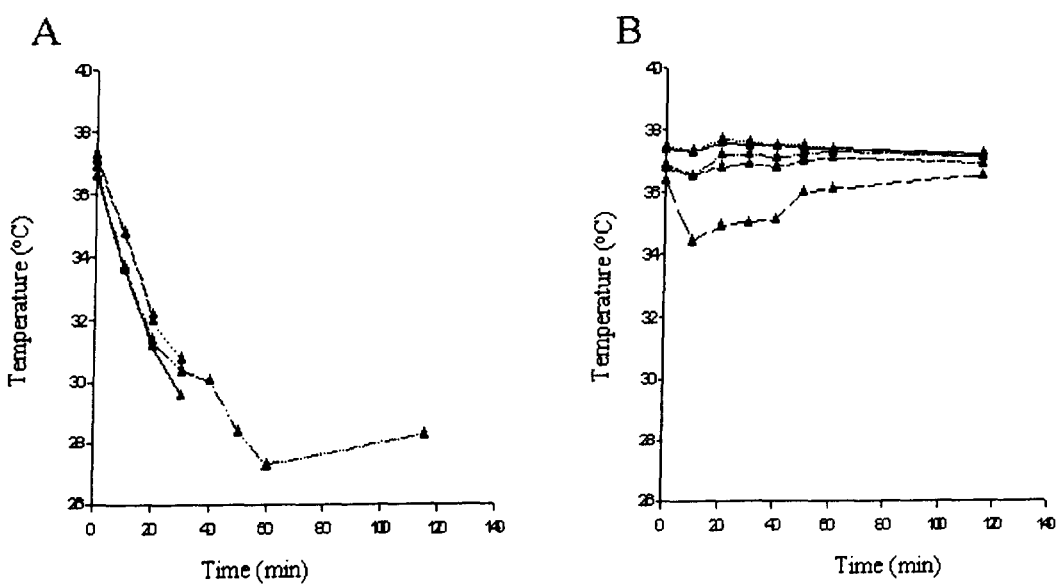

Fig. 4.21: Passive anaphylaxis in $kit^{+/+}$ and W/W^v mice.

Groups of mice were subjected to passive OVA-induced anaphylaxis and rectal temperature was measured. A) Passive anaphylaxis in $kit^{+/+}$ mice (n = 4). One mouse died after 20 minutes, another two died after 30 minutes. B) Passive anaphylaxis in W/W^v mice (n = 5).

4.2.7.2. Lectin pathway activity in kit$^{+/+}$ and W/W^{v} mice

Serum samples of W/W^{v} mice showed no decrease in C4 activation capacity (Fig.3.28 B). Serum samples of kit$^{+/+}$ mice, on the other hand, displayed the expected decrease in C4 activation capacity after anaphylactic shock. 30 minutes after challenge activity was significantly diminished (1.99 $\pm$ 0.36 U/ml, $p < 0.001$) when compared to samples from untreated mice (4.22 $\pm$ 0.66 U/ml) (Fig. 4.22 A).

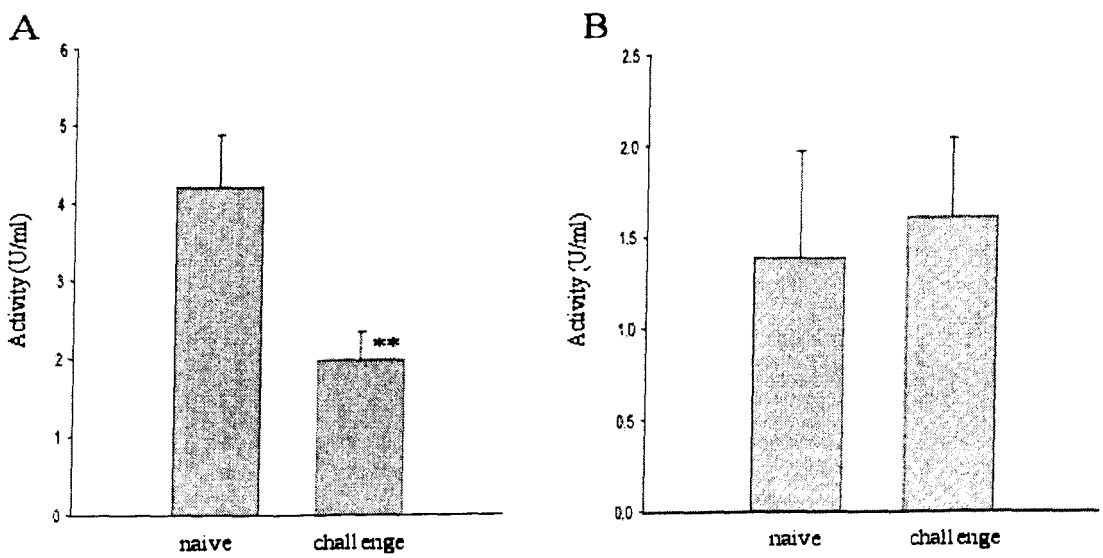

Fig. 4.22: C4 activation after passive anaphylaxis in kit$^{+/+}$ and W/W^{v} mice.
Mice were subjected to passive BSA-induced anaphylaxis; blood was taken 30 minutes after challenge and compared to samples from untreated control mice. A) C4 activation capacity of naive (n = 4) and challenged (n = 5) kit$^{+/+}$ mice. **$p < 0.005$ B) C4 activation capacity of naive (n = 5) and challenged (n = 5) W/W^{v} mice.

Normal serum levels in $kit^{+/+}$ were 24.10 ± 4.87 µg/ml for MBL-A and 88.40 ± 18.39 µg/ml for MBL-C. These were also markedly reduced 30 minutes after challenge to 13.89 ± 4.09 µg/ml ($p = 0.011$; Fig. 4.23 A) for MBL-A and to 55.10 ± 5.23 µg/ml ($p = 0.006$; Fig. 4.24 A) for MBL-C, whereas W/W^v mice had undiminished MBL-A (Fig. 4.23 B) and MBL-C (Fig. 4.24 B) serum levels.

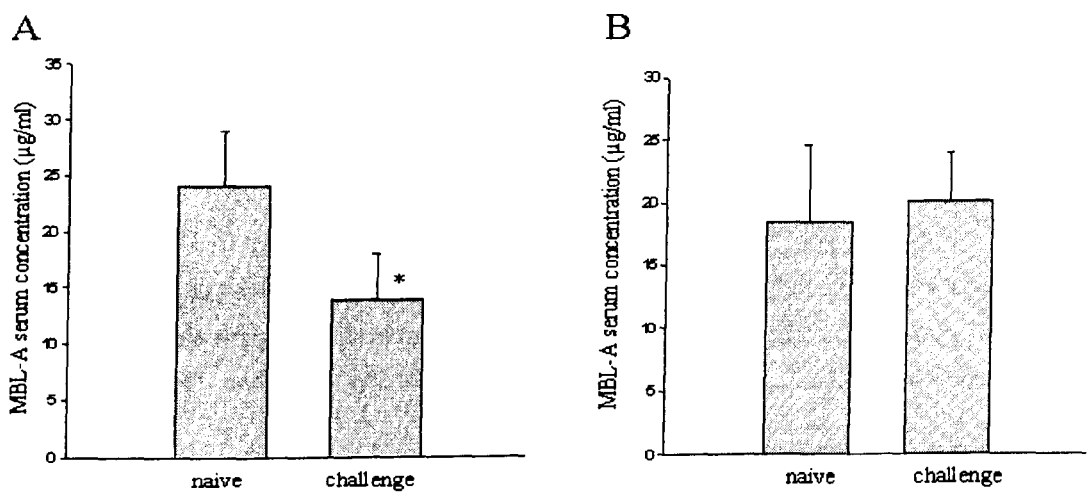

Fig. 4.23: Serum MBL-A levels after passive anaphylaxis in $kit^{+/+}$ and W/W^v mice.
Mice were subjected to passive BSA-induced anaphylaxis and blood was taken 30 minutes after challenge. MBL levels were determined by TRIFMA. A) Serum MBL-A levels of naive (n = 4) and challenged (n = 5) $kit^{+/+}$ mice. *$p < 0.05$ B) Serum MBL-A levels of naive (n = 5) and challenged (n = 5) W/W^v mice.

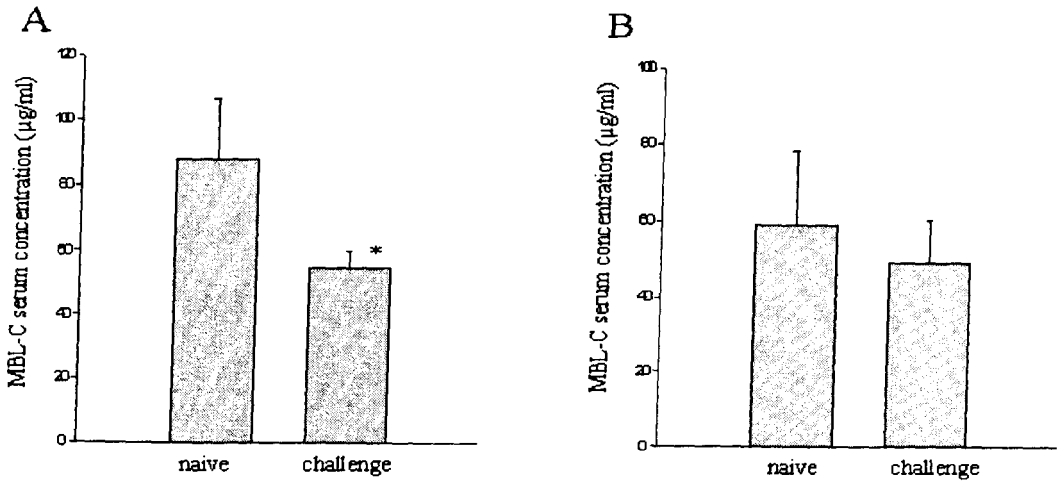

Fig. 4.24: Serum MBL-C levels after passive anaphylaxis in $kit^{+/+}$ and W/W^{v} mice.
Mice were subjected to passive BSA-induced anaphylaxis and blood was taken 30 minutes after challenge. MBL levels were determined by TRIFMA. A) Serum MBL-C levels of naive (n = 4) and challenged (n = 5) $kit^{+/+}$ mice. *$p < 0.05$ B) Serum MBL-C levels of naive (n = 5) and challenged (n = 5) W/W^{v} mice.

4.2.7.3. MBL-binding to proteoglycans

Mast cell degranulation evidently is necessary for the development of anaphylactic symptoms in the model used in this work, and also for the decrease in serum MBL levels. Therefore a closer look at the granule contents was justified. Since the mast cell proteoglycans heparin and chondroitin sulfate contain glycosaminoglycan side chains, their potential role as MBL ligands was investigated. Decreasing amounts of either heparin or chondroitin sulfate were added to mouse serum (diluted 1:400 in MBL-binding-buffer) and incubated for 45 minutes at room temperature prior to adding the mixture to mannan-coated ELISA plates. After allowing the remaining free MBL-MASP-complexes to bind to the plate, C4 activation was measured. In addition, bound

MBL was detected with monoclonal rat anti-mouse MBL-A and MBL-C antibodies. Serial dilutions of mouse serum served as positive control. For negative controls serum was omitted. Preincubation with heparin had no inhibitory effect. Chondroitin sulfate, on the other hand, distinctly inhibited C4 cleavage capacity of samples (Fig. 4.25), as well as MBL-A and MBL-C binding. At a concentration of 100 µg/ml the signal for MBL-A was decreased by 50% (Fig. 4.26), whereas 800 µg/ml were needed for inhibiting 50% of the MBL-C signal (Fig. 4.27).

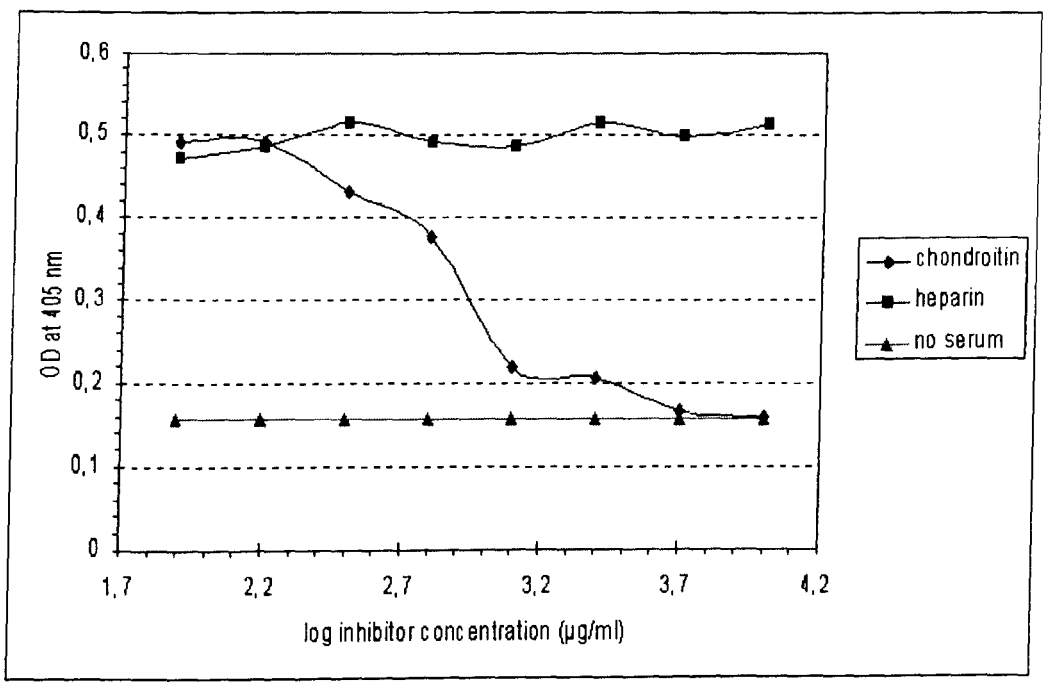

Fig. 4.25: Inhibition of C4 cleavage.
Normal mouse serum was incubated with increasing concentrations of either heparin or chondroitin sulfate (concentrations ranging from 78 µg/ml to 10 mg/ml) prior to performing a C4 cleavage assay as described in Materials and Methods. The x-axis represents the logarithm of the heparin/chondroitin sulfate concentration. The y-axis represents the optical density at 405 nm.

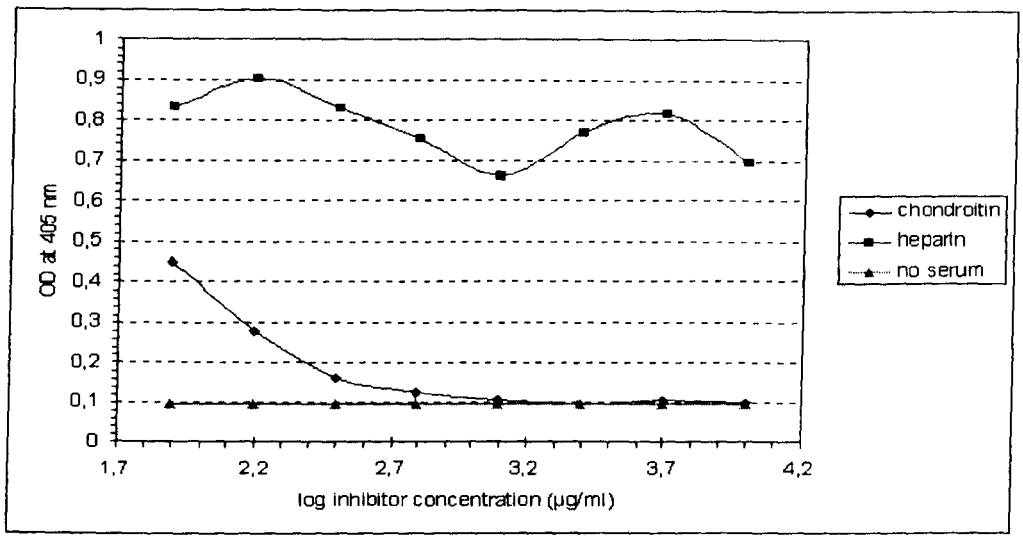

Fig. 4.26: Inhibition of MBL-A-binding.

Normal mouse serum was incubated with increasing concentrations of either heparin or chondroitin sulfate (concentrations ranging from 78 µg/ml to 10 mg/ml) prior to adding it to a mannan-coated plate. Bound MBL-A was detected. The x-axis represents the logarithm of the heparin/chondroitin sulfate concentration. The y-axis represents the optical density at 405 nm.

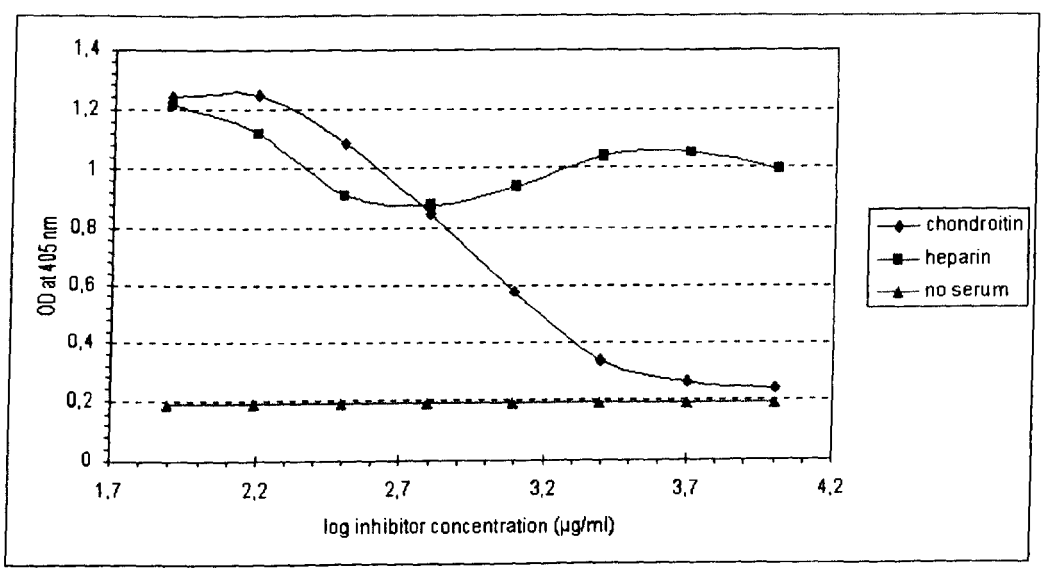

Fig. 4.27: Inhibition of MBL-C binding.

Normal mouse serum was incubated with increasing concentrations of either heparin or chondroitin sulfate (concentrations ranging from 78 µg/ml to 10 mg/ml) prior to adding it to a mannan-coated plate. Bound MBL-C was detected. The x-axis represents the logarithm of the heparin/chondroitin sulfate concentration. The y-axis represents the optical density at 405 nm.

4.2.8. Role of complement activation in anaphylactic shock

To study the pathophysiological consequences of selective complement deficiency, several available complement-deficient mouse strains were used. *C1q a*$^{-/-}$ mice are deficient in the antibody-mediated classical pathway of complement activation (Botto *et al.*, 1998), while the lectin and alternative pathway are functionally active. *H2-Bf/C2*$^{-/-}$ mice lack all three complement activation routes (Celik *et al.*, 2001). Both mouse strains showed the same decrease in body temperature as the background controls (Fig. 4.28). Mice with a double deficiency for MBL-A and MBL-C (MBL$^{-/-}$) lack lectin pathway activation via MBL but not via ficolins, while the classical and alternative pathway are fully functional (Shi *et al.*, 2004). Also these mice were not protected from anaphylactic shock (Fig. 4.29). Mice made deficient in the C5a receptor (Hopken *et al.*, 1996) (C5aR$^{-/-}$; Fig. 4.30 A) and naturally C5-deficient mice (van den Berg *et al.*, 1991) (DBA/2; Fig. 4.30 B) displayed a rapid decrease in body temperature as well.

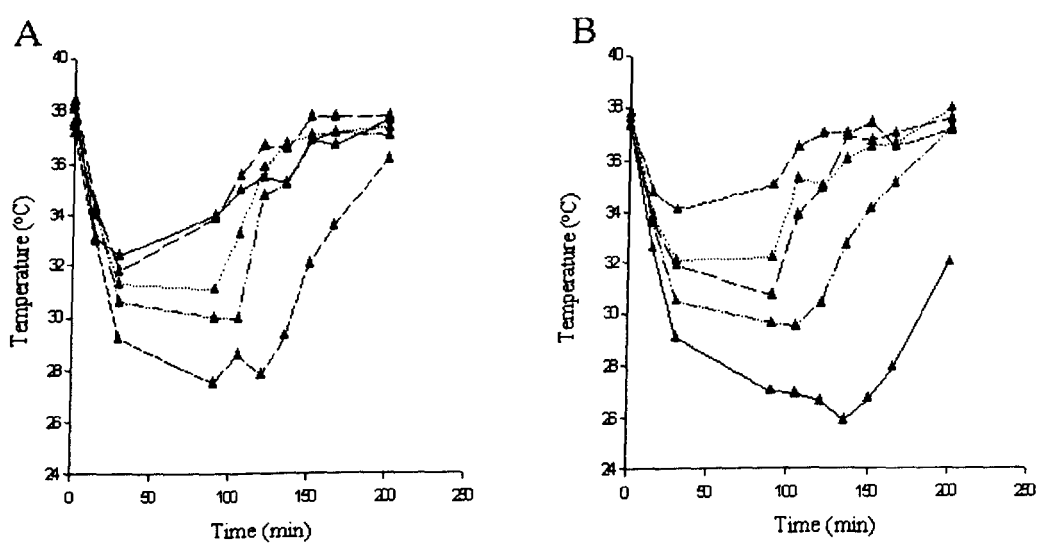

Fig. 4.28: passive anaphylaxis in *C1q a*$^{-/-}$ and *H2-Bf/C2*$^{-/-}$ mice.
Groups of five *C1q a*$^{-/-}$ (A) and *H2-Bf/C2*$^{-/-}$ (B) mice were subjected to passive OVA-induced anaphylaxis and rectal temperature was recorded.

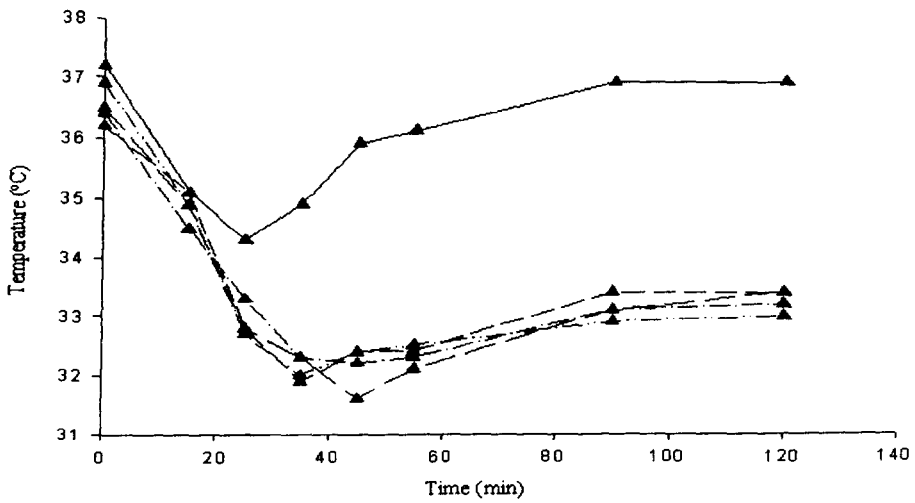

Fig. 4.29: passive anaphylaxis in $MBL^{-/-}$ mice.
Mice with a double deficiency for MBL-A and MBL-C (n = 5) were subjected to passive BSA-induced anaphylaxis and rectal temperature was recorded.

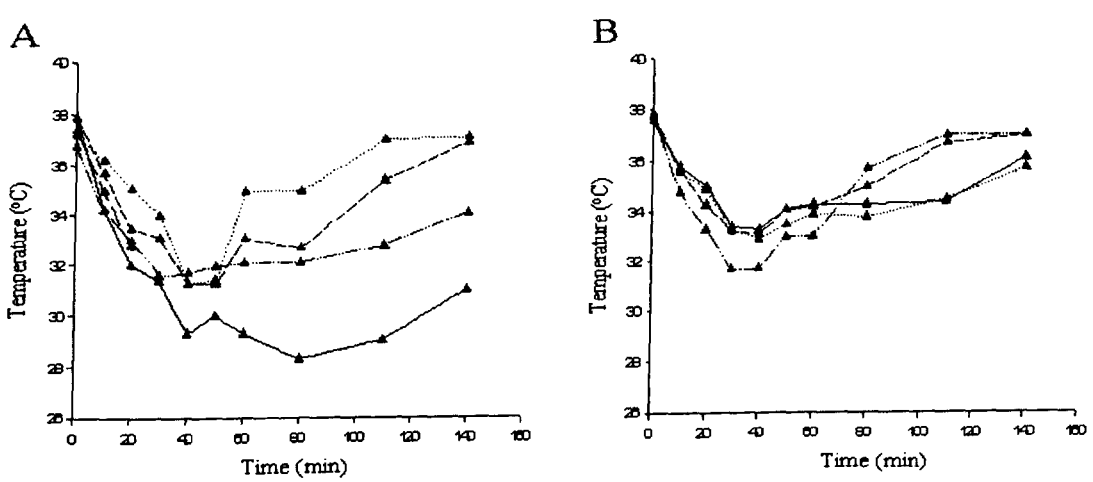

Fig. 4.30: Passive anaphylaxis in $C5aR^{-/-}$ and DBA/2 mice.
A) $C5aR^{-/-}$ mice (n = 5) were subjected to passive BSA-induced anaphylaxis and rectal temperature was recorded. One mouse died after 20 minutes. B) Body temperature of DBA/2 mice (n = 4) after passive BSA-induced anaphylaxis.

4.3. Discussion

The term anaphylaxis describes a rapid, generalised, and often unanticipated immunologically mediated reaction that occurs after exposure to foreign substances in previously sensitised individuals. Some of the most common causative agents are foods (e.g. peanuts), drugs (e.g. β-Lactam antibiotics), and venoms (e.g. insect stings) (Kemp and Lockey, 2002). Reexposure of sensitised persons to the antigen results in a sudden release of mast cell- and basophil-derived mediators into the circulation, leading to hypotension, bronchospasm, angioedema, urticaria and pruritus (Kemp and Lockey, 2002). The classic anaphylactic reaction is mediated by allergen binding to IgE on mast cells (Serafin and Austen, 1987). However, other mechanisms are involved as well, since anaphylaxis can also be induced in IgE-deficient mice (Oettgen *et al.*, 1994).

In mice anaphylaxis can be elicitated either via active immunisation and subsequent antigen challenge or passively through transfer of immune sera followed by antigen challenge. Both models lead to the same pathophysiological changes, but the passive model has the advantage that effects of complement deficiency on antibody production are eliminated.

During anaphylactic shock the complement system is activated (Tannenbaum *et al.*, 1975; Smith *et al.*, 1980; del Balzo *et al.*, 1988; van der Linden *et al.*, 1990). It is believed that this complement activation occurs via the classical pathway, as serum C1q levels are diminished after antigen challenge (Tannenbaum *et al.*, 1975; van der Linden *et al.*, 1990). We observed that also the lectin pathway of complement activation is involved in anaphylactic shock, as sera taken after antigen challenge from NMRI mice (Figs. 4.5, 4.6, 4.13 and 4.14) and from *C1q* $a^{-/-}$ (Figs. 4.15 A as well as 4.16 A and B) and *H2-Bf/C2*$^{-/-}$ mice (Figs. 4.15 B as well as 4.16 C and D) showed a decreased capacity to cleave C4 and diminished MBL levels, suggesting lectin pathway activation or, at least, MBL consumption during anaphylactic shock.

It has been shown for C1q that increased vasopermeability and loss of proteins into the interstitial space do not explain the decreased serum levels (Tannenbaum *et al.*, 1975; van der Linden *et al.*, 1990). This is also true for MBL, as total serum protein content after anaphylactic shock was unchanged and total IgG content of sera decreased only marginally.

The suggestion that MBL may have lost its ability to bind to carbohydrates could be ruled out by confirming the results obtained in the TRIFMA with a Sandwich-ELISA (Fig. 4.7). This ELISA is not dependent on the ability of MBL to bind to mannan-coated wells, as it is trapped between two monoclonal antibodies, both recognising different epitopes on the collagenous stalks. Also in this test serum MBL levels were decreased after anaphylactic shock when compared to samples from untreated controls.

The decrease in serum MBL levels is noticeable within minutes after antigen challenge and therefore unlikely to be due to decreased protein expression. To confirm this, mRNA was isolated from livers at several time points after challenge, transcribed into cDNA and PCR was performed for MBL-A, MBL-C and β-actin as housekeeper. Like after CLP (Windbichler *et al.*, 2004), no change in mRNA expression could be observed (Fig. 4.17), indicating that the decreased serum levels are due to MBL consumption.

To investigate the possibility of MBL binding to antigen or immune complexes, ELISA-plates were coated with OVA, a glycoprotein with one N-linked high mannose group. Both MBL-A and MBL-C were found to bind to solid phase OVA via their carbohydrate recognition domains, since this binding was Ca^{++}-dependent and could be inhibited by adding the known MBL-ligand mannan (Fig. 4.8. and 4.9). Solid-phase BSA, which is not glycosylated, showed no MBL-binding under the same conditions (diamonds in Fig. 4.10. and 4.11. and data not shown). Previous reports have suggested that MBL is able to bind to IgA (Roos *et al.*, 2001) and to IgG glycoforms produced in rheumatoid arthritis patients (Malhotra *et al.*, 1995). To see whether MBL binds to the

immunoglobulin-constituent of the immune complexes, anti-BSA antibodies were affinity-purified from immune sera and immobilised on BSA-coated ELISA-plates to form solid-phase immune complexes. Also under these conditions no MBL-binding could be observed (Fig. 4.10. and 4.11.). This finding is in accordance with a recent report showing that although oligomannose structures are present on human serum IgD and IgE, they are not accessible for MBL-binding (Arnold *et al.*, 2004). However, the binding of MBL to the protein antigen OVA does not explain the decreased serum MBL levels after anaphylactic shock, as this phenomenon could also be observed after BSA-induced anaphylaxis (Fig. 4.14).

It has been established that complement activation via the classical pathway (Collard *et al.*, 1997) or the alternative pathway (Stahl *et al.*, 2003; Thurman *et al.*, 2003) contributes to tissue damage after ischemia-reperfusion injury (Yasuda *et al.*, 1990; Collard *et al.*, 1999). Also the lectin pathway is involved, since MBL has been shown to bind to hypoxic and reoxygenated HUVECs (Collard *et al.*, 1999) as well as to heart (Collard *et al.*, 2000) and kidney (de Vries *et al.*, 2004) in an ischemia-reperfusion model. Administration of an anti-MBL-A antibody reduced postischemic myocardial reperfusion injury (Jordan *et al.*, 2001). Since endothelial cells are also severely affected in anaphylactic shock, this might also lead to the exposure of MBL ligands. Immunhistochemical staining of cryostat sections from heart, liver, spleen, kidney, lung, muscle, thymus and small intestine confirmed previously reported extrahepatical expression of MBL with staining of endothelial cells in the small intestine for MBL-C (Uemura *et al.*, 2002) and glomerular cells of the kidney for MBL-A and MBL-C (Wagner *et al.*, 2003; de Vries *et al.*, 2004). C3 staining could be observed on endothelial cells (Ueki *et al.*, 1987), epithelial cells of the lung (Strunk *et al.*, 1988) and intestine (Andoh *et al.*, 1993) as well as along the tubular basement membrane, the Bowman's capsule and on mesangial cells in the kidney (Thurman *et al.*, 2003).

Negative controls (without primary antibody or with an irrelevant rat IgG_{2a} as isotype control) showed no reactivity. However, the staining pattern of tissues taken 30 minutes after antigen challenge did not differ from untreated controls (Figs. 4.18 to 4.20). This might either be because no neoepitopes are exposed or because the lesions are too spread out to be detected by immunhistochemical methods. Unlike in other species, where characteristic tissue lesions can be observed (Tizard, 2000b), mice which died from anaphylactic shock show no histological changes (Lei *et al.*, 1996), since the cause of death is a generalised hypotension resulting in cardiac failure (Munoz and Bergman, 1965).

Although it has been shown that anaphylaxis can also be induced in mast cell-deficient mice (Ha *et al.*, 1986; Choi *et al.*, 1998; Okunuki *et al.*, 2000), it is still widely believed that mast cells play a central role in triggering anaphylactic shock (Serafin and Austen, 1987; Martin *et al.*, 1989; Oettgen *et al.*, 1994; Nagai *et al.*, 2000). In patients the severity of symptoms shows a positive correlation with serum histamine levels (Tannenbaum *et al.*, 1975; Smith *et al.*, 1980) and treatment with a histamine antagonist was protective in a mouse model (Lei *et al.*, 1996). Also the mouse model used in this study clearly is mast cell dependent, as W/W^v mice were completely protected (Fig. 4.21). A possible explanation for this might be that immunisation with protein and alum as adjuvant as done in this work leads to high IgE levels in serum (Grunewald et al., 1998). Antigen challenge would then lead to crosslinking of IgE, a process that is critical for mast cell activation (Martin et al., 1989; Strait et al., 2002).

Activated mast cells release a variety of mediators considered important in anaphylactic reactions, e.g. histamine, proteases and PAF. Histamine causes smooth muscle contraction and increases vascular permeability; PAF contributes to platelet activation, airway constriction, hypotension, and vascular permeation.

Mast cell granules also contain proteoglycans. Proteoglycans are macromolecules composed of glycosaminoglycan chains bound to a protein core. The glycosaminoglycan component consists of an alternating sequence of hexosamines (D-glucosamine or D-galactosamine), sulphated at various positions, and a hexuronic acid (D-glucuronic acid or L-iduronic acid) (Ruoslahti, 1988). Two types of proteoglycans occur in the secretory granules of mast cells and basophils. Rat mucosal mast cells (Enerback *et al.*, 1985), mouse bone marrow derived mast cells (Razin *et al.*, 1982) and human basophils (Metcalfe *et al.*, 1984) mainly contain chondroitin sulfate, which has galactosaminoglycan side chains, whereas the major proteoglycan produced by human skin mastocytoma cells (Metcalfe *et al.*, 1980), mouse tissue mast cells (Bland *et al.*, 1982) and rat peritoneal mast cells (Yurt *et al.*, 1977) is heparin, which contains *N*-acetyl-D-glucosamine.

The presence of glycosaminoglycan side chains, along with the fact that mast cell-deficient W/W^v mice showed no anaphylactic symptoms (Fig. 4.21) and no decrease in serum MBL levels (Figs. 4.23 and 4.24) hinted at a potential role of proteoglycans as MBL ligands.

Heparin has been shown to bind a number of other complement components (Almeda *et al.*, 1983; Sahu and Pangburn, 1993) and inhibits complement activation via the classical (Almeda *et al.*, 1983) and the alternative pathway (Kazatchkine *et al.*, 1981) both in vitro (Dubreuil *et al.*, 2004) and in vivo (Weiler *et al.*, 1992). Also MASP-2 activity is inhibited by heparin (Presanis *et al.*, 2004). Interaction of the soybean lectin Concanavalin A (Con A) with heparin has also been documented (Monge *et al.*, 1989). Despite this, serum samples incubated with heparin prior to adding them to mannan-coated ELISA-plates showed no decrease in C4-activation or MBL-binding (Figs. 4.25 to 4.27), possibly because the conditions used in this assay did not favour an interaction of heparin with MBL, since the strongest binding of heparin to Con A was observed at

pH 4.6, whereas the presence of 100 mM NaCl inhibited this interaction (Monge *et al.*, 1989). Chondroitin sulfate, on the other hand, showed a distinct inhibitory effect under the conditions used above. At a concentration of 100 μg/ml the signal for MBL-A was decreased by 50% (Fig. 4.26), whereas 800 μg/ml were needed for inhibiting 50% of the MBL-C signal (Fig. 4.27). Whether this corresponds to a concentration that can be reached in vivo is difficult to say, as there is no information to be found in the literature regarding the number of mast cells that contain chondroitin sulfate and the amount of chondroitin sulfate that they contain in normal mice. It was estimated that human basophils, isolated from patients with chronic myelogenous leukemia, contain approximately 5.1 to 12.0 μg of chondroitin sulfate per 10^6 cells, whereas eosinophils contain 0.03 μg chondroitin sulfate per 10^6 cells (Metcalfe *et al.*, 1984). Human skin mast cells isolated from a patient with mastocytosis were found to contain approximately 1.2 to 4.5 μg heparin per 10^6 cells (Metcalfe *et al.*, 1980).

Mice have roughly $2.4x10^5$ eosinophils and $8x10^4$ basophils per ml blood. Assuming that these cells contain as much chondroitin sulfate as human leukemic cells, they could release roughly 1 μg/ml chondroitin sulfate. To generate the amount needed to halve the MBL concentration, a further $2x10^8$ mast cells would be needed, again assuming that a normal mouse mast cell contains as much chondroitin sulfate as a human mast cell from a patient with mastocytosis contains heparin. Because of the reasons outlined above, it is not possible to say whether the interaction of MBL with chondroitin sulfate explains the decrease in MBL serum levels, especially as concentrations and binding conditions might be different in the area surrounding a degranulated mast cell.

It was attempted to further characterise the mechanisms of the MBL/chondroitin sulfate interaction and to determine the optimal binding conditions with ELISA methods, but the attempt to coat plates directly with chondroitin sulfate gave no satisfactory results;

therefore it is not possible to say whether this binding is mediated via the carbohydrate recognition domain. However, the fact that the main sugar component in chondroitin sulfate is *N*-acetyl-D-galactosamine, which is not an MBL ligand (Holmskov *et al.*, 2003), suggests that this interaction is of an ionic nature, as has been proposed for the Con A-heparin interaction (Monge *et al.*, 1989). Chondroitin sulfate interaction with other proteins has been less well studied. However, it inhibits binding of heparin to C1q (Almeda *et al.*, 1983). Like heparin, it serves to package the proteases within the secretory granules of mast cells and also remains attached after release of the granule content into the extracellular environment. This might prevent extensive diffusion of proteases, protect them from inhibition, or both (Serafin and Austen, 1987). That the released heparin also inhibits complement activation may provide a negative feedback loop for further mast cell activation by anaphylatoxins (Kazatchkine *et al.*, 1981). A similar role for chondroitin sulfate might be conceivable.

However, it is also possible (and probably more likely) that mast cell-deficient mice show no decrease in MBL serum levels not because they do not release proteoglycans, but because they are protected from anaphylactic shock altogether.

To find out whether MBL consumption is of pathophysiological consequence in anaphylactic shock and to determine the relative importance of other mechanisms of complement activation, several mouse strains with selective deficiencies in complement components were tested.

C1q a$^{-/-}$ mice are unable to activate the complement cascade via the classical pathway, while the lectin and alternative pathways are fully functional (Botto *et al.*, 1998). These mice showed the same symptoms as complement-sufficient controls, demonstrating that lack of classical pathway activation is not protective (Fig. 4.28 A).

MBL$^{-/-}$ mice were made deficient in MBL-A and MBL-C. They are unable to activate the lectin pathway, while the classical and alternative pathways are still functional (Shi

et al., 2004). Complement activation via ficolins is also possible in these mice. When challenged, they showed a rapid decrease in body temperature, indicating that MBL does not contribute to the anaphylactic symptoms (Fig. 4.29).

Mice made deficient in factors B and C2 lack complement activation via the classical, lectin, and alternative pathway downstream of C4 cleavage (Celik *et al.*, 2001). These mice were not protected from anaphylactic shock in this model, either (Fig. 4.28 B). This is in clear contrast with a report from Regal *et al.* (1993), where pretreatment with soluble complement receptor 1, which binds to C3b and C4b and inhibits further anaphylatoxin generation, ameliorated shock symptoms in a similar model in the guinea pig. However, it is possible that complement activation occurred independently of the normal pathways. It has been shown that allergens can directly activate the complement system (Nagata and Glovsky, 1987). Also enzymes released from mast cells (Schwartz *et al.*, 1983) and neutrophils (Ward and Hill, 1970) can cleave complement components. These mechanisms could still lead to C5a generation in *H2-Bf/C2*$^{-/-}$ mice, even in the absence of functional activation pathways. Therefore we used the naturally C5-deficient DBA/2 mouse strain to investigate the importance of these mechanisms, but also these mice showed the usual anaphylactic symptoms (Fig. 4.30 B).

The anaphylatoxins C3a and C5a can directly activate mast cells (Johnson *et al.*, 1975; Schulman *et al.*, 1988; el Lati *et al.*, 1994) via the C5a receptor (C5aR). Also, C5aR activation on smooth muscle cells and endothelial cells leads to smooth muscle contraction, increased vasopermeability, and increased expression of adherence proteins (Frank and Fries, 1991). Therefore we used a C5aR-deficient mouse strain (Hopken *et al.*, 1996). Also these mice displayed symptoms of anaphylactic shock that were indistinguishable from those shown by the complement-sufficient controls (Fig. 4.30 A).

In accordance with reports showing that decomplementation with cobra venom factor prior to antigen challenge was not protective (Oettgen *et al.*, 1994; Strait *et al.*, 2002), our results indicate that complement activation is not essential for the generation of anaphylactic shock, and that mast cell derived mediators are more important for elicitation of the symptoms, as W/W^v mice were completely protected from onset of anaphylactic shock.

Taken together, these results show that, in contrast to the complement system, mast cells are required for manifestation of anaphylaxis in our mouse model. However, even though MBL and complement activation are not causative of the shock symptoms, serum MBL levels decrease in anaphylactic shock. The exact mechanism and the biological relevance of this decrease remain unclear.

5. Summary

In this work it was attempted to further elucidate the role of MBL and the lectin pathway of complement activation in both a septic and non-septic model of shock.

As a model for septic shock peritoneal bacterial infection and sepsis induced by CLP was chosen, which can be considered as a relevant animal model to study the importance of different mechanisms of host innate and adaptive immune responses to combat bacterial infections (Wichterman *et al.*, 1980). To study the role of MBL in a non-septic shock situation, a mouse model of anaphylaxis was used. Anaphylaxis is a rapid and systemic immunologically mediated reaction that occurs after exposure to foreign substances in previously sensitised individuals, and that is caused by the sudden release of mast cell- and baspohil-derived mediators (Kemp and Lockey, 2002).

As already shown recently in a different model of experimentally induced polymicrobial peritonitis (Celik *et al.*, 2001), the results from this work demonstrate the need for an intact complement system for survival after CLP. Mice lacking factors B and C2 and therefore all complement activation downstream of C4, succumbed very rapidly to the infection, with 90% mortality after three days, and also showed an impaired bacterial clearance from livers and spleens 24 hours after CLP. This underlines the importance of complement activation for surviving CLP and indicates that the opsonising activity of C1q and MBL alone is not sufficient to successfully combat bacterial infections.

Also mice lacking only the classical pathway were more susceptible to CLP than the wild type mice, with a survival rate of 40% after 12 days. In the first few days after CLP, however, their mortality was not much different from that of wild type mice, possibly because an intact lectin pathway was sufficient to give them protection. At that time also the bacterial counts in livers and spleens of mice deficient in C1q and wild type mice were similar. Only after 7 days, at a time when the first specific antibodies

against the invading microorganisms would appear, did the mortality of *C1q* $a^{-/-}$ mice increase.

To study the involvement of the lectin pathway, MBL-mediated C4 cleavage capacity of sera taken after CLP was measured. C4 cleavage capacity was found to decrease significantly within hours after CLP and stayed low for more than 10 days. This decrease is most likely due to a lower number of functional MBL complexes and not caused by their inability to cleave C4, as also MBL-A and MBL-C serum levels were decreased during that time when measured directly with monoclonal antibodies. Transcription of MBL was unaffected by CLP as judged by Northern Blot, indicating that the decrease in MBL serum levels is caused by consumption rather than decreased production.

While binding of MBL to microorganisms may account for some of this decrease, especially in the first stages after CLP, it is unlikely that bacterial numbers are high enough to explain this long lasting depletion. Immunhistological staining of the abscess that is formed as a consequence of CLP, revealed specific staining for MBL on necrotic parts of its wall. This is in agreement with previous reports demonstrating binding of MBL to apoptotic (Ogden *et al.*, 2001) and necrotic cells (Nauta *et al.*, 2003). This binding of MBL might lead to complement activation on dead tissue, as staining for C3 could be observed in the same areas. As the resolution of these peritoneal abscesses takes weeks, continuous consumption of MBL during this process may explain the long lasting depletion. However, it is possible that other, as yet unidentified mechanisms also contribute to this effect.

Likewise, after anaphylactic shock a rapid decrease in both C4 cleavage activity and MBL serum levels could be observed, suggesting lectin pathway activation or, at least, MBL consumption in this model as well. However, unlike after CLP, this decrease was

short lived and lasted not more than 24 hours. Again, mRNA transcription was not altered.

It has been shown previously that the complement system is activated during anaphylactic shock (Tannenbaum *et al.*, 1975; Smith *et al.*, 1980; del Balzo *et al.*, 1988; van der Linden *et al.*, 1990). However, it was believed that this activation occurs via the classical pathway, as C1q serum levels were diminished after antigen challenge (Tannenbaum *et al.*, 1975; van der Linden *et al.*, 1990), but, surprisingly, as there are no bacteria involved, also the lectin pathway seems to be activated. As in the case of C1q (Tannenbaum *et al.*, 1975; van der Linden *et al.*, 1990), increased vasopermeability and loss of proteins into the interstitial space cannot explain the decreased serum levels, as total serum protein content after anaphylactic shock was unchanged and total IgG content of sera decreased only marginally.

Interaction of MBL with immune complexes might be another possible explanation for the decreased serum levels. MBL binding via the CRD could be shown on solid-phase OVA, which has an N-linked high mannose group, but not on BSA, which is unglycosylated. Also solid-phase immune complexes did not fix MBL. As MBL levels were decreased likewise after OVA- and BSA-induced anaphylaxis, MBL binding to OVA does not account for MBL consumption in this model.

Since it has been shown previously that MBL binds to hypoxic and reoxygenated endothelial cells (Collard *et al.*, 1999), and these cells are also severely affected in anaphylaxis, we speculated that perhaps the systemic vascular changes after antigen challenge might lead to the exposure of MBL-fixing neoepitopes. Immunhistochemical stainings of heart, lung, kidney, liver, spleen, thymus, muscle and small intestine, however, could not confirm this. Although previously reported extrahepatical MBL expression (Uemura *et al.*, 2002; Wagner *et al.*, 2003; de Vries *et al.*, 2004) was seen, the staining pattern on tissues taken 30 minutes after antigen challenge was not different

from untreated control tissues. This might be either because no neoepitopes are exposed or because the lesions are too spread out to be detected by immunhistochemical methods.

Another possibility that might explain the decreased MBL serum levels could be the interaction of MBL with mast cell contents that are released after degranulation. Preincubation of serum samples with chondroitin sulfate, but not heparin, reduced subsequent MBL binding to ELISA plates. The exact nature of the interaction of MBL with chondroitin sulfate, however, remains unclear. Nevertheless it would seem that this is of little biological relevance, as mice deficient in MBL showed the same symptoms after antigen challenge as MBL-sufficient mice.

Furthermore, to evaluate the importance of other mechanisms of complement activation in anaphylaxis, mice lacking C1q, C5, the C5aR or *H2-Bf/C2*$^{-/-}$ mice were tested. Again, these mice displayed a decrease in body temperature similar to complement-sufficient controls, indicating that lack of complement activation is not protective in anaphylactic shock. Mast cell-deficient mice, on the other hand, were completely protected from onset of shock, showing that the release of mast cell mediators is causative of the observed symptoms.

In mast cell-deficient mice also no decrease in MBL serum levels was seen. Nevertheless it remains to be clarified whether mast cell degranulation leads to MBL consumption directly via interaction of MBL with granule contents, or indirectly via other, as yet unidentified pathophysiological mechanisms.

Taken together, these results demonstrate a clear need for the complement system in combating bacterial infections. In anaphylactic shock, however, in contrast to mast cell-derived mediators, it is not causative of the symptoms, even though complement is activated. The lectin pathway is involved in both models, as both CLP and anaphylaxis lead to a rapid decrease in MBL serum levels with little or no effect on MBL

biosynthesis. In CLP this decrease is long lasting and binding of MBL to necrotic parts of the abscess wall during this time could be shown. After anaphylactic shock, on the other hand, this decrease is short lived and its exact mechanism remains unclear.

6. Future work

Although MBL does not seem to play a major role in anaphylactic shock, the exact mechanism that leads to a decreased MBL serum level after antigen challenge is still far from being clear at the moment. Injecting mice with radioactively labelled or green-fluorescence protein-tagged recombinant MBL prior to subjecting them to anaphylaxis might give a hint as to whether MBL is redistributed. However, the amount that can be administered to the mouse is limited, and since anaphylaxis is a systemic event, the labelled material might be distributed evenly throughout the whole body and not give a clear signal.

The problem that chondroitin sulfate or heparin cannot be used to coat ELISA plates can be overcome by biotinylating these proteoglycans. Then they can be bound to streptavidin-coated plates and it can be tested under which conditions MBL interacts with proteoglycans and whether this binding is dependent on the CRD.

Immunhistochemical methods could be employed to see whether MBL accumulates in the vicinity of degranulated mast cells. Since the dyes used for mast cell staining, like Giemsa or toluidine blue, stain the granule contents and therefore make detection of degranulated mast cells difficult, it would be preferable to use antibodies against surface markers, e.g. c-kit.

To exclude that the decrease in MBL serum levels after CLP is due to a loss of function, MBL levels can be determined using a Sandwich-ELISA. In this case detection of MBL is not dependent on functional CRDs. Using a non-reducing SDS-PAGE gel, possibly followed by Western Blot, will give a hint as to whether the amount of MBL monomers increases after CLP.

To see whether MBL does indeed lead to complement activation on the abscess wall, antibodies labelled with different fluorescent dyes can be used on the same section to

show colocalisation. Also other injured tissues, e.g. after burn injuries, could be stained to confirm binding of MBL to necrotic tissue.

Furthermore it would be interesting to see how mice deficient in both MBL-A and MBL-C perform in the model of CLP. Also the kinetics of abscess resolution in wild type and MBL-deficient mice need to be studied. Infusions of recombinant or purified MBL into MBL-deficient mice will show whether the wild type phenotype can be restored.

Since most carriers of MBL variant alleles are healthy, an MBL-replacement therapy for MBL-deficient individuals does not seem justified. Under certain circumstances, however, it can be envisaged that MBL-deficient patients could benefit from this therapy. Possible scenarios would be e.g. chemotherapy, cystic fibrosis or recurrent infections.

A clear understanding of the role that MBL plays in certain diseases and of the mechanisms involved in its action will give hints as to whether and when MBL-replacement therapy might be beneficial.

7. Acknowledgements

I am grateful to Prof. Wilhelm Schwaeble for offering to supervise my PhD thesis and for organisatorial assistance.

I am indebted to Prof. Daniela Maennel for giving me the opportunity to do the work in her institute and for excellent supervision.

I gratefully acknowledge Dr. Bernd Echtenacher and Karin Holz for assistance with the animal experiments.

Furthermore I would like to thank Prof. Jens Jensenius for teaching me the TRIFMA method and for helpful discussions.

Special thanks go to Silvia Seegers and Valeria Runza for critically reading the manuscript.

Thanks to everyone in the lab for making me feel at home and for being good friends.

8. References

Almeda, S., Rosenberg, R. D., and Bing, D. H. (1983). The binding properties of human complement component C1q. Interaction with mucopolysaccharides. *J Biol.Chem.* **258**, 785-791.

Ambrus, G., Gal, P., Kojima, M., Szilagyi, K., Balczer, J., Antal, J., Graf, L., Laich, A., Moffatt, B. E., Schwaeble, W., Sim, R. B., and Zavodszky, P. (2003). Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. *The Journal of Immunology* **170**, 1374-1382.

Andoh, A., Fujiyama, Y., Bamba, T., and Hosoda, S. (1993). Differential cytokine regulation of complement C3, C4, and factor B synthesis in human intestinal epithelial cell line, Caco-2. *The Journal of Immunology* **151**, 4239-4247.

Arnold, J. N., Radcliffe, C. M., Wormald, M. R., Royle, L., Harvey, D. J., Crispin, M., Dwek, R. A., Sim, R. B., and Rudd, P. M. (2004). The glycosylation of human serum IgD and IgE and the accessibility of identified oligomannose structures for interaction with mannan-binding lectin. *The Journal of Immunology* **173**, 6831-6840.

Atkinson, A. P., Cedzynski, M., Szemraj, J., St Swierzko, A., Bak-Romaniszyn, L., Banasik, M., Zeman, K., Matsushita, M., Turner, M. L., and Kilpatrick, D. C. (2004). L-ficolin in children with recurrent respiratory infections. *Clin.Exp.Immunol* 138, 517-520.

Bellamy, R., Ruwende, C., McAdam, K. P., Thursz, M., Sumiya, M., Summerfield, J., Gilbert, S. C., Corrah, T., Kwiatkowski, D., Whittle, H. C., and Hill, A. V. (1998). Mannose binding protein deficiency is not associated with malaria, hepatitis B carriage nor tuberculosis in Africans. *QJM.* **91**, 13-18.

Bland, C. E., Ginsburg, H., Silbert, J. E., and Metcalfe, D. D. (1982). Mouse heparin proteoglycan. Synthesis by mast cell-fibroblast monolayers during lymphocyte-dependent mast cell proliferation. *J Biol.Chem.* **257**, 8661-8666.

Botto, M., Dell'Agnola, C., Bygrave, A. E., Thompson, E. M., Cook, H. T., Petry, F., Loos, M., Pandolfi, P. P., and Walport, M. J. (1998). Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat.Genet.* **19**, 56-59.

Brown, J. S., Hussell, T., Gilliland, S. M., Holden, D. W., Paton, J. C., Ehrenstein, M. R., Walport, M. J., and Botto, M. (2002). The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proc.Natl.Acad.Sci.U.S.A* **99**, 16969-16974.

Cara, D. C., Ebbert, K. V., and McCafferty, D. M. (2004). Mast cell-independent mechanisms of immediate hypersensitivity: a role for platelets. *J.Immunol.* **172**, 4964-4971.

Carlsson, M., Sjoholm, A. G., Eriksson, L., Thiel, S., Jensenius, J. C., Segelmark, M., and Truedsson, L. (2005). Deficiency of the mannan-binding lectin pathway of complement and poor outcome in cystic fibrosis: bacterial colonization may be decisive for a relationship. *Clin.Exp.Immunol* **139**, 306-313.

Carroll, M. C. and Fischer, M. B. (1997). Complement and the immune response. *Curr.Opin.Immunol* **9**, 64-69.

Carroll, M. C. (2004). The complement system in B cell regulation. *Mol.Immunol* **41**, 141-146.

Cedzynski, M., Szemraj, J., Swierzko, A. S., Bak-Romaniszyn, L., Banasik, M., Zeman, K., and Kilpatrick, D. C. (2004). Mannan-binding lectin insufficiency in children with recurrent infections of the respiratory system. *Clin.Exp.Immunol* **136**, 304-311.

Celik, I., Stover, C., Botto, M., Thiel, S., Tzima, S., Kunkel, D., Walport, M., Lorenz, W., and Schwaeble, W. (2001). Role of the classical pathway of complement activation in experimentally induced polymicrobial peritonitis. *Infect.Immun.* **69**, 7304-7309.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., and Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature* **335**, 88-89.

Chen, C. B. and Wallis, R. (2001). Stoichiometry of complexes between mannose-binding protein and its associated serine proteases. Defining functional units for complement activation. *J Biol.Chem.* **276**, 25894-25902.

Chen, C. B. and Wallis, R. (2004). Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases. *J Biol.Chem.* **279**, 26058-26065.

Choi, I. H., Shin, Y. M., Park, J. S., Lee, M. S., Han, E. H., Chai, O. H., Im, S. Y., Ha, T. Y., and Lee, H. K. (1998). Immunoglobulin E-dependent active fatal anaphylaxis in mast cell-deficient mice. *The Journal of Experimental Medicine* **188**, 1587-1592.

Christiansen, O. B., Kilpatrick, D. C., Souter, V., Varming, K., Thiel, S., and Jensenius, J. C. (1999). Mannan-binding lectin deficiency is associated with unexplained recurrent miscarriage. *Scand.J Immunol* **49**, 193-196.

Collard, C. D., Vakeva, A., Bukusoglu, C., Zund, G., Sperati, C. J., Colgan, S. P., and Stahl, G. L. (1997). Reoxygenation of hypoxic human umbilical vein endothelial cells activates the classic complement pathway. *Circulation* **96**, 326-333.

Collard, C. D., Lekowski, R., Jordan, J. E., Agah, A., and Stahl, G. L. (1999). Complement activation following oxidative stress. *Mol.Immunol* **36**, 941-948.

Collard, C. D., Vakeva, A., Morrissey, M. A., Agah, A., Rollins, S. A., Reenstra, W. R., Buras, J. A., Meri, S., and Stahl, G. L. (2000). Complement activation after oxidative stress: role of the lectin complement pathway. *Am.J.Pathol.* **156**, 1549-1556.

Collard, C. D., Montalto, M. C., Reenstra, W. R., Buras, J. A., and Stahl, G. L. (2001). Endothelial oxidative stress activates the lectin complement pathway: role of cytokeratin 1. *Am.J Pathol.* **159**, 1045-1054.

Cook, M.J. (1965). The Anatomy of the Laboratory Mouse. *Academic Press.*

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

Dahl, M., Tybjaerg-Hansen, A., Schnohr, P., and Nordestgaard, B. G. (2004). A population-based study of morbidity and mortality in mannose-binding lectin deficiency. *J Exp.Med.* **199**, 1391-1399.

de Vries, B., Walter, S. J., Peutz-Kootstra, C. J., Wolfs, T. G., van Heurn, L. W., and Buurman, W. A. (2004). The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am.J Pathol.* **165**, 1677-1688.

del Balzo, U., Polley, M. J., and Levi, R. (1988). Activation of the third complement component (C3) and C3a generation in cardiac anaphylaxis: histamine release and associated inotropic and chronotropic effects. *J Pharmacol.Exp.Ther.* **246**, 911-916.

Dodds, A. W. (1993). Small-scale preparation of complement components C3 and C4. *Methods Enzymol.* **223**, 46-61.

Dubreuil, J. D., Ruggiero, P., Rappuoli, R., and Del Giudice, G. (2004). Effect of heparin binding on Helicobacter pylori resistance to serum. *J Med.Microbiol.* **53**, 9-12.

Echtenacher, B., Falk, W., Mannel, D. N., and Krammer, P. H. (1990). Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. *The Journal of Immunology* **145**, 3762-3766.

Echtenacher, B., Weigl, K., Lehn, N., and Mannel, D. N. (2001). Tumor necrosis factor-dependent adhesions as a major protective mechanism early in septic peritonitis in mice. *Infect.Immun.* **69**, 3550-3555.

Echtenacher, B. and Mannel, D. N. (2002). Requirement of TNF and TNF receptor type 2 for LPS-induced protection from lethal septic peritonitis. *J.Endotoxin.Res.* **8**, 365-369.

Echtenacher, B., Urbaschek, R., Weigl, K., Freudenberg, M. A., and Mannel, D. N. (2003). Treatment of experimental sepsis-induced immunoparalysis with TNF. *Immunobiology* **208**, 381-389.

Eisen, D. P. and Minchinton, R. M. (2003). Impact of mannose-binding lectin on susceptibility to infectious diseases. *Clin.Infect.Dis.* **37**, 1496-1505.

el Lati, S. G., Dahinden, C. A., and Church, M. K. (1994). Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells. *J.Invest Dermatol.* **102**, 803-806.

Enerback, L., Kolset, S. O., Kusche, M., Hjerpe, A., and Lindahl, U. (1985). Glycosaminoglycans in rat mucosal mast cells. *Biochem.J* **227**, 661-668.

Ezekowitz, R. A., Day, L. E., and Herman, G. A. (1988). A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins [published erratum appears in J Exp Med 1991 Sep 1;174(3):753]. *The Journal of Experimental Medicine* **167**, 1034-1046.

Ezekowitz, R. A. (1998). Genetic heterogeneity of mannose-binding proteins: the Jekyll and Hyde of innate immunity? *Am.J Hum.Genet.* **62**, 6-9.

Fischer, P. B., Ellermann-Eriksen, S., Thiel, S., Jensenius, J. C., and Mogensen, S. C. (1994). Mannan-binding protein and bovine conglutinin mediate enhancement of herpes simplex virus type 2 infection in mice. *Scand.J Immunol* **39**, 439-445.

Frank, M. M. and Fries, L. F. (1991). The role of complement in inflammation and phagocytosis. *Immunol Today* **12**, 322-326.

Gadjeva, M., Takahashi, K., and Thiel, S. (2004a). Mannan-binding lectin--a soluble pattern recognition molecule. *Mol.Immunol* **41**, 113-121.

Gadjeva, M., Paludan, S. R., Thiel, S., Slavov, V., Ruseva, M., Eriksson, K., Lowhagen, G. B., Shi, L., Takahashi, K., Ezekowitz, A., and Jensenius, J. C. (2004b). Mannan-binding lectin modulates the response to HSV-2 infection. *Clin.Exp.Immunol* **138**, 304-311.

Galli, S. J. and Kitamura, Y. (1987). Genetically mast-cell-deficient W/Wv and Sl/Sld mice. Their value for the analysis of the roles of mast cells in biologic responses in vivo. *Am.J.Pathol.* **127**, 191-198.

Garred, P., Madsen, H. O., Balslev, U., Hofmann, B., Pedersen, C., Gerstoft, J., and Svejgaard, A. (1997a). Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. *Lancet* **349**, 236-240.

Garred, P., Richter, C., Andersen, A. B., Madsen, H. O., Mtoni, I., Svejgaard, A., and Shao, J. (1997b). Mannan-binding lectin in the sub-Saharan HIV and tuberculosis epidemics. *Scand.J Immunol* **46**, 204-208.

Garred, P., Madsen, H. O., Halberg, P., Petersen, J., Kronborg, G., Svejgaard, A., Andersen, V., and Jacobsen, S. (1999a). Mannose-binding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. *Arthritis Rheum.* **42**, 2145-2152.

Garred, P., Pressler, T., Madsen, H. O., Frederiksen, B., Svejgaard, A., Hoiby, N., Schwartz, M., and Koch, C. (1999b). Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. *J Clin.Invest* **104**, 431-437.

Garred, P., Madsen, H. O., Marquart, H., Hansen, T. M., Sorensen, S. F., Petersen, J., Volck, B., Svejgaard, A., Graudal, N. A., Rudd, P. M., Dwek, R. A., Sim, R. B., and Andersen, V. (2000). Two edged role of mannose binding lectin in rheumatoid arthritis: a cross sectional study. *J Rheumatol.* **27**, 26-34.

Garred, P., Pressler, T., Lanng, S., Madsen, H. O., Moser, C., Laursen, I., Balstrup, F., Koch, C., and Koch, C. (2002). Mannose-binding lectin (MBL) therapy in an MBL-deficient patient with severe cystic fibrosis lung disease. *Pediatr.Pulmonol.* **33**, 201-207.

Garred, P., Larsen, F., Madsen, H. O., and Koch, C. (2003a). Mannose-binding lectin deficiency--revisited. *Mol.Immunol* **40**, 73-84.

Garred, P., Strom, J., Quist, L., Taaning, E., and Madsen, H. O. (2003b). Association of mannose-binding lectin polymorphisms with sepsis and fatal outcome, in patients with systemic inflammatory response syndrome. *J.Infect.Dis.* **188**, 1394-1403.

Ghiran, I., Barbashov, S. F., Klickstein, L. B., Tas, S. W., Jensenius, J. C., and Nicholson-Weller, A. (2000). Complement receptor 1/CD35 is a receptor for mannan-binding lectin. *The Journal of Experimental Medicine* **192**, 1797-1808.

Graudal, N. A., Madsen, H. O., Tarp, U., Svejgaard, A., Jurik, G., Graudal, H. K., and Garred, P. (2000). The association of variant mannose-binding lectin genotypes with radiographic outcome in rheumatoid arthritis. *Arthritis Rheum.* **43**, 515-521.

Gruden-Movsesijan, A., Petrovic, M., and Sofronic-Milosavljevic, L. (2003). Interaction of mannan-binding lectin with Trichinella spiralis glycoproteins, a possible innate immune mechanism. *Parasite Immunol* **25**, 545-552.

Grunewald, S. M., Werthmann, A., Schnarr, B., Klein, C. E., Brocker, E. B., Mohrs, M., Brombacher, F., Sebald, W., and Duschl, A. (1998). An Antagonistic IL-4 Mutant Prevents Type I Allergy in the Mouse: Inhibition of the IL-4/IL-13 Receptor System Completely Abrogates Humoral Immune Response to Allergen and Development of Allergic Symptoms In Vivo. *The Journal of Immunology* **160**, 4004-4009.

Guo, Y., Hedqvist, P., and Gustafsson, L. E. (2001). Absence of mast cell involvement in active systemic anaphylaxis in rats. *Eur.J.Pharmacol.* **430**, 305-310.

Ha, T. Y., Reed, N. D., and Crowle, P. K. (1986). Immune response potential of mast cell-deficient W/Wv mice. *Int.Arch.Allergy Appl.Immunol.* **80**, 85-94.

Hajela, K., Kojima, M., Ambrus, G., Wong, K. H., Moffatt, B. E., Ferluga, J., Hajela, S., Gal, P., and Sim, R. B. (2002). The biological functions of MBL-associated serine proteases (MASPs). *Immunobiology* **205**, 467-475.

Hansen, S. and Holmskov, U. (1998). Structural aspects of collectins and receptors for collectins. *Immunobiology* **199**, 165-189.

Hansen, S., Thiel, S., Willis, A., Holmskov, U., and Jensenius, J. C. (2000). Purification and Characterization of Two Mannan-Binding Lectins from Mouse Serum. *The Journal of Immunology* **164**, 2610-2618.

Holmskov, U., Thiel, S., and Jensenius, J. C. (2003). Collections and ficolins: humoral lectins of the innate immune defense. *Annu.Rev.Immunol* **21**, 547-578.

Hopken, U. E., Lu, B., Gerard, N. P., and Gerard, C. (1996). The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature* **383**, 86-89.

Hopken, U. E., Lu, B., Gerard, N. P., and Gerard, C. (1997). Impaired inflammatory responses in the reverse arthus reaction through genetic deletion of the C5a receptor. *J Exp.Med.* **186**, 749-756.

Ihara, S., Takahashi, A., Hatsuse, H., Sumitomo, K., Doi, K., and Kawakami, M. (1991). Major component of Ra-reactive factor, a complement-activating bactericidal protein, in mouse serum. *The Journal of Immunology* **146**, 1874-1879.

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

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

Johnson, A. R., Hugli, T. E., and Muller-Eberhard, H. J. (1975). Release of histamine from rat mast cells by the complement peptides C3a and C5a. *Immunology* **28**, 1067.

Jordan, J. E., Montalto, M. C., and Stahl, G. L. (2001). Inhibition of mannose-binding lectin reduces postischemic myocardial reperfusion injury. *Circulation* **104**, 1413-1418.

Kazatchkine, M. D., Fearon, D. T., Metcalfe, D. D., Rosenberg, R. D., and Austen, K. F. (1981). Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. *J Clin.Invest* **67**, 223-228.

Kemp, S. F. and Lockey, R. F. (2002). Anaphylaxis: a review of causes and mechanisms. *J Allergy Clin.Immunol* **110**, 341-348.

Kildsgaard, J., Hollmann, T. J., Matthews, K. W., Bian, K., Murad, F., and Wetsel, R. A. (2000). Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. *The Journal of Immunology* **165**, 5406-5409.

Kilpatrick, D. C., Starrs, L., Moore, S., Souter, V., and Liston, W. A. (1999). Mannan binding lectin concentration and risk of miscarriage. *Hum.Reprod.* **14**, 2379-2380.

Kilpatrick, D. C., McLintock, L. A., Allan, E. K., Copland, M., Fujita, T., Jordanides, N. E., Koch, C., Matsushita, M., Shiraki, H., Stewart, K., Tsujimura, M., Turner, M. L., Franklin, I. M., and Holyoake, T. L. (2003). No strong relationship between mannan binding lectin or plasma ficolins and chemotherapy-related infections. *Clin.Exp.Immunol* **134**, 279-284.

Kilpatrick, D. C., Stewart, K., Allan, E. K., McLintock, L. A., Holyoake, T. L., and Turner, M. L. (2005). Successful haemopoietic stem cell transplantation does not correct mannan-binding lectin deficiency. *Bone Marrow Transplant.* **35**, 179-181.

Kim, Y. U., Kinoshita, T., Molina, H., Hourcade, D., Seya, T., Wagner, L. M., and Holers, V. M. (1995). Mouse complement regulatory protein Crry/p65 uses the specific mechanisms of both human decay-accelerating factor and membrane cofactor protein. *J Exp.Med.* **181**, 151-159.

Kimura, S., Nagata, M., Takeuchi, M., Takano, K., and Harada, M. (1997). Anti-granulocyte antibody suppression of active and passive anaphylactic shock in WBB6F1-W/Wv mice. *Cell Mol.Life Sci.* **53**, 663-666.

Klabunde, J., Berger, J., Jensenius, J. C., Klinkert, M. Q., Zelck, U. E., Kremsner, P. G., and Kun, J. F. (2000). Schistosoma mansoni: adhesion of mannan-binding lectin to surface glycoproteins of cercariae and adult worms. *Exp.Parasitol.* **95**, 231-239.

Klabunde, J., Uhlemann, A. C., Tebo, A. E., Kimmel, J., Schwarz, R. T., Kremsner, P. G., and Kun, J. F. (2002). Recognition of plasmodium falciparum proteins by mannan-binding lectin, a component of the human innate immune system. *Parasitol.Res.* **88**, 113-117.

Klickstein, L. B., Barbashov, S. F., Liu, T., Jack, R. M., and Nicholson-Weller, A. (1997). Complement receptor type 1 (CR1, CD35) is a receptor for C1q. *Immunity.* **7**, 345-355.

Larsen, F., Madsen, H. O., Sim, R. B., Koch, C., and Garred, P. (2004). Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. *J Biol.Chem.* **279**, 21302-21311.

Lee, S. J., Gonzalez-Aseguinolaza, G., and Nussenzweig, M. C. (2002). Disseminated candidiasis and hepatic malarial infection in mannose-binding-lectin-A-deficient mice. *Mol.Cell Biol.* **22**, 8199-8203.

Lei, H. Y., Lee, S. H., and Leir, S. H. (1996). Antigen-induced anaphylactic death in mice. *Int.Arch.Allergy Immunol.* **109**, 407-412.

Liu, H., Jensen, L., Hansen, S., Petersen, S. V., Takahashi, K., Ezekowitz, A. B., Hansen, F. D., Jensenius, J. C., and Thiel, S. (2001). Characterization and quantification of mouse mannan-binding lectins (MBL-A and MBL-C) and study of acute phase responses. *Scand.J Immunol* **53**, 489-497.

Lu, J. H., Thiel, S., Wiedemann, H., Timpl, R., and Reid, K. B. (1990). Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. *The Journal of Immunology* **144**, 2287-2294.

Lu, J., Teh, C., Kishore, U., and Reid, K. B. (2002). Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. *Biochim.Biophys.Acta* **1572**, 387-400.

Luty, A. J., Kun, J. F., and Kremsner, P. G. (1998). Mannose-binding lectin plasma levels and gene polymorphisms in Plasmodium falciparum malaria. *J Infect.Dis.* **178**, 1221-1224.

Lynch, N. J., Khan, S. U., Stover, C. M., Sandrini, S. M., Marston, D., Presanis, J. S., and Schwaeble, W. J. (2005). Composition of the lectin pathway of complement in Gallus gallus: absence of mannan-binding lectin-associated serine protease-1 in birds. *The Journal of Immunology* **174**, 4998-5006.

Madsen, H. O., Garred, P., Thiel, S., Kurtzhals, J. A., Lamm, L. U., Ryder, L. P., and Svejgaard, A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *The Journal of Immunology* **155**, 3013-3020.

Maier, S., Traeger, T., Entleutner, M., Westerholt, A., Kleist, B., Huser, N., Holzmann, B., Stier, A., Pfeffer, K., and Heidecke, C. D. (2004). Cecal ligation and puncture versus colon ascendens stent peritonitis: two distinct animal models for polymicrobial sepsis. *Shock* **21**, 505-511.

Makabe-Kobayashi, Y., Hori, Y., Adachi, T., Ishigaki-Suzuki, S., Kikuchi, Y., Kagaya, Y., Shirato, K., Nagy, A., Ujike, A., Takai, T., Watanabe, T., and Ohtsu, H. (2002). The control effect of histamine on body temperature and respiratory function in IgE-dependent systemic anaphylaxis. *J Allergy Clin.Immunol* **110**, 298-303.

Malhotra, R., Wormald, M. R., Rudd, P. M., Fischer, P. B., Dwek, R. A., and Sim, R. B. (1995). Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat.Med.* **1**, 237-243.

Martin, T. R., Galli, S. J., Katona, I. M., and Drazen, J. M. (1989). Role of mast cells in anaphylaxis. Evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. *J Clin.Invest* **83**, 1375-1383.

Matsushita, M. and Fujita, T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *The Journal of Experimental Medicine* **176**, 1497-1502.

Matsushita, M., Endo, Y., and Fujita, T. (2000a). Cutting Edge: Complement-Activating Complex of Ficolin and Mannose-Binding Lectin-Associated Serine Protease. *The Journal of Immunology* **164**, 2281-2284.

Matsushita, M., Thiel, S., Jensenius, J. C., Terai, I., and Fujita, T. (2000b). Proteolytic activities of two types of mannose-binding lectin-associated serine protease. *The Journal of Immunology* **165**, 2637-2642.

Matsushita, M. and Fujita, T. (2001). Ficolins and the lectin complement pathway. *Immunol Rev.* **180**, 78-85.

Matsushita, M., Kuraya, M., Hamasaki, N., Tsujimura, M., Shiraki, H., and Fujita, T. (2002). Activation of the Lectin Complement Pathway by H-Ficolin (Hakata Antigen). *The Journal of Immunology* **168**, 3502-3506.

Maurer, M., Echtenacher, B., Hultner, L., Kollias, G., Mannel, D. N., Langley, K. E., and Galli, S. J. (1998). The c-kit ligand, stem cell factor, can enhance innate immunity through effects on mast cells. *The Journal of Experimental Medicine* **188**, 2343-2348.

Meri, S. and Jarva, H. (1998). Complement regulation. *Vox Sang.* **74 Suppl 2**, 291-302.

Metcalfe, D. D., Soter, N. A., Wasserman, S. I., and Austen, K. F. (1980). Identification of sulfated mucopolysaccharides including heparin in the lesional skin of a patient with mastocytosis. *J Invest Dermatol.* **74**, 210-215.

Metcalfe, D. D., Bland, C. E., and Wasserman, S. I. (1984). Biochemical and functional characterization of proteoglycans isolated from basophils of patients with chronic myelogenous leukemia. *The Journal of Immunology* **132**, 1943-1950.

Miller, G. W. and Nussenzweig, V. (1974). Complement as a regulator of interactions between immune complexes and cell membranes. *The Journal of Immunology* **113**, 464-469.

Mombo, L. E., Ntoumi, F., Bisseye, C., Ossari, S., Lu, C. Y., Nagel, R. L., and Krishnamoorthy, R. (2003). Human genetic polymorphisms and asymptomatic Plasmodium falciparum malaria in Gabonese schoolchildren. *Am.J Trop.Med.Hyg.* **68**, 186-190.

Monge, J. C., Recondo, E. F., and Fernandez de Recondo, M. E. (1989). Heparin and concanavalin A interaction as a model for studying the mechanism of the anticoagulant activity. *Thromb.Res.* **54**, 237-243.

Mulligan, M. S., Schmid, E., Beck-Schimmer, B., Till, G. O., Friedl, H. P., Brauer, R. B., Hugli, T. E., Miyasaka, M., Warner, R. L., Johnson, K. J., and Ward, P. A. (1996). Requirement and role of C5a in acute lung inflammatory injury in rats. *J Clin.Invest* **98**, 503-512.

Mullighan, C. G., Heatley, S., Doherty, K., Szabo, F., Grigg, A., Hughes, T. P., Schwarer, A. P., Szer, J., Tait, B. D., Bik, T. L., and Bardy, P. G. (2002). Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* **99**, 3524-3529.

Munoz, J. and Bergman, R. K. (1965). Mechanism of anaphylactic death in the mouse. *Nature* **205**, 199-200.

Nagai, H., Abe, T., Yamaguchi, I., Mito, K., Tsunematsu, M., Kimata, M., and Inagaki, N. (2000). Role of mast cells in the onset of IgE-mediated late-phase cutaneous response in mice. *J Allergy Clin.Immunol* **106**, S91-S98.

Nagata, S. and Glovsky, M. M. (1987). Activation of human serum complement with allergens. I. Generation of C3a, C4a, and C5a and induction of human neutrophil aggregation. *J Allergy Clin.Immunol* **80**, 24-32.

Naito, H., Ikeda, A., Hasegawa, K., Oka, S., Uemura, K., Kawasaki, N., and Kawasaki, T. (1999). Characterization of human serum mannan-binding protein promoter. *J.Biochem.(Tokyo)* **126**, 1004-1012.

Nauta, A. J., Raaschou-Jensen, N., Roos, A., Daha, M. R., Madsen, H. O., Borrias-Essers, M. C., Ryder, L. P., Koch, C., and Garred, P. (2003). Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur.J.Immunol.* **33**, 2853-2863.

Nauta, A. J., Roos, A., and Daha, M. R. (2004). A regulatory role for complement in innate immunity and autoimmunity. *Int.Arch.Allergy Immunol* **134**, 310-323.

Neth, O., Jack, D. L., Johnson, M., Klein, N. J., and Turner, M. W. (2002). Enhancement of complement activation and opsonophagocytosis by complexes of mannose-binding lectin with mannose-binding lectin-associated serine protease after binding to Staphylococcus aureus. *The Journal of Immunology* **169**, 4430-4436.

Nielsen, S. L., Andersen, P. L., Koch, C., Jensenius, J. C., and Thiel, S. (1995). The level of the serum opsonin, mannan-binding protein in HIV-1 antibody-positive patients. *Clin.Exp.Immunol* **100**, 219-222.

Oettgen, H. C., Martin, T. R., Wynshaw-Boris, A., Deng, C., Drazen, J. M., and Leder, P. (1994). Active anaphylaxis in IgE-deficient mice. *Nature* **370**, 367-370.

Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A., and Henson, P. M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *The Journal of Experimental Medicine* **194**, 781-795.

Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Yamazaki, H., Shimada, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., and Wakamiya, N. (1999). Molecular cloning of a novel human collectin from liver (CL-L1). *J Biol.Chem.* **274**, 13681-13689.

Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Itabe, H., Suzutani, T., Ogasawara, M., Yoshida, I., and Wakamiya, N. (2001). The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J Biol.Chem.* **276**, 44222-44228.

Okunuki, H., Teshima, R., Sakushima, J., Akiyama, H., Goda, Y., Toyoda, M., and Sawada, J. I. (2000). Induction of active systemic anaphylaxis by oral sensitization with ovalbumin in mast-cell-deficient mice. *Immunol.Lett.* **74**, 233-237.

Petersen, S. V., Thiel, S., Jensen, L., Vorup-Jensen, T., Koch, C., and Jensenius, J. C. (2000). Control of the classical and the MBL pathway of complement activation. *Mol.Immunol* **37**, 803-811.

Petersen, S. V., Thiel, S., Jensen, L., Steffensen, R., and Jensenius, J. C. (2001a). An assay for the mannan-binding lectin pathway of complement activation. *J.Immunol.Methods* **257**, 107-116.

Petersen, S. V., Thiel, S., and Jensenius, J. C. (2001b). The mannan-binding lectin pathway of complement activation: biology and disease association. *Mol.Immunol* **38**, 133-149.

Peterslund, N. A., Koch, C., Jensenius, J. C., and Thiel, S. (2001). Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. *Lancet* **358**, 637-638.

Presanis, J. S., Hajela, K., Ambrus, G., Gal, P., and Sim, R. B. (2004). Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. *Mol.Immunol* **40**, 921-929.

Razin, E., Stevens, R. L., Akiyama, F., Schmid, K., and Austen, K. F. (1982). Culture from mouse bone marrow of a subclass of mast cells possessing a distinct chondroitin sulfate proteoglycan with glycosaminoglycans rich in N-acetylgalactosamine-4,6-disulfate. *J Biol.Chem.* **257**, 7229-7236.

Regal, J. F., Fraser, D. G., and Toth, C. A. (1993). Role of the complement system in antigen-induced bronchoconstriction and changes in blood pressure in the guinea pig. *J Pharmacol.Exp.Ther.* **267**, 979-988.

Roos, A., Bouwman, L. H., Gijlswijk-Janssen, D. J., Faber-Krol, M. C., Stahl, G. L., and Daha, M. R. (2001). Human IgA activates the complement system via the mannan-binding lectin pathway. *The Journal of Immunology* **167**, 2861-2868.

Ruoslahti, E. (1988). Structure and biology of proteoglycans. *Annu.Rev.Cell Biol.* **4**, 229-255.

Saevarsdottir, S., Vikingsdottir, T., Vikingsson, A., Manfredsdottir, V., Geirsson, A. J., and Valdimarsson, H. (2001). Low mannose binding lectin predicts poor prognosis in patients with early rheumatoid arthritis. A prospective study. *J Rheumatol.* **28**, 728-734.

Saevarsdottir, S., Vikingsdottir, T., and Valdimarsson, H. (2004). The potential role of mannan-binding lectin in the clearance of self-components including immune complexes. *Scand.J Immunol* **60**, 23-29.

Sahu, A. and Pangburn, M. K. (1993). Identification of multiple sites of interaction between heparin and the complement system. *Mol.Immunol* **30**, 679-684.

Santos, I. K., Costa, C. H., Krieger, H., Feitosa, M. F., Zurakowski, D., Fardin, B., Gomes, R. B., Weiner, D. L., Harn, D. A., Ezekowitz, R. A., and Epstein, J. E. (2001). Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. *Infect.Immun.* **69**, 5212-5215.

Sastry, K., Zahedi, K., Lelias, J. M., Whitehead, A. S., and Ezekowitz, R. A. (1991). Molecular characterization of the mouse mannose-binding proteins. The mannose-binding protein A but not C is an acute phase reactant. *The Journal of Immunology* **147**, 692-697.

Schafranski, M. D., Stier, A., Nisihara, R., and Messias-Reason, I. J. (2004). Significantly increased levels of mannose-binding lectin (MBL) in rheumatic heart disease: a beneficial role for MBL deficiency. *Clin.Exp.Immunol* **138**, 521-525.

Schagat, T. L., Wofford, J. A., and Wright, J. R. (2001). Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. *The Journal of Immunology* **166**, 2727-2733.

Schulman, E. S., Post, T. J., Henson, P. M., and Giclas, P. C. (1988). Differential effects of the complement peptides, C5a and C5a des Arg on human basophil and lung mast cell histamine release. *J.Clin.Invest* **81**, 918-923.

Schwartz, L. B., Kawahara, M. S., Hugli, T. E., Vik, D., Fearon, D. T., and Austen, K. F. (1983). Generation of C3a anaphylatoxin from human C3 by human mast cell tryptase. *The Journal of Immunology* **130**, 1891-1895.

Serafin, W. E. and Austen, K. F. (1987). Mediators of immediate hypersensitivity reactions. *N.Engl.J.Med.* **317**, 30-34.

Shi, L., Takahashi, K., Dundee, J., Shahroor-Karni, S., Thiel, S., Jensenius, J. C., Gad, F., Hamblin, M. R., Sastry, K. N., and Ezekowitz, R. A. (2004). Mannose-binding Lectin-deficient Mice Are Susceptible to Infection with Staphylococcus aureus. *The Journal of Experimental Medicine* **199**, 1379-1390.

Siassi, M., Hohenberger, W., and Riese, J. (2003). Mannan-binding lectin (MBL) serum levels and post-operative infections. *Biochem.Soc.Trans.* **31**, 774-775.

Smith, P. L., Kagey-Sobotka, A., Bleecker, E. R., Traystman, R., Kaplan, A. P., Gralnick, H., Valentine, M. D., Permutt, S., and Lichtenstein, L. M. (1980). Physiologic manifestations of human anaphylaxis. *J Clin.Invest* **66**, 1072-1080.

Stahl, G. L., Xu, Y., Hao, L., Miller, M., Buras, J. A., Fung, M., and Zhao, H. (2003). Role for the alternative complement pathway in ischemia/reperfusion injury. *Am.J Pathol.* **162**, 449-455.

Stengaard-Pedersen, K., Thiel, S., Gadjeva, M., Moller-Kristensen, M., Sorensen, R., Jensen, L. T., Sjoholm, A. G., Fugger, L., and Jensenius, J. C. (2003). Inherited deficiency of mannan-binding lectin-associated serine protease 2. *N.Engl.J Med.* **349**, 554-560.

Stover, C. M., Thiel, S., Thelen, M., Lynch, N. J., Vorup-Jensen, T., Jensenius, J. C., and Schwaeble, W. J. (1999a). Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. *The Journal of Immunology* **162**, 3481-3490.

Stover, C. M., Thiel, S., Lynch, N. J., and Schwaeble, W. J. (1999b). The rat and mouse homologues of MASP-2 and MAp19, components of the lectin activation pathway of complement. *The Journal of Immunology* **163**, 6848-6859.

Stover, C. M., Lynch, N. J., Dahl, M. R., Hanson, S., Takahashi, M., Frankenberger, M., Ziegler-Heitbrock, L., Eperon, I., Thiel, S., and Schwaeble, W. J. (2003). Murine serine proteases MASP-1 and MASP-3, components of the lectin pathway activation complex of complement, are encoded by a single structural gene. *Genes Immun.* **4**, 374-384.

Strait, R. T., Morris, S. C., Yang, M., Qu, X. W., and Finkelman, F. D. (2002). Pathways of anaphylaxis in the mouse. *J.Allergy Clin.Immunol.* **109**, 658-668.

Strunk, R. C., Eidlen, D. M., and Mason, R. J. (1988). Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. *J Clin.Invest* **81**, 1419-1426.

Stuart, G. R., Lynch, N. J., Day, A. J., Schwaeble, W. J., and Sim, R. B. (1997). The C1q and collectin binding site within C1q receptor (cell surface calreticulin). *Immunopharmacology* **38**, 73-80.

Summerfield, J. A., Ryder, S., Sumiya, M., Thursz, M., Gorchein, A., Monteil, M. A., and Turner, M. W. (1995). Mannose binding protein gene mutations associated with unusual and severe infections in adults. *Lancet* **345**, 886-889.

Summerfield, J. A., Sumiya, M., Levin, M., and Turner, M. W. (1997). Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. *BMJ* **314**, 1229-1232.

Summerfield, J. A. (2003). Clinical potential of mannose-binding lectin-replacement therapy. *Biochem.Soc.Trans.* **31**, 770-773.

Super, M., Thiel, S., Lu, J., Levinsky, R. J., and Turner, M. W. (1989). Association of low levels of mannan-binding protein with a common defect of opsonisation. *Lancet* **2**, 1236-1239.

Takahashi, K., Gordon, J., Liu, H., Sastry, K. N., Epstein, J. E., Motwani, M., Laursen, I., Thiel, S., Jensenius, J. C., Carroll, M., and Ezekowitz, R. A. (2002). Lack of mannose-binding lectin-A enhances survival in a mouse model of acute septic peritonitis. *Microbes.Infect.* **4**, 773-784.

Takeda, K., Hamelmann, E., Joetham, A., Shultz, L. D., Larsen, G. L., Irvin, C. G., and Gelfand, E. W. (1997). Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *The Journal of Experimental Medicine* **186**, 449-454.

Tannenbaum, H., Ruddy, S., and Schur, P. H. (1975). Acute anaphylaxis associated with serum complement depletion. *J Allergy Clin.Immunol* **56**, 226-234.

Taylor, P. R., Nash, J. T., Theodoridis, E., Bygrave, A. E., Walport, M. J., and Botto, M. (1998). A Targeted Disruption of the Murine Complement Factor B Gene Resulting in Loss of Expression of Three Genes in Close Proximity, Factor B, C2, and D17H6S45. *Journal of Biological Chemistry* **273**, 1699-1704.

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

Thiel, S., Holmskov, U., Hviid, L., Laursen, S. B., and Jensenius, J. C. (1992). The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. *Clin.Exp.Immunol.* **90**, 31-35.

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

Thiel, S., Petersen, S. V., Vorup-Jensen, T., Matsushita, M., Fujita, T., Stover, C. M., Schwaeble, W. J., and Jensenius, J. C. (2000). Interaction of C1q and Mannan-Binding Lectin (MBL) with C1r, C1s, MBL-Associated Serine Proteases 1 and 2, and the MBL-Associated Protein MAp19. *The Journal of Immunology* **165**, 878-887.

Thielens, N. M., Tacnet-Delorme, P., and Arlaud, G. J. (2002). Interaction of C1q and mannan-binding lectin with viruses. *Immunobiology* **205**, 563-574.

Thurman, J. M., Ljubanovic, D., Edelstein, C. L., Gilkeson, G. S., and Holers, V. M. (2003). Lack of a functional alternative complement pathway ameliorates ischemic acute renal failure in mice. *The Journal of Immunology* **170**, 1517-1523.

Tizard, I. R. (2000a). Veterinary Immunology: An Introduction, 6th ed. *W. B. Saunders Company*, p 176.

Tizard, I. R. (2000b). Veterinary Immunology: An Introduction, 6th ed. *W. B. Saunders Company*, pp 317-318.

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

Turnberg, D. and Botto, M. (2003). The regulation of the complement system: insights from genetically-engineered mice. *Mol.Immunol* **40**, 145-153.

Turner, M. W. (1996). Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Immunol Today* **17**, 532-540.

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

Ueki, A., Sai, T., Oka, H., Tabata, M., Hosokawa, K., and Mochizuki, Y. (1987). Biosynthesis and secretion of the third component of complement by human endothelial cells in vitro. *Immunology* **61**, 11-14.

Uemura, K., Saka, M., Nakagawa, T., Kawasaki, N., Thiel, S., Jensenius, J. C., and Kawasaki, T. (2002). L-MBP Is Expressed in Epithelial Cells of Mouse Small Intestine. *The Journal of Immunology* **169**, 6945-6950.

Urbaschek, B., Ditter, B., Becker, K. P., and Urbaschek, R. (1984). Protective effects and role of endotoxin in experimental septicemia. *Circ.Shock* **14**, 209-222.

Valdimarsson, H., Stefansson, M., Vikingsdottir, T., Arason, G. J., Koch, C., Thiel, S., and Jensenius, J. C. (1998). Reconstitution of opsonizing activity by infusion of mannan-binding lectin (MBL) to MBL-deficient humans. *Scand.J Immunol* **48**, 116-123.

Valdimarsson, H., Vikingsdottir, T., Bang, P., Saevarsdottir, S., Gudjonsson, J. E., Oskarsson, O., Christiansen, M., Blou, L., Laursen, I., and Koch, C. (2004). Human plasma-derived mannose-binding lectin: a phase I safety and pharmacokinetic study. *Scand.J Immunol* **59**, 97-102.

van den Berg, C. W., Hofhuis, F. M., Rademaker, P. M., and van Dijk, H. (1991). Induction of active immunological hypo/non-responsiveness to C5 in adult C5-deficient DBA/2 mice. *Immunology* **74**, 380-385.

van der Linden, P. W., Hack, C. E., Kerckhaert, J. A., Struyvenberg, A., and van der Zwan, J. C. (1990). Preliminary report: complement activation in wasp-sting anaphylaxis. *Lancet* **336**, 904-906.

Vandivier, R. W., Ogden, C. A., Fadok, V. A., Hoffmann, P. R., Brown, K. K., Botto, M., Walport, M. J., Fisher, J. H., Henson, P. M., and Greene, K. E. (2002). Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J.Immunol.* **169**, 3978-3986.

Volk, H. D., Reinke, P., Krausch, D., Zuckermann, H., Asadullah, K., Muller, J. M., Docke, W. D., and Kox, W. J. (1996). Monocyte deactivation--rationale for a new therapeutic strategy in sepsis. *Intensive Care Med.* **22 Suppl 4**, S474-S481.

Vorup-Jensen, T., Petersen, S. V., Hansen, A. G., Poulsen, K., Schwaeble, W., Sim, R. B., Reid, K. B. M., Davis, S. J., Thiel, S., and Jensenius, J. C. (2000). Distinct Pathways of Mannan-Binding Lectin (MBL)- and C1-Complex Autoactivation Revealed by Reconstitution of MBL with Recombinant MBL-Associated Serine Protease-2. *The Journal of Immunology* **165**, 2093-2100.

Wagner, S., Lynch, N. J., Walter, W., Schwaeble, W. J., and Loos, M. (2003). Differential Expression of the Murine Mannose-Binding Lectins A and C in Lymphoid and Nonlymphoid Organs and Tissues. *The Journal of Immunology* **170**, 1462-1465.

Wallis, R. (2002). Structural and functional aspects of complement activation by mannose-binding protein. *Immunobiology* **205**, 433-445.

Wallis, R., Shaw, J. M., Uitdehaag, J., Chen, C. B., Torgersen, D., and Drickamer, K. (2004). Localization of the serine protease-binding sites in the collagen-like domain of mannose-binding protein: indirect effects of naturally occurring mutations on protease binding and activation. *J Biol.Chem.* **279**, 14065-14073.

Ward, P. A. and Hill, J. H. (1970). C5 chemotactic fragments produced by an enzyme in lysosomal granules of neutrophils. *The Journal of Immunology* **104**, 535-543.

Weiler, J. M., Edens, R. E., Linhardt, R. J., and Kapelanski, D. P. (1992). Heparin and modified heparin inhibit complement activation in vivo. *The Journal of Immunology* **148**, 3210-3215.

Whaley, K. and Schwaeble, W. (1997). Complement and complement deficiencies. *Semin.Liver Dis.* **17**, 297-310.

Wichterman, K. A., Baue, A. E., and Chaudry, I. H. (1980). Sepsis and septic shock--a review of laboratory models and a proposal. *J.Surg.Res.* **29**, 189-201.

Windbichler, M., Echtenacher, B., Hehlgans, T., Jensenius, J. C., Schwaeble, W., and Mannel, D. N. (2004). Involvement of the lectin pathway of complement activation in antimicrobial immune defense during experimental septic peritonitis. *Infect.Immun.* **72**, 5247-5252.

Wong, N. K., Kojima, M., Dobo, J., Ambrus, G., and Sim, R. B. (1999). Activities of the MBL-associated serine proteases (MASPs) and their regulation by natural inhibitors. *Mol.Immunol* **36**, 853-861.

Yasuda, M., Takeuchi, K., Hiruma, M., Iida, H., Tahara, A., Itagane, H., Toda, I., Akioka, K., Teragaki, M., and Oku, H. (1990). The complement system in ischemic heart disease. *Circulation* **81**, 156-163.

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

Yurt, R. W., Leid, R. W., Jr., and Austen, K. F. (1977). Native heparin from rat peritoneal mast cells. *J Biol.Chem.* **252**, 518-521.

Zundel, S., Cseh, S., Lacroix, M., Dahl, M. R., Matsushita, M., Andrieu, J. P., Schwaeble, W. J., Jensenius, J. C., Fujita, T., Arlaud, G. J., and Thielens, N. M. (2004). Characterization of recombinant mannan-binding lectin-associated serine protease (MASP)-3 suggests an activation mechanism different from that of MASP-1 and MASP-2. *The Journal of Immunology* **172**, 4342-4350.

Publications

Windbichler, M., Echtenacher, B., Hehlgans, T., Jensenius, J. C., Schwaeble, W., and Mannel, D. N. (2004). Involvement of the lectin pathway of complement activation in antimicrobial immune defense during experimental septic peritonitis. *Infect.Immun.* **72**, 5247-5252.

Stover, C. M., Lynch, N. J., Hanson, S. J., Windbichler, M., Gregory, S. G., and Schwaeble, W. J. (2004). Organization of the *MASP2* locus and its expression profile in mouse and rat. *Mamm.Genome* **15**, 887-900.

Windbichler, M., Echtenacher, B., Jensenius, J. C., Schwaeble, W., and Mannel, D. N. Investigations on the involvement of the lectin pathway of complement activation in anaphylaxis. Manuscript submitted.