Oligomerization of mannan-binding lectin dictates binding properties and complement activation

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Non-standard abbreviations
CL-K1: collectin kidney 1
CL-L1: collectin liver 1
HSA: human serum albumin
K_D: dissociation constant
MAp: MBL-associated protein
MASP: MBL-associated serine protease
MBL: mannan-binding lectin
NHS: normal human serum
O/N: over night
PRM: pattern recognition molecule
rMBL: recombinant MBL
RT: room temperature

ABSTRACT

The complement system is part of the innate immune system and is involved in recognition and clearance of pathogens and altered-self structures The lectin pathway of the complement system is initiated when soluble collagen-region containing pattern recognition molecules (PRMs) bind to foreign or altered self-surfaces. Associated with the collagen stems of these PRMs are three mannan-binding lectin (MBL)-associated serine proteases (MASPs) and two MBL-associated proteins (MAps). The most studied of the PRMs, MBL, is present in serum mainly as trimeric and tetrameric oligomers of the structural subunit. We hypothesized that oligomerization of MBL may influence both the potential to bind to microorganisms and the interaction with the MASPs and MAps, thus influencing the ability to initiate complement activation. When testing binding at 37°C, we found higher binding of tetrameric MBL to *Staphylococcus aureus* than trimeric and dimeric MBL. In serum, we found that tetrameric MBL was the main oligomeric form present in complexes with the MASPs and MAp44. Such preference was confirmed using purified forms of recombinant MBL (rMBL) oligomers, where tetrameric rMBL interacted stronger with all of the MASPs and MAp44, compared to trimeric MBL. As a direct consequence of the weaker interaction with the MASPs, we found that trimeric rMBL was inferior to tetrameric rMBL in activating the complement system. Our data suggests that the oligomeric state of MBL is crucial both for the binding properties and the effector function of MBL.

INTRODUCTION

The complement system is involved in recognition and clearance of pathogens and altered-self structures (1). It is thus an important and integral part of the innate immune system and deficiencies of the complement system can lead to diseases. The complement system may be viewed as a cascade enzymatic system composed of multiple serine proteases as well as proteins enhancing or inhibiting these proteases. The complement system is divided into three initiating pathways: the lectin, the classical, and the alternative pathway. Although different in the initiating processes, the ultimate outcome is to eradicate dangerous material such as bacteria, viruses or to remove altered-self to sustain homeostasis (1). A major function of the alternative pathway is to potentiate the other pathways, while the lectin and the classical pathway actively recognize pathogen-associated molecular patterns via pattern recognition molecules (PRMs). The lectin pathway encompasses a number of PRMs including four collectins (mannan-binding lectin (MBL), collectin kidney 1 (CL-K1), collectin liver 1 (CL-L1) (2) and heterocomplexes of CL-K1 and CL-L1 (CL-LK) (3)) and three ficolins (M-, L- and H-ficolin, also known as Ficolin-1, -2 and -3, respectively) (4). All of these PRMs interact with three different proteases, originally named MBL-associated serine proteases, MASPs (MASP-1, MASP-2 and MASP-3) and two so-called MBL-associated proteins, MAps (MAp44 and MAp19) (4). In contrast, the classical system only contains one PRM, the C1q molecule, which together with its associated serine proteases C1r and C1s forms the C1 complex (5).

Clustering of PRM/MASP complexes on ligand surfaces leads to activation of the lectin pathway. Thus the zymogen MASP molecules must be positioned close to each other (juxta-positioning) for efficient activation to take place (6). Activated, MASP-1 efficiently cleaves and activates MASP-2 (7-9), which then cleaves the complement components C4 and C4-bound C2, generating the C3 convertase (C4bC2a). MASP-1 also contributes by cleaving C4-bound C2 (10).

The polypeptide chains of the collectins and ficolins are composed of similar motifs: a short N-terminal containing cysteines involved in oligomerization of the polypeptide chain followed by a collagen-like region responsible for formation of a collagen-like triple helix providing for MASP/MAp interaction, and finally a C-terminal ligand recognition domain (carbohydrate recognition domains for the collectins, and fibrinogen-like domains for the ficolins). For MBL and the ficolins three identical polypeptide chains trimerize into structural subunits, which further oligomerizes into higher oligomer (11). CL-LK is an exception as it is composed of two different polypeptide chains (CL-K1 and CL-L1) forming the structural subunit and the higher oligomers (3).

The dissociation constant (K_D) of the single carbohydrate recognition domain of MBL towards a target is in the low millimolar region (12), whereas the avidity of the whole molecule for larger patterns of carbohydrates is dependent on the degree of oligomerization, reaching low nanomolar K_D values for the higher oligomers (13, 14). Also the interaction between MBL and the MASPs and MAps might be dependent on the oligomerization of MBL. It has previously been suggested that, e.g. the trimeric oligomer of MBL interacts mainly with MASP-1 (15, 16), although this is not evident in another study where the same affinity was observed for interaction with the trimer and the tetramer (17).

Here we investigate how oligomerization influences the ability of MBL to bind to *Staphylococcus aureus*, how oligomerization of MBL influences the interaction with the MASPs and MAps, and how oligomerization affect the ability to activate the lectin pathway of the complement system. In contrast to certain previous studies, we observe a significant difference between the interactions of trimeric and tetrameric MBL with the MASPs and MAps, and as a likely consequence, we also observe a clear difference in activation potential.

MATERIALS AND METHODS

Buffers and antibodies. TBS was 10 mM Tris-HCl, 140 mM NaCl, 15 mM NaN_3, pH 7.4. TBS/Tw was TBS, 0.05% (v/v) Tween-20. TBS/Ca^{2+} was TBS, 5 mM $CaCl_2$. Calcicum containing running buffer for anion exchange chromatography was 10 mM Tris, 5 mM $CaCl_2$, pH 8.5. EDTA running buffer was 10 mM Tris, 50 mM NaCl, 10 mM EDTA, pH 8.5. PBS was 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4. Blocking buffer was TBS with 1 mg human serum albumin (HSA) per ml. TBS/Tw/Ca^{2+} was TBS/Tw, 5 mM $CaCl_2$. Dilution buffer for time resolved immune fluorometric assay (TRIFMA) was 10 mM Tris-HCl, 1 M NaCl, 5 mM $CaCl_2$, 15 mM NaN_3, pH 7.4, containing 1 mg HSA/ml, 0.05% (v/v) Triton X-100 and 100 µg/ml of of rat IgG (Lampire), mouse IgG (Lampire), bovine IgG (Lampire) and heat-aggregated human IgG (Beriglobulin, CSL Behring; heat-aggregated by incubating at 63^{o}C for 30 min, followed by centrifugation at 3,000*g* and recovery of the supernatant). Europium buffer was TBS/Tw, 25 µM EDTA. Carbonate coating buffer was 15 mM Na_2CO_3, 35 mM, $NaHCO_3$, 15 mM NaN_3, pH 9.6. Elution buffer was 10 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, 100 mM GlcNAc (Sigma), 0.05% (v/v) Tween-20, 15 mM NaN_3, pH 7.4. Barbital buffer was 4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4. SDS-PAGE sample buffer was 30 mM Tris-HCL, 10% (v/v) glycerol, 8 M urea, 3% (w/v) SDS, 0.1% (w/v) bromphenol blue, pH 8.9. Western Blotting (WB) buffer was TBS/Tw, 1 mM EDTA with 1 mg HSA/ml. HEPES buffer was 10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4.
Mouse monoclonal antibody 5F5 reacts with a domain (the CCP1 domain) shared by MASP-1, -3 and MAp44 (18). Mouse monoclonal antibody 8B5 reacts specifically with MASP-2 (19). Mouse monoclonal antibody 2D5B6 reacts specifically with MAp44 (18) (Hycult Biotech). Rat monoclonal antibody 38:12-3 reacts specifically with MASP-3 (10) (Hycult Biotech). Mouse/rat monoclonal antibody 4D12H2 reacts specifically with MAp19 (20).

The affinity purified rabbit anti-MASP-1 reacts specifically with MASP-1 and was produced by immunization with the serine protease (SP) part of MASP-1 as follows. Briefly, the SP domain of MASP-1 was expressed in E. coli and purified to homogeneity analogously to the larger catalytic fragment of MASP-1 (21). Rabbits were immunized with 400, then 3-times 200 µg doses of MASP-1 SP, and the antiserum was collected. Antibodies of the antiserum were purified by protein A chromatography followed by affinity purification on a column with the immobilized antigen.

Mouse monoclonal antibody 131-1 (BioPorto) reacts specifically with MBL (21+1).

Fractionation of MBL oligomers. rMBL was produced and purified as described previously (22+1). To fractionate the product into the different oligomeric forms we first precipitated the protein with 4.5% or 5.5% (depending on the specific procedure – see below) PEG-6000 in TBS/Tw for 1 hour at room temperature (RT) followed by centrifugation at 10,000*g* for 10 min. The supernatant was diluted in calcicum running buffer and oligomers of MBL were fractionated on a MonoQ 5/50GL column using calcicum running buffer and applying a linear gradient to 0.5 M NaCl. Dimer and trimer-containing fractions were pooled, made 10 mM in EDTA by adding 100^{th} volume of 1 M EDTA and run again on the MonoQ now using EDTA running buffer and applying a linear gradient to 0.5 M NaCl. For the preparation of tetrameric MBL, the 5.5% PEG-6000 pellet was redissolved and large oligomers precipitated with 4% PEG-6000 as above. The supernatant was run on MonoQ under calcium-containing conditions as described above. Tetramer-containing fractions were pooled, made 10 mM in EDTA by adding 100^{th} volume of 1 M EDTA and fractionated as above in EDTA containing buffer. The oligomeric state of the rMBL in the fractions was visualized on silver stained SDS-PAGE gels. The protein concentrations of the rMBL oligomers were measured on a Nanodrop 1000 spectrophotometer.

Assay for MBL. Microtiter plate wells were coated with 1 µg monoclonal anti-MBL antibody 93C (23) in PBS. After incubation over night (O/N) at RT, the wells were blocked with blocking buffer, washed thrice with TBS/Tw/Ca^{2+}, and samples diluted in the dilution buffer were subsequently added. After incubation O/N at 4°C, the wells were washed thrice with TBS/Tw/Ca^{2+}, and added biotinylated monoclonal anti-MBL antibody (biotin-131-1) at 10 ng per ml TBS/Tw. Following incubation for 2 hours at RT, the wells were washed and incubated with europium-labelled streptavidin (Perkin Elmer) at 0.1 µg per ml europium buffer. After 1 hour of incubation, the wells were washed, added enhancement solution (Perkin Elmer) and time-resolved fluorescence was read on a VICTOR5 plate reader (Perkin Elmer).

C4 activation assay. Microtitre plate wells were coated with mannan (Sigma) at 10 µg per ml carbonate coating buffer, blocked and washed. rMBL oligomers were diluted in dilution buffer containing MBL deficient serum at a final serum concentration of 0.4% (v/v). The samples were incubated on the mannan coat O/N at 4°C. After wash, the wells were eluted with 100 µl elution buffer. The eluate was diluted 10 fold in TBS/Tw and the concentration of rMBL was measured in the MBL assay as described above. In a parallel setup the capacity of the MBL/MASP complexes to activate C4 were estimated (24). Instead of eluting MBL complexes purified C4 at 2 µg per ml barbital buffer was added to the wells. After incubation for 1.5 hours at 37°C, the wells were washed and added 1 µg biotin-anti-C4 (Sigma) per ml TBS/Tw/Ca^{2+}, incubated for 2 hours at RT, washed and developed with europium-labelled streptavidin as above.

rMBL binding to mannan at 4°C and 37°C. rMBL oligomers were diluted to 100 ng/ml in TBS/Tw/Ca^{2+}, added to mannan coated microtiter wells and incubated O/N at 4°C or 30 min, 1 hour, 2 hours, 5 hours or O/N at 37 °C. After wash, proteins in the wells were eluted as described above and the eluted MBL measured in the MBL assay.

Binding of MBL to *Staphylococcus aureus*. *S. aureus* (serotype WOOD) was cultured in Todd-Hewitt broth O/N at 37°C under 5% CO_2. The bacteria were fixed with formaldehyde at a final concentration of 1% (v/v).

Residual aldehyde groups were blocked with 0.1 M ethanolamine (pH 9). The bacteria were sedimented (2000*g*, 15 min, 5°C), washed thrice in TBS and stored at 4°C. rMBL oligomers were diluted to 450 ng/ml in TBS/Tw/Ca^{2+} containing MBL deficient serum at a final serum concentration of 10% (v/v). One hundred µl of diluted rMBL oligomers were added to 10^9 bacteria and incubated on rotation for 2 hours at 37°C. The bacteria were sedimented and MBL in the supernatant was quantified as described above.

Immunoblotting of MBL/MASP complexes. 50 µl of normal human serum (NHS) was diluted with an equal volume of dilution buffer and added to microtiter wells coated with 1 µg 5F5 (monoclonal antibody reacting with the common CCP1 domain of MASP-1, -3 and MAp44), 8B5 (anti-MASP-2), 2D5B6 (anti-MAp44), 38:12-3 (anti-MASP-3), 4D12H2 (anti-MAp19), 131-1 (anti-MBL), rabbit anti-MASP-1, normal mouse IgG, normal rat IgG or normal rabbit IgG per ml carbonate coating buffer and incubated O/N at 4°C. After incubation and wash, the bound complexes were eluted consecutively from 23 identical wells in a total of 100 µl 50% (v/v) SDS-PAGE sample buffer in TBS. The proteins in the eluates were fractionated by SDS-PAGE, blotted onto nitrocellulose (Bio-Rad), the membrane blocked with TBS, 0.1% (v/v) Tween-20, washed and incubated with biotin-131-1 (anti-MBL) in WB buffer, followed by streptavidin-HRP (Sigma) in WB buffer without NaN_3. Blots were developed with Supersignal West Dura extended duration substrate (Pierce) and the signal recorded by a CCD camera.

MAp44, MASP-1, MASP-2 and MASP-3 interaction with rMBL oligomers in serum. rMBL oligomers were diluted to 500 ng/ml in TBS/Tw/Ca^{2+} containing 100 µg/ml of each of the following (for reduction of background): rat IgG, mouse IgG, bovine IgG and heat-aggregated human IgG, and also containing 5% (v/v) MBL deficient serum. Samples were incubated for 2 hours at RT and added to microtitre wells coated with 1 µg/ml 2D5B6 (anti-MAp44), 8B5 (anti-MASP-2), 38:12-3 (anti-MASP-3) or rabbit anti-MASP-1. Following incubation O/N at 4°C, the wells were washed thrice in TBS/Tw/Ca^{2+} and incubated for 2 hours at RT with 1 µg/ml biotinylated MAb 93C (anti-MBL) in dilution buffer. The plates were developed as above. Data was compared using one-way ANOVA with Tukey's post hoc test (GraphPad Prism 5.0).

RESULTS

MBL oligomers, binding and activation. Using a combination of PEG precipitation and ion exchange chromatography we separated rMBL into distinct oligomers (**Fig. 1A**). In the dimeric MBL preparation (Fig. 1A, lane 2) we observed doublet bands: a band of approximately 150 kDa, fitting with the theoretical weight of 144 kDa for 6 non-modified polypeptide chains of 24 kDa each, and a band with slightly higher molecular weight. The two bands may represent variations in post-translational modifications. This was not examined any further. Trimeric MBL (Fig. 1A, lane 3) migrated with an apparent weight of ~250 kDa and contained only traces of dimer MBL. Tetrameric MBL (Fig. 1A, lane 4) migrated with a higher weight than trimeric MBL, but also contained traces of trimeric and pentameric MBL. We were not able to produce pure preparations of any higher oligomeric forms.

During the above described purification the concentrations of the various MBL oligomers were determined by OD_{280}, i.e., the concentration of polypeptide chains. To be able to compare the complement activating potential (see below) of the various MBL oligomers on mannan coated microtiter wells it was essential to be able to estimate the amount of MBL actually bound in the wells (and not merely express the capacity as compared to how much MBL was added to the well). To be able to estimate the concentration bound in mannan coated microtiter wells we eluted the bound MBL with buffer containing EDTA and GlcNAc and used a solid phase MBL assay based on two anti-MBL antibodies to measure the concentration of MBL in the eluate. The assay we used measures the concentration of MBL polypeptide chains independently of the oligomerization (**Fig. 1B**). As previously described, the selection of the monoclonal anti-MBL antibodies used is crucial for the equal estimation of the different oligomers (19).

The complement activation potential of the different oligomers was tested on a mannan surface using MBL deficient serum as MASP source (**Fig. 1C**). Interaction with the mannan surface was at 4^{o}C O/N in order to allow the likely lower avidity interactions of the smaller oligomers with the ligand. At the chosen conditions, the amount of dimeric MBL bound to the mannan surface was equivalent to that of the higher oligomeric forms (**Fig. 1D**). On the other hand, when binding was estimated at 37°C, a clear difference was seen between the different oligomeric forms, i.e. much lower binding was seen for trimeric and dimeric rMBL as compared to tetrameric rMBL (**Fig. 1E**). This binding was time dependent, reaching a maximum signal after approximately 5 hours. At the conditions used here, ensuring quantitatively equivalent binding of the various oligomers at the starting point of the C4 deposition assay, much less C4 fragment deposition was accomplished by the trimeric MBL compared to the tetrameric MBL. Dimeric MBL was not able to procure C4 deposition on the mannan surface (**Fig. 1C**).

To compare the binding of the various forms of MBL to a physiological relevant surface, we added rMBL oligomers to MBL deficient serum and detected the level of MBL binding to *S. aureus*. As can be seen in **Fig. 1F** more tetrameric rMBL bound to the bacteria compared to the less oligomeric forms. Approximately 30% of the added tetrameric rMBL was bound, whereas less than 10% of the trimeric rMBL was bound. Dimeric rMBL did not bind to the bacteria.

Analysis of MBL/MASP complexes in serum. MBL/MASP (and MBL/MAp) complexes in serum were affinity purified on microtiter wells coated with anti-MASP or anti-MAp specific antibodies. The complexes were eluted, separated on SDS-PAGE, Western blotted and the blot was developed for MBL. Wells coated with anti-MBL bound the three main forms, i.e. trimer, tetramer and higher forms, found in serum (Fig. 2A and Fig. 2B, right lane). Remarkably, the results illustrated by the other lanes demonstrate that only miniscule amounts of trimeric MBL was present in the eluates from wells coated with anti-MASP-2, anti-MASP-3, anti-MAp44 or antibody reacting with the common part of MASP-1/MASP-3/MAp44) (Fig. 2A) or from wells coated with anti-MASP-1 (Fig. 2B), indicating that in serum, the trimeric MBL interacts only weakly with all of the MASPs and MAp44 as compared to tetrameric and pentameric MBL. Microtiter wells coated with normal mouse or rat Ig served as negative controls for our monoclonal mouse (anti-MAp44, anti-MASP-1/-3/MAp44, anti-MBL) and rat (anti-MASP-2, anti-MASP-3) antibodies (Fig. 2A) and wells coated with normal rabbit as control for the rabbit anti-MASP-1 (Fig. 2B). No signals were seen in the negative controls.

Formation of MBL/MASP complexes. The ability of dimeric, trimeric and tetrameric rMBL to associate with MASPs in MBL deficient serum was tested. The various forms were added to MBL deficient serum and subsequently added to microtiter wells coated with specific antibodies. Capturing complexes using antibodies recognizing MAp44 (**Fig. 3A**), MASP-2 (**Fig. 3B**), MASP-1 (**Fig. 3C**) or MASP-3 (**Fig. 3D**) and subsequently developing for MBL, showed a clear difference between trimeric and tetrameric rMBL, with less complexes of trimeric rMBL being captured (**Fig. 3A-D**). Neither MAp44 nor the MASPs were found in complex with the added dimeric rMBL.

DISCUSSION

Variable oligomerization of structural subunits is an inherent feature of all the complement activating PRMs of the lectin pathway of complement. The prominent MBL forms found in human serum are trimers and tetramers (as also demonstrated in **Fig. 2A**, right lane), which together comprise about 70% of the serum MBL (6, 17). The remaining MBL is mainly present as higher oligomers. It is known for human MBL, that higher oligomerization leads to higher ligand avidity (14) and the possibility to associate with more than one MASP or MAp dimer (6, 25). In the present report we examine in more detail the effects of oligomerization of MBL on ligand interaction, association with MASPs and MAp, and complement activation.

We observe that tetrameric rMBL activates complement more efficiently on a mannan surface compared to trimeric and dimeric rMBL (**Fig. 1C**). To ensure equivalent levels of the different rMBL oligomers bound to the mannan surface at the initiation of our C4 deposition assay, the incubation was done at 4°C O/N. The subsequent incubation with C4 is conducted at 37°C for 1.5 hours. Thus, the initial rMBL surface levels are the same but the attachment of the various forms at the following step may be affected, and this may be a contributing factor to the lower complement activation seen by dimeric and trimeric rMBL compared to tetrameric rMBL (**Fig. 1C**).

The mannan surface used in the microtiter-well-based assays is a substitute for yeast surfaces. The dependence of oligomerization for binding to ligands was also seen when studying whole bacteria, where tetrameric rMBL also binds stronger compared to the lower oligomer forms (**Fig. 1F**).

In MBL assays based on catching MBL on a mannan surface and developing for bound MBL, higher oligomer forms are more efficiently measured, which would skew the measurement of the lower forms investigated in this study (23). The combination of monoclonal antibodies used in the present study was previously found capable of determining not only functional MBL but also the aberrant MBL in persons with MBL allotypes associated with MBL deficiency (19). In our study we find that this combination also measure different recombinant MBL oligomers with equal efficiency (**Fig. 1B**).

MASPs and MAp form homodimers and both the trimeric and the tetrameric MBL oligomers form 1:1 complexes with the MASP dimers or the MAp dimers (17). Theoretically, this corresponds to 100% occupancy of the four collagen binding sites present in a MASP dimer (26) for tetramic MBL, and 75% for trimeric MBL. Intuitively, this suggests a difference in the interaction properties of tetrameric and trimeric MBL towards the associated enzymes and proteins.

We observe a clear dependence of oligomerization on the ability of MBL to interact with MASPs. We were able to purify MBL/MASP and MBL/MAp44 complexes from NHS using specific antibodies targeting the MASPs or MAp44 and visualize the oligomer forms of the bound MBL by eluting the MBL and analysis via Western blotting (**Fig. 2A and Fig. 2B**). Even though trimers constitute a significant proportion of serum MBL (~35% (6)), and as seen in eluates from the anti-MBL coated wells, only minimal amounts of complexes containing trimeric MBL were purified on MASP or MAp44 specific antibodies. This finding was substantiated when adding purified oligomeric forms of rMBL to MBL deficient serum followed by detection of the MAp44, MASP-2, MASP-3 or MASP-1 in complex with the added rMBL (**Fig. 3A-D**). We found that significantly less trimeric MBL could be recovered in complex with MAp44, MASP-2, MASP-3 or MASP-1 compared to that of tetrameric MBL. Dimeric rMBL did not lead to complexes with MASPs or MAp44.

Trimeric and tetrameric MBL purified from human serum were previously shown to interact equally well with the MASPs when analyzed by surface plasmon resonance, i.e., measured by interaction of MASP to immobilized MBL (17). Also, ^{35}S-labeled rat MBP-A binding to coated recombinant CUB1-EGF-CUB2 domains of MASP-1 or MASP-2 was equally well inhibited by trimeric and tetrameric MBL-A, but less by dimeric MBL (27). In the present report we test the interaction properties in serum which may give results closer to the physiological situation.

As an obvious consequence of the observed binding properties, trimeric rMBL was less effective in activating the lectin pathway, whereas dimeric was nearly completely incapable of activating (**Fig. 1C**). This contradicts the rat scenario where trimers activates complement similar to tetramers (28), but agrees with studies using plasma purified MBL where a preparation of mainly tetrameric MBL activated better than a preparation of trimeric and tetrameric MBL (29).

In conclusion, tetrameric MBL seems to be a more effective PRM compared to trimeric MBL, as tetrameric MBL not only shows superior binding to carbohydrate patterns, but also interact stronger with the MASPs. One is left wondering what might be the purpose of the lower oligomeric forms. Different oligomeric forms are also seen for the other recognition molecules of the lectin pathway, e.g. a range of oligomers is seen for H-ficolin (30) and CL-LK (3) and two forms of oligomers is seen for L-ficolin (Krarup A, Thiel S, Hansen A, Fujita T, Jensenius JC. L-ficolin is a pattern recognition molecule specific for acetyl groups. J Biol Chem. 2004;279(46):47513-9). Thus, our conclusions might be relevant for all of the lectin pathway recognition molecules.

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Figure 1. Preparations of different oligomers of MBL and estimation of their binding and complement activating efficacy. (A) rMBL was fractionated into distinct oligomeric forms using PEG precipitation and anion exchange chromotography. Molecular weight markers are seen in lane 1, dimeric rMBL in lane 2, trimeric rMBL in lane 3, and tetrameric rMBL in lane 4. **(B)** The different oligomers were detected equally well based on weight in a sandwich type TRIFMA MBL assay. Representative of two individual experiments. **(C)** Activation potential of the oligomeric MBL forms shows that tetrameric rMBL activates complement stronger than the lower oligomeric forms. The amount of MBL eluted (i.e. the MBL that had actually bound on the mannan) from the wells is shown on the x-axis and the signal from C4b deposited in the corresponding wells are on the y-axis. Representative of two individual experiments. **(D)** The different oligomeric rMBL forms bound equally well to mannan at 4°C O/N. Mean and SD of three experiments. **(E)** At 37°C rMBL binding to mannan is dependent on oligomerization and time of incubation in the mannan-coated wells (time given on x-axis). Mean and SEM of two experiments. **(F)** Binding of the MBL oligomers to *S. aureaus*. The MBL oligomers were added to MBL deficient serum and the amount of MBL bound at 37°C to the bacteria were analysed. Mean and SEM of two experiments.

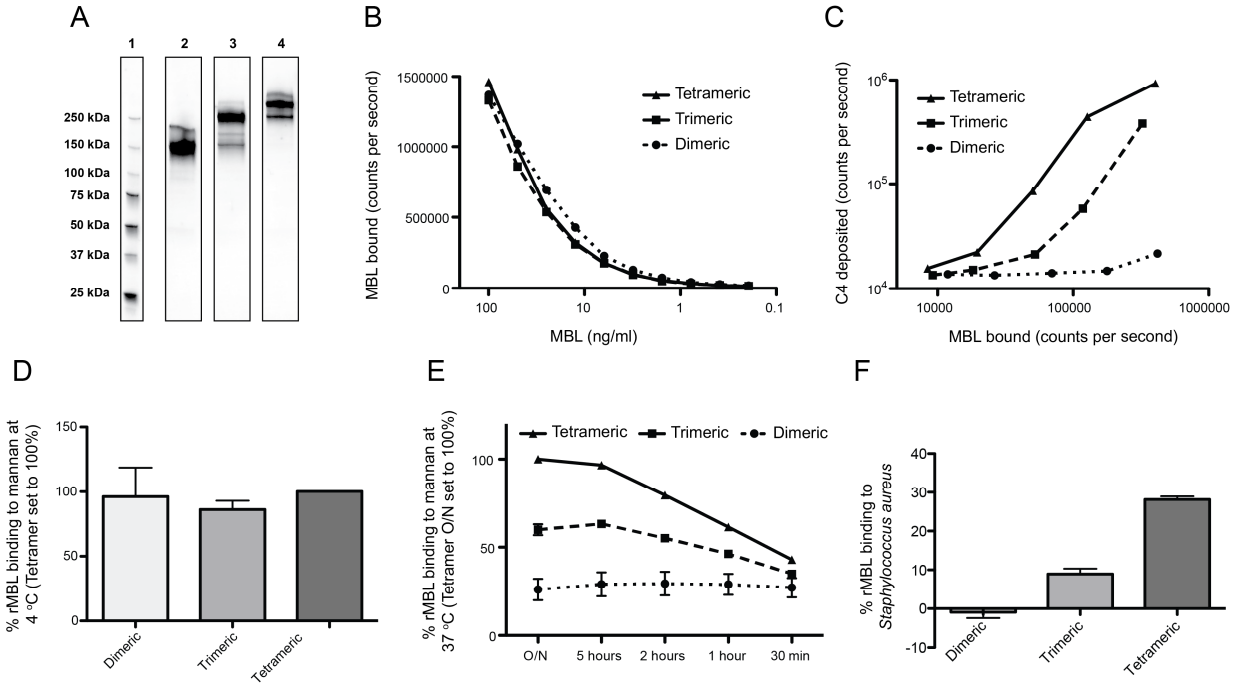

Figure 2. MBL/MASP or MBL/MAp complexes affinity purified from serum. Microtiter wells coated with anti-MASP or anti-MAp antibodies were incubated with serum. After wash any bound MBL/MASP or MBL/MAp complexes were eluted and examined by Western blotting developed with anti-MBL antibody**. (A)** Complexes were purified on anti-MAp44, anti-MASP-2, anti-MASP-1/-3/MAp44 (targeting the CCP1 domain in common for MASP-1, MASP-3 and MAp44) and anti-MASP-3. Normal mouse and rat IgG coats served as negative controls. Eluate from wells coated with anti-MBL antibody (right hand side of the blot) show the total of MBL oligomers present in the serum. As compared to the anti-MBL coated wells no complexes containing trimeric MBL were eluted from wells coated with the anti-MASP and anti-MAp antibodies. Representative of two individual experiments using serum and one experiment using re-calcified plasma**. (B)** Complexes were purified on wells coated with rabbit anti-MASP-1 and rabbit IgG coat served as negative control. The signal from anti-MBL coats are shown on the right hand side of the blot. As compared to the anti-MBL coated wells no complexes containing trimeric MBL were eluted from wells coated with the anti-MASP-1 antibody. Representative of two individual experiments using serum.

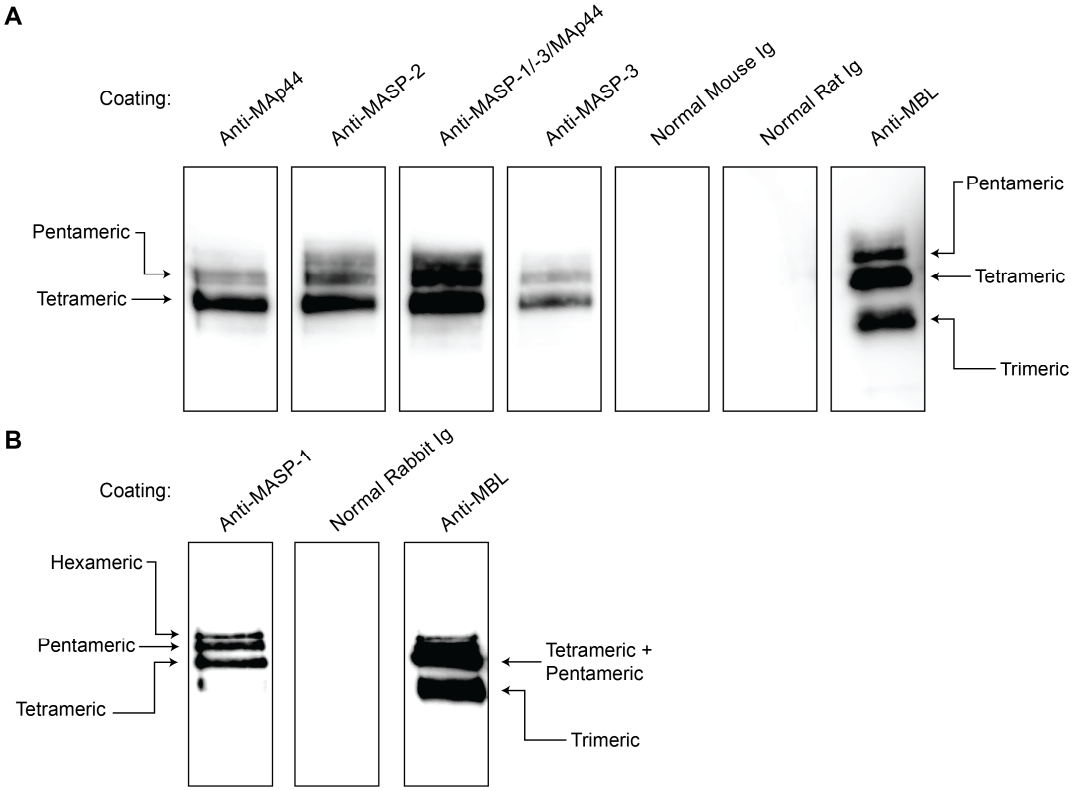

Figure 3. Tetrameric rMBL binds more MASPs and MAp. (A-D) Oligomeric forms of rMBL were added to MBL deficient serum. The formed complexes were caught in wells coated with anti-MAp44 (A), anti-MASP-2 (B), anti-MASP-1 (C) or anti-MASP-3 (D) and developed with anti-MBL antibody. For comparison, the signal obtained with the tetramer rMBL is set to 100%. Data are shown as means with SD of three separate experiments. Data was analysed using one-way ANOVA with Tukey's post hoc test. ** p <0.01, *** p <0.001.