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Co-Complexes of MASP-1 and MASP-2 Associated with the Soluble Pattern-Recognition Molecules Drive Lectin Pathway Activation in a Manner Inhibitable by MAp44

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The lectin pathway of complement is an integral component of innate immunity. It is activated upon binding of mannan-binding lectin (MBL) or ficolins (H-, L-, and M-ficolin) to suitable ligand patterns on microorganisms. MBL and ficolins are polydisperse homo-oligomeric molecules, found in complexes with MBL-associated serine proteases (MASP-1, -2, and -3) and MBL-associated proteins (MAp19 and MAp44). This scenario is far more complex than the well-defined activation complex of the classical pathway, C1qC1r2C1s2, and the composition of the activating complexes of the lectin pathway is ill defined. We and other investigators recently demonstrated that both MASP-1 and MASP-2 are crucial to lectin pathway activation. MASP-1 transactivates MASP-2 and, although MASP-1 also cleaves C2, MASP-2 cleaves both C4 and C2, allowing formation of the C3 convertase, C4bC2a. Juxtaposition of MASP-1 and MASP-2 during activation must be required for transactivation. We previously presented a possible scenario, which parallels that of the classical pathway, in which MASP-1 and MASP-2 are found together in the same MBL or ficolin complex. In this study, we demonstrate that, although MASP's do not directly form heterodimers, the addition of MBL or ficolins allows the formation of MASP-1–MASP-2 co-complexes. We find that such co-complexes have a functional role in activating complement and are present in serum at varying levels, impacting on the degree of complement activation. This raises the novel possibility that MAp44 may inhibit complement, not simply by brute force displacement of MASP-2 from MBL or ficolins, but by disruption of co-complexes, hence impairing transactivation. We present support for this contention. The Journal of Immunology, 2013, 191: 1334–1345.

The complement system is a crucial component of innate immunity that is central to health and disease (1). It consists of three pathways of activation, which converge at the level of C3 convertase formation, leading to generation of C5 convertase and the subsequent formation of the terminal membrane attack complex (2). The three pathways of activation are the classical pathway, the alternative pathway, and the lectin pathway.

The classical and the lectin pathways are conceptually very similar. The classical pathway is initiated by a defined complex, C1, composed of the recognition molecule C1q with a tetramer of two serine proteases, C1r2C1s2. C1q itself is a monodisperse homo-oligomeric molecule composed of six heterotrimeric subunits, each comprising an A-chain, a B-chain, and a C-chain. Upon binding of the C1 complex to immune complexes, the two C1r autoactivate and, in turn, activate the two C1s, which can then cleave C4 and C2, forming the C3 convertase C4bC2a.

Although the lectin pathway is conceptually similar, the composition of the activating complexes is far more complex and less well characterized than that of the classical pathway. Four pattern-recognition molecules (PRMs), mannan-binding lectin (MBL), H-ficolin, L-ficolin, and M-ficolin, associate with three proteases, MBL-associated serine protease (MASP)-1, MASP-2, and MASP-3, as well as two MBL-associated proteins (MAps), MAp19 (also termed sMAP) and MAp44 (also known as MAP-1) (3, 4). MBL and ficolins are highly polydisperse homo-oligomers of homotrimeric subunits. Thus, MBL is found in a number of oligomeric forms in serum, ranging from dimers to hexamers and even higher-order oligomers, the most predominant being trimers (9 polypeptide chains) and tetramers (12 polypeptide chains) (5). A similar scenario presents for the ficolins, although the degree of oligomerization varies among the three.

The MASP and MAp are generated from two genes: MASP-1, MASP-3, and MAp44 are alternative splice products of MASP1, whereas MASP-2 and MAp19 are alternative splice products of MASP2 (3, 4). It is known that MASP-2 is necessary for lectin pathway function, by virtue of its ability to cleave both C4 and C2 (6). Although MASP-2 was also reported to be sufficient in itself, as a result of its ability to autoactivate (7, 8), it was recently found that MASP-1 is crucial in transactivating MASP-2 under physiological circumstances (9–11). Indeed, MASP-1 was found to autoactivate and to cleave MASP-2 much more efficiently than MASP-2 itself (12). Additionally, MASP-1 cleaves auxiliary C2 for the C3 convertase formation (10, 13). Based on studies in knockout mice, MASP-1 and MASP-3 were suggested to be responsible for cleavage of pro-iD to active factor D; the latter was
also proposed to cleave fB directly (14, 15). However, the exact role is still unclear, because we recently found that a patient deficient in both MASP-1 and MASP-3 retained a functional alternative pathway (9). MASP-3 has an important function during development because its absence causes the so-called 3MC syndrome (16, 17). A suggested complement-regulatory role for MAp19 (18) could not be confirmed by us (19), whereas we found that MAp44 competitively inhibited binding of MASP-2 to MBL and, hence, attenuated lectin pathway activity (3). This activity of MAp44 was subsequently confirmed by other investigators, both in vitro (4) and in vivo (20).

A number of possible scenarios allowing transactivation of MASP-2 by MASP-1 present themselves. The first possibility, and the most simple, would be for MASP-1 and MASP-2 to interact directly. However, the MASPs and MAps reportedly only form homodimers, antiparallel tail-to-tail, by virtue of interactions in their CUB–EGF–CUB regions (21). This is somewhat surprising, considering the high homology of this region between MASP-1 and MASP-2 and its identity among MASP-1, MASP-3, and MAp44 (Supplemental Fig. 1). To our knowledge, no data have been presented to document that MASPs cannot, or do not, form heterodimers. The second possibility would be for MASP-1 and MASP-2 to associate in the same MBL or ficolin complex. This scenario is partially analogous to that of the classical pathway, with the important difference that a tetramer composed of two heterodimers of C1r and C1s occur in the C1 complex. We recently demonstrated that MASP-1 and MASP-2 can indeed form co-complexes with MBL and that co-complexes with MBL and/or ficolins can be detected in serum (9), despite previous suggestions that this was not the case (22, 23). The third possible scenario is that MASP-1 and MASP-2 could be found in distinct complexes with MBL and/or ficolins and that such complexes would be brought into close proximity upon binding to ligand surfaces, as conceptually suggested previously (24). Of note, these scenarios are not necessarily mutually exclusive. In this study, we examined the first and second scenarios.

We find that coexpression of MASP-1 and MASP-2 results in a detectable level of MASP-1/2 heterodimer. However, we do not detect significant levels of such heterodimers in serum. Presumably, the dimers are very stable, and exclusive expression in distinct tissues may cause significant formation of only homodimers in vivo. In contrast, the addition of MBL or ficolins allows the formation of MASP-1 and MASP-2 co-complexes, as well as the other combinations of MASPs and MAps. We demonstrate that these complexes have a functional role, because co-complexes of MASP-1 and MASP-2 efficiently activate complement. Such co-complexes of MASP-1 and MASP-2 are present in serum, and their abundance varies from individual to individual, influencing the degree of complement activation. Intriguingly, this raises the novel possibility that MAp44 inhibits complement, not simply by complete displacement of MASP-2 from MBL, but by displacement of either MASP-1 or MASP-2, disrupting such co-complexes and, hence, impairing transactivation. Indeed, we present evidence supporting this idea.

Materials and Methods

Blood samples

Blood was obtained from apparently healthy Danish blood donors after informed consent and according to the requirements of the Helsinki Declaration. Similarly, blood was obtained from a MASP-2–deficient patient, homozygous for the D120G mutation, which was described previously in another patient (25).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 software.

Recombinant and purified proteins

Recombinant MASPs and MAps were produced by Lipofectamine 2000–mediated plasmid transfection of HEK293F FreeStyle cells in 293F Expression Medium (Invitrogen), as described in detail (26), using the constructs presented (3, 9, 19). For coexpressions, plasmids were mixed 1:1 before incubation with Lipofectamine 2000 and subsequent transfection. Recombinant MBL was produced as described (27). L-ficolin devoid of MASPs was purified from plasma according to a previously published method, using polyethylene glycol precipitation, affinity chromatography, and anion-exchange chromatography (28). H-ficolin from H-ficolin/MASP complexes, purified as described (29), was separated from MASPs by two consecutive rounds of size-exclusion chromatography on a Superose 6 HR 10/30 column in a buffer containing EDTA and high ionic strength (10 mM Tris, 10 mM EDTA, 1 M NaCl, 0.01% Tween 20 [pH 7.4]), followed by concentration and buffer exchange to TBS/Ca−2 (10 mM Tris, 5 mM CaCl2 [pH 7.4]) on Vivaspin 6, 10,000 MWCO spin concentrators (Sartorius).

Abs

The reactivities of Abs used in the following sections are illustrated in Supplemental Fig. 1.

Analysis of MASP-2 in complex with other MASPs and MAps in serum

FluoroNunc microtiter wells (Nunc) were coated with anti–MASP-2 B-chain Ab (mAb 8B5 [30]; Hycult Biotech) at a concentration of 2 μg/ml PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8.1 mM Na2HPO4 [pH 7.4]) overnight at room temperature. The wells were blocked with TBS (10 mM Tris, 140 mM NaCl, 15 mM Na2SO4 [pH 7.4]), containing 0.05% v/v Tween 20) and then washed thrice in TBS. The wells were then added a pool of normal human serum (NHS), diluted 5-fold in serum dilution buffer: TBS/Ca2+ (TBS containing 5 mM CaCl2) containing 100 μg/ml each rat Ig (Lampire), mouse Ig (Lampire), bovine Ig, and heat-aggregated human IgG (Beringulbin; CSL Behring; heat-aggregated by incubating the IgG at 56°C for 30 min, followed by centrifugation at 10,000 × g and recovery of the supernatant). Serum dilution buffer alone, as a control, was added to parallel wells. The samples were incubated in wells overnight at 4°C, and wells were washed thrice with TBS/Ca2+. This was followed by incubation with biotinylated anti–MASP-1–terminal Ab (polyclonal Ab rat 3) (31), anti–MASP-3 C-terminus Ab (mAb 38.12.3) (32), anti–MAp44 C-terminal Ab (mAb 2D5) (32), or anti–MASP-2 MAp19 Ab (mAb 6G12) (30), all at 1 μg/ml TBS/Ca2+. Following a 2-h incubation at room temperature, the wells were washed thrice with TBS/Ca2+ and europium-labeled streptavidin, 0.1 μg/ml TBS containing 25 μM EDTA, was added. After a 1-h incubation and washing thrice with TBS/Ca2+, the amount of bound europium-streptavidin was measured following the addition of enhancement solution (Perkin-Elmer) by reading time-resolved fluorescence on a VICTOR3 plate reader.

Analysis of heterodimer formation upon coexpression of MASPs and MAps

Microtiter wells were coated with either anti–MASP-1–/3/MAp44 Ab (mAb 5F5) (32) or anti–MASP-2–B-chain Ab (8B5), 2 μg/ml PBS. After overnight incubation, the wells were blocked with TBS. Supernatants from recombinant expression of MASP-2 alone or MASP-2 coexpressed with MASP-1, MASP-3, or MAp44 were diluted 5-fold in TBS/Ca2+, with or without 100 mM mannose and with or without 1 μg recombinant MBL/ml. Inactive versions of the MASPs (recombinant catalytically inactive [active-site serine-to-alanine mutant], in the following referred to as rMASP-1, rMASP-2i, and rMASP-3i) were used to exclude interference from catalytic activities. Following overnight incubation at 4°C, wells were washed thrice with TBS/Ca2+. Wells coated with anti–MASP-2 (8B5) were developed with either biotinylated anti–MASP-2 (8B5) or anti–MASP-1–/3/MAp44 (5F5). Wells coated with anti–MASP-1–/3/MAp44 (5F5) were developed with either biotinylated anti–MASP-2 (8B5) or anti–MASP-2–MAp19 (6G12), followed by europium-labeled streptavidin.

Assay for MAp44 and MASP homodimers and higher-order oligomers formed through MBL binding

Microtiter wells were coated with anti-MAp44 (2D5), 2 μg/ml PBS. The wells were blocked with TBS. Supernatants from recombinant expression of the inactive versions of MASP-1 (16.8 μg/ml), MASP-2 (1.5 μg/ml), MASP-3 (2.6 μg/ml), and MAp44 (7.5 μg/ml) were mixed in a ratio of 1:1 and then diluted 5-fold in TBS/Ca2+, with or without 1 μg recombinant MBL/ml, and incubated for 2 h at room temperature. The samples were
then added to the microtiter wells and incubated overnight at 4°C. The wells were washed thrice with TBST/Ca2+ and then biotinylated anti–MASP-1 (rat 3), anti–MASP-3 (38.12.3), anti–MAP44 (2D5), or anti–MASP-2/MAP19 (6G12), all at 1 μg/ml TBST/Ca2+. was added. Following incubation for 2 h at room temperature, the wells were washed and developed with europium-labeled streptavidin, as before.

Measurement of heterodimers in serum
Four normal sera and a MASP-2−deficient serum were diluted 10-fold in either TBST/Ca2+ or in a buffer dissociating MASPs and MAPs from MBL and ficolins, TBST/1 M NaCl/10 mM EDTA (TBST added NaCl to 1 M total). The samples were incubated in anti–MASP-2 Ab (8B5)-coated microtiter wells, and the wells were washed and subsequently developed with biotinylated anti–MASP-1/-3/3/3/3 MAP44 (5FS), followed by europium-labeled streptavidin, as above.

Analysis of heterocomplex formation mediated by MBL and L-ficolin
Supernatants from recombinant expression of MAP44 and inactive versions of the MASPs (tMAP44 [7.5 μg/ml], tMAP3 [2.6 μg/ml], tMAP2i [1.5 μg/ml], and tMAP2i [16.8 μg/ml]) were mixed 1:1 and then with 2-fold dilution series of recombinant MBL or purified L-ficolin in TBST/Ca2+. Following a 2-h incubation at room temperature, assays for co-complexes were performed as described above.

Analysis of heterocomplex formation mediated by H-ficolin
Supernatants from recombinant expression of inactive versions of MASP-2 (1.5 μg/ml) and MASP-1 (16.8 μg/ml) were mixed 1:1 and then with 2-fold dilution series of purified H-ficolin in TBST/Ca2+. Following a 2-h incubation at room temperature, assays for MASP-2−MASP-1 co-complexes were performed as described above. The oligomerization pattern of the purified H-ficolin was verified by immunoblotting (as described below) and developed with in-house biotinylated polyconal goat anti-human H-ficolin Ab (AF2367; R&D Systems). The amount of endogenous MASP-2 remaining in the purified preparation was <0.2% w/w compared with H-ficolin.

Ab capture of complexes and analysis by immunoblotting
Twenty-four microtiter wells were coated with anti–MASP-2 Ab (8B5), and 24 wells were coated with anti–MASP-1/-3/3/3 MAP44 Ab (5FS), at 4 μg/ml carbonate coating buffer (15 mM Na2CO3, 35 mM NaHCO3 [pH 9.6]) and 3 μg/ml PBS, respectively. The wells were blocked with TBST for 1 h at room temperature and then washed with this buffer. A serum pool was diluted 10-fold in TBST/Ca2+ and added to 12 wells for each Ab coat, and, in parallel, in the complex-dissociating buffer TBST/1 M NaCl/10 mM EDTA, and added to the remaining 12 wells for each Ab coat, at 100 μl/well. The plate was then incubated overnight at 4°C. After incubation, the microtiter wells were emptied and washed thrice with TBST/Ca2+ and then 200 μl TBST/Ca2+ was added. Each set of 12 wells was then serially eluted, first removing the 200 μl TBST, adding 100 μl PBS sample buffer (62.5 mM Tris, 8 M urea, 10% [v/v] glycercerol, 3% [w/v] SDS, 0.001% [w/v] bromophenol blue [pH 6.7]) diluted 1:1 with PBS, incubating for 10 min and then emptying the next well of PBS and transferring the elution buffer to this well, repeating until 12 wells had been serially eluted for each sample. Forty-five microliters of the eluate was loaded per well on Criterion XT 4–12% gels (Bio-Rad). The gel was run in XT MOPS running buffer before being semiodylzed onto a polyvinilidene difluoride membrane (Bio-Rad). The membrane was blocked in 0.1% Tween 20 in TBS and then incubated with primary Ab in primary buffer (TBS, 0.05% Tween 20, 1 mM EDTA, 1 mg human serum albumin [CSL Behring]/ml, and 100 μg human IgG/ml). The membrane was washed, incubated with secondary Ab in secondary buffer (TBST, no azide, 1 mM EDTA, and 100 μg human IgG/ml) and washed again before being developed with SuperSignal West Dura Extended Duration Substrate (Pierce). Images were taken using a charge-coupled device camera (LAS-3000; Fuji) and analyzed with the Image Analysis Software supplied with the camera. The primary Abs were biotinylated anti–MASP-2/MAP19 (1.3B7; 1 μg/ml) (33), followed by HRP-streptavidin (P0397; DAKO) or affinity-purified rabbit anti-human-MAP44 (R74B; 1 μg/ml) (3), followed by HRP-goat anti-rabbit Ig (P0448; DAKO).

Titration of co-complexes in serum and analysis by size-exclusion chromatography
Microtiter wells were coated with anti-MAP44 (2D5) or anti-MAP-2 (8B5), at 2 μg/ml PBS, and then blocked with TBST, as before. An NHS pool was diluted 5-fold and then serially 2-fold in serum dilution buffer. The serial dilutions and a serum dilution buffer−only control were incubated in wells overnight at 4°C. The wells were then washed thrice in TBST/Ca2+, and biotinylated anti–MASP-3 (38.12.3), anti–MASP-2/MAP19 (6G12), or anti–MASP-1 (rat 3), at 1 μg/ml TBST/Ca2+, was added. Following incubation for 2 h at room temperature, the wells were washed and developed as before.

Two hundred microliters of serum from two individuals were centrifuged briefly at 10,000 × g and passed through a Superoxide 6 10/30 JR column (GE) in TBS. Fractions of 250 μl were collected in Tween-20−preblocked microtiter plates. A 2-fold dilution series, from 1/5 to 1/80, for each parent serum, as well as the fractions diluted 1:1 in serum dilution buffer, were tested as before for MAP44−MASP-3 complexes (anti–MAP44 [2D5] capture, biotinylated anti–MASP-3 [38.12.3] development) or MASP-2−MASP-1 complexes (anti–MASP-2 [8B5] capture, biotinylated anti–MASP-1 [rat 3 development]). Using previously described assays, fractions were also tested for MBL (fractions diluted 1:5) (34), H-ficolin (1:100) (35), L-ficolin (1:5) (35), MASP-2 (1:2) (30), and/or MAP44 (1:4) (32).

Analysis of co-complex levels in sera
Microtiter wells were coated with anti–MASP-2 (8B5) or anti–MAP44 (2D5), at 2 μg/ml PBS, and blocked with TBST. Supernatants from recombinant expression of inactive forms of MASP-1 (16.8 μg/ml), MASP-2 (1.5 μg/ml), MASP-2 (2.6 μg/ml), and MAP44 (7.5 μg/ml) were mixed in a ratio of 1:1 and then diluted 5-fold in TBST/Ca2+ containing a 2-fold dilution series of recombinant MBL, starting at 1 μg/ml. The samples were incubated for 2 h at room temperature and then added to microtiter wells and incubated overnight at 4°C. The wells were washed thrice with TBST/Ca2+ and developed with biotinylated anti–MASP-1/-3/3/3 MAP44 (5FS) for anti–MASP-2 (8B5) coat and biotinylated anti–MASP-1 (rat 3) or biotinylated anti–MASP-3 (38.12.3) for anti–MAP44 (2D5) coat, at 1 μg/ml TBST/Ca2+. Matched samples of serum, EDTA plasma, heparin plasma, and citrate plasma were included for five individuals to compare the different sampling methods.

MBL concentration−dependent formation of co-complexes
Microtiter wells were coated with anti–MASP-2 (8B5) or anti–MAP44 (2D5), at 2 μg/ml PBS, and blocked with TBST. Supernatants from recombinant expression of inactive forms of MASP-1 (16.8 μg/ml), MASP-2 (1.5 μg/ml), MASP-3 (2.6 μg/ml), and MAP44 (7.5 μg/ml) were mixed in a ratio of 1:1 and then diluted 5-fold in TBST/Ca2+ containing a 2-fold dilution series of recombinant MBL, starting at 1 μg/ml. The samples were incubated for 2 h at room temperature and then added to microtiter wells and incubated overnight at 4°C. The wells were washed thrice with TBST/Ca2+ and developed with biotinylated anti–MASP-1/-3/3/3 MAP44 (5FS) for anti–MASP-2 (8B5) coat and biotinylated anti–MASP-1 (rat 3) or biotinylated anti–MASP-3 (38.12.3) for anti–MAP44 (2D5) coat, followed by incubation with europium-labeled streptavidin and measurement of bound europium.

Analysis of C4 deposition as a function of MASP-1−MASP-2 co-complexes
Microtiter wells were coated either with anti–MASP-1/-3/3/3 MAP44 Ab (5FS), as described previously, or with mannan (a mannose-rich mem-
[v/v] Triton X-100 (pH 7.4), containing heat-aggregated human Ig, mouse Ig, rat Ig, and bovine Ig, all at 100 μg/ml, were added to the wells and incubated overnight at 4°C. After washing, the wells were incubated, in parallel, with C4 for 2 h and developed with biotinylated anti-C4c, as described, or developed with biotinylated anti--MASP-2 (8B5), at 1 μg/ml TBST/Ca2+.

**MAp44 inhibition of co-complex formation and C4 deposition**

Microtiter wells were coated with anti--MASP-2 (8B5), at 2 μg/ml PBS, blocked, and washed with TBST as before. Wild-type zymogen MASP-1 was mixed with wild-type zymogen MASP-2 for final concentrations of 60 ng/ml each and then mixed with a dilution series of recombinant MAp44 to give final concentrations ranging from 2.7 μg/ml to 2.6 ng/ml, and including 0 ng/ml. Recombinant MBL was added to the mixtures to a final concentration of 50 ng/ml. Following incubation for 2 h at room temperature, the mixtures were added to the anti--MASP-2 (8B5)-coated wells. The wells were developed as described above for deposition of C4 fragments, MASP-1, and MAp44.

**Analysis of MAp44 competition with pre-existing co-complexes in serum**

Serum was diluted 5- or 2.5-fold in serum dilution buffer and then combined 1:1 with a 2-fold dilution series of purified recombinant MAp44 in serum dilution buffer, ranging from 4 μg/ml down to 31 ng/ml, and including a serum dilution buffer-only control. The samples were incubated for 24 h end-over-end at 4°C, added to microtiter wells coated with anti--MASP-1/-3/MAp44 Ab (5F5; 2 μg/ml PBS, blocked with TBST), and incubated overnight at 4°C. Following incubation, the wells were washed, and parallel wells were developed with biotinylated anti--MASP-2 Ab (8B5), followed by europium-streptavidin as before, or incubated with purified C4, at 2 μg/ml B1 buffer, for 2 h at 37°C. The latter wells were washed and developed with biotinylated anti-C4c Ab (162-2), followed by europium-labeled streptavidin, as before.

**Results**

We previously reported preliminary data indicating that MASP-1 and MASP-2 may form MBL-dependent co-complexes in vitro, as well as that co-complexes of MASP-1 and MASP-2, with MBL, ficolins, or both, can be detected in serum (9).

**MASPs and MAps in serum**

We first sought to examine whether MASP-2 might also be found in complex with the other MASPs and MAps. We incubated a serum pool diluted 5-fold in buffer, or buffer alone, in wells coated with a specific anti--MASP-2 Ab (8B5), washed the wells, and then developed them using either an Ab specific for MASP-1 (rat 3), MASP-3 (mAb 38.12.3), or MAp44 (mAb 2D5) or an Ab reacting with both MASP-2 and MAp19 (mAb 6G12). Of note, the buffers used throughout for experiments with serum included a large excess of rat Ig, mouse Ig, bovine Ig, and heat-aggregated human Ig to prevent any heterophilic Ab, contaminating bovine Ig, or rheumatoid factor interference [from any human sera, animal sera, polyclonal Abs, or mAbs used (37)]. An overview of the domain structure of MASPs and MAps, as well as the specificities of the Abs used, is shown in Supplemental Fig. 1. MASP-1, MASP-3, and MAp44 were all found in complex with MASP-2 (Fig. 1). The signal seen for MASP-2–MASP-1 complexes confirms our previous preliminary findings (9). The strong signal seen when developing with anti--MASP-2/MAp19 (6G12) is expected from complexes of MASP-2, but a contribution of co-complex with MAp19 cannot be excluded. Importantly, one cannot directly compare the magnitudes of the responses seen for the different combinations of Abs used. Nonetheless, it appears that MASP-2 may be found in complex with each of the other MASPs and MAps in serum. Two possible scenarios present themselves, as outlined in the introduction: MASP-2 might, to some extent, form heterodimers with the other MASPs and MAps or MASP-2 and the other MASPs and MAps might form MBL- and/or ficolin-dependent co-complexes, with the PRM harboring more than one MASP dimer. These two scenarios are not mutually exclusive.

**Coexpression of MASP-2 with MASP-1, MASP-3, or MAp44 leads to detectable levels of heterodimers**

We sought to address the most simple scenario for colocalization of MASP-1 and MASP-2 in connection with transactivation: heterodimer formation. We also included MASP-3 and MAp44 in the analysis, thus including all three splice products from MASP1. These three have identical dimerization domains (CUB–EGF–CUB), but the possibility remained that the configuration of the remainder of the molecule could influence dimerization. We also sought to examine the effect of MBL in this scenario. To control for unwanted cross-linking due to MBL binding to potential carbohydrates on the MASPs, we also included a mannose-containing buffer as control. Mannose, being a ligand for MBL, would compete out any such interaction. Supernatants from cells expressing MASP-2 alone, MASP-2 and MASP-1, MASP-2 and MASP-3, or MASP-2 and MAp44, as well as a buffer-only control, were preincubated alone, with mannose, with MBL, or with MBL and mannose. The samples were then applied to microtiter wells coated with either anti--MASP-2 (8B5) or anti--MASP-1/-3/MAp44 (5F5). The MASP-2 capture wells were developed with either the same anti--MASP-2 (8B5) or with anti--MASP-1/-3/MAp44 (5F5). The MASP-1 capture wells were developed with two different anti--MASP-2 Abs (8B5 and 6G12, the latter is additionally able to recognize MAp19, which is not present in this set-up).

Upon capture of MASP-2 in the absence of MBL, there is a negligible signal when developing for MASP-2 for all of the samples, similar to buffer only (Fig. 2A). Presumably, this is because the two epitopes present in dimeric MASP-2 are insufficient for capture and development using the same Ab (8B5). However, when adding MBL, there is a marked (3-fold) increase in the signal, indicating MBL-dependent formation of complexes harboring more than one MASP-2 dimer. The signals are similar in the absence and presence of mannose, both with and without MBL. This indicates that the complex formation is not influenced significantly.
by the binding activity of MBL, and mannose, in itself, has no effect. Still, the possibility remained that the reason for the increase in the observed signal in the anti–MASP-2–anti–MASP-2 sandwich assay upon addition of MBL was simply a steric effect. One could imagine that both epitopes in the free MASP-2 dimer could easily be bound by the capture Ab, preventing binding of the developing Ab, whereas in the presence of MBL, steric constraints would prevent the capture Ab from binding both epitopes, freeing one for interaction with the developing Ab.

The result of capturing MASP-2 and developing for MASP-1, MASP-3, or MAp44 is shown in Fig. 2B. As expected, supernatant from cells expressing MASP-2 alone does not yield an appreciable signal but is similar to buffer only. On the contrary, following coexpression, MASP-1, MASP-3, and MAp44 all appear to have formed heterodimers with MASP-2, because there is a signal independent of the presence of MBL. However, when adding MBL, there is a significant increase in the signal, indicating MBL-dependent formation of complexes containing more than one MASP–MASP-2 dimer and/or a combination with MASP-1 and MASP-2 homodimers. Again, the observed complex formation was independent of the ligand-binding activity of MBL, as evidenced by similar signals when including mannose to compete out the carbohydrate recognition–domain binding. In this instance, the above-mentioned steric considerations cannot be invoked to explain the observed effect of MBL.

We see a very similar scenario when reversing the assay set-up, capturing using anti–MASP-1/MASP-3/MAp44 and developing for MASP-2 using either of two anti–MASP-2 Abs (8B5 in Fig. 2C, 6G12 in Fig. 2D). Note, however, that the relative magnitude of the different complexes detected when capturing with 8B5 as opposed to 5F5 change, likely reflecting a difference in the capacity of the coated mAbs to capture Ag.

We conclude that coexpression of MASP-2 with MASP-1, MASP-3, or MAp44 leads to detectable levels of heterodimers and that the addition of MBL causes the formation of larger heterocomplexes.

**MASP and MAp**44** homodimers are stable and do not interchange upon admixture**

The possibility remained that MASP and MAp homodimers are not stable entities but are able to dissociate and reassociate, leading to dynamic formation of heterodimers. To examine this, we incubated MAp44 by itself or with MASP-3, MASP-2, or MASP-1, including a buffer control, all either in the presence or absence of MBL. We added the samples to wells coated with anti–MAp44-specific Ab (2D5), washed, and developed for MASP-3, MASP-2, MASP-1, or MAp44. As can be seen from Fig. 3, in the absence of MBL, no signal was detectable for heterocomplexes in any combination, indicating that the MASP and MAp44 homodimers are stable and do not interchange. Of note, when capturing MAp44 and developing for MAp44, there was a signal markedly higher than background under all conditions in the absence of MBL, although it was not statistically significant. In the presence of MBL, MAp44 was able to form complexes with MASP-3, MASP-2, and MASP-1, as well as with itself (Fig. 3). Markedly lower levels of MBL-dependent MAp44–MAp44 complexes were observed in the presence of each of the MASPs (Fig. 3D), indicating that these compete for complex formation.

Thus, homodimers, and presumably the heterodimers generated by coexpression, were stable entities that did not interchange once

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** Cotransfection of MASP-2 with MASP-1, MASP-3, or MAp44 leads to detectable levels of heterodimers, whereas the addition of MBL causes formation of larger heterocomplexes independent of ligand-binding activity. (A) Capture of recombinant inactive versions of MASP-2, MASP-2 coexpressed with MASP-1, MASP-3, or MAp44, or buffer alone in microtiter wells (8B5), developed for MASP-2 (8B5). Dilution buffer without MBL (white bars), with 100 mM mannose (horizontal stripes), with 1 μg recombinant MBL/ml (black bars), or with 1 μg recombinant MBL/ml and 100 mM mannose (diagonal stripes). Data are mean and SD of duplicates. (B) As in (A), but capture of MASP-2 (8B5) and development for MASP-1/MASP-3/MAp44 (5F5). (C) As in (A), but capture of MASP-1/MASP-3/MAp44 (5F5) and development for MASP-2/MAp44 (6G12).

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** MAp44 and MASP dimers are stable and do not interchange upon admixture, and MAp44 can form MBL-dependent heterocomplexes with each of the MASPs. (A) Capture of MAp44 (2D5) developed for MASP-3 (38,12.3), using MAp44 (0.75 μg/ml final) mixed 1:1 with catalytically inactive versions of MASP-3 (0.26 μg/ml final), MASP-2 (0.15 μg/ml final), or MASP-1 (1.68 μg/ml final); MAp44 alone (0.75 μg/ml final); or buffer only in the absence (white bars) or presence (black bars) of MBL (1 μg/ml final). Data are mean with SD of four measurements in two experiments. Data were analyzed using two-way ANOVA, followed by the Dunnett posttest, comparing each column pairwise with buffer control in either the absence or presence of MBL. (B) As in (A), but developing for MASP-2 (6G12). (C) As in (A), but developing for MASP-1 (rat 3). (D) As in (A), but developing for MAp44 (2D5). The p values were corrected for multiple comparisons, and a cutoff α of 0.05 (*) was used. ****p < 0.0001.
formed, under the conditions of the assays presented in this study. Again, we saw the formation of MBL-dependent co-complexes of MASPs and MAp44.

**Heterodimers are not present in serum at significant levels**

Having confirmed that MASP dimers are stable entities and that heterodimers can be generated by coexpression of MASPs, we proceeded to examine whether heterodimers might be present in serum. Complexes of MASPs and MAps with MBL and ficolins are known to be calcium dependent and to dissociate in the presence of EDTA and high ionic strength (33). In contrast, MASP and MAp dimers reportedly are not sensitive to EDTA and high ionic strength (21). Using our assay, capturing with anti–MASP-2 (8B5) and developing with anti–MASP-1/-3/MAp44 (5F5), we examined four sera for the presence of calcium-dependent co-complexes of MASP-2 with MASP-1/-3/MAp44 (MBL/ficolin-dependent) versus calcium-independent co-complexes (this would include, but not necessarily be limited to, heterodimers). We included a background control in the form of a MASP-2–deficient serum. In the presence of Ca2+, we saw high signals in the assay in the four normal sera, which were significantly different from the background signal of the MASP-2–deficient serum (two-way ANOVA, followed by pairwise comparison with MASP-2–deficient serum using the Dunnett posttest) (Fig. 4). This agreed well with our observation of MBL/ficolin-dependent complex formation. Conversely, in the absence of Ca2+, sera 1–3 did not give a signal above the MASP-2–deficient serum background (*p > 0.05 for each). Meanwhile, serum 4, in the absence of Ca2+, gave a signal that was approximately four times lower than in the presence of Ca2+ and only slightly higher than background (*p < 0.01). Taken together, this indicates that the formation of MASP heterodimers is insignificant compared with co-complex formation through interaction with MBL and ficolins.

**Co-complexes can be formed with MBL, L-ficolin, and H-ficolin**

Thus, it appears that MBL- and/or ficolin-dependent co-complex formation was the major driving force behind the association of different MASPs and MAps, including MASP-1 and MASP-2, in serum. We provided evidence for this above with regard to MBL. We next investigated whether similar co-complexes could be formed with ficolins. As a proof of principle, we compared the ability of recombinant MBL and purified L-ficolin to generate co-complexes of MASP-2 with MAp44, MASP-2 with MASP-3, MAp44 with MASP-1, and MAp44 with MASP-3. Both MBL and L-ficolin possessed the capacity to form such co-complexes (Fig. 5). This indicates that L-ficolin purified from human plasma also harbors multiple binding sites for MASPs and MAps. The prominent prozone effect seen for MBL at 1 μg/ml (Fig. 5A, 5B) might be explained as an effect of too much MBL compared with MASP-2 and MAp44, and MASP-2 and MASP-3, respectively, because this would cause the formation of MBL complexes with only single MASP dimers.

Because H-ficolin is the predominant humoral complement-activating PRM in serum, we also examined the ability of purified H-ficolin to generate co-complexes of MASP-1 with MASP-2 (Supplemental Fig. 2).

**Pull-down from serum and analysis by immunoblotting confirms the presence of calcium-dependent co-complexes**

To further evaluate the existence of the co-complexes detected in the solid-phase assays, we performed pull-down from serum, followed by immunoblotting. We coated anti–MASP-2 (8B5) or anti–MASP-1/-3/MAp44 (5F5) in microtiter wells and then incubated with a serum pool either diluted in buffer containing 5 mM calcium (associating conditions) or in buffer containing 10 mM EDTA and 1 M NaCl (dissociating conditions). After incubation, the wells were washed and then serially eluted using SDS-PAGE sample buffer. Resulting samples were run on SDS-PAGE, blotted to polyvinylidene difluoride membrane, and then probed with anti-MAp44 (R74B) and anti–MASP-2/MAp19 (1.3B7).

As can be seen from Fig. 6A, when capturing with anti–MASP-2 (8B5) under associating conditions (calcium-containing buffer, lane 1) and probing the blot for MAp44, we saw a clear band corresponding to the size of MAp44 (arrow) (the prominent background band ~150 kDa is presumably IgG, either 8B5 eluted from the capture coat or heterophilic Ab and/or rheumatoid factor from the serum pool binding to the former; cross-reactive with the HRP-labeled secondary Ab). On the contrary, we did not see a band when capturing MASP-2 under dissociating conditions (buffer containing EDTA and high ionic strength, lane 2). This confirmed the existence of MBL- and/or ficolin-dependent co-complexes of MASP-2 and MAp44 in serum, whereas no heterodimers were detected.

Similarly, in Fig. 6B, we pulled down either MASP-2–containing (8B5) or MASP-1/-3/MAp44–containing (5F5) complexes under either associating or dissociating conditions and then developed for MASP-2/MAp19 (1.3B7). We detected MASP-19 in MASP-2 complexes under associating conditions (lane 1), as well as in MASP-1/-3/MAp44 complexes under both associating (lane 3) and, albeit at a much lower level, dissociating conditions (lane 4). This indicates the existence of MBL- and/or ficolin-dependent MASP-2 and MASP-1/-3/MAp44 co-complexes with MAp19 and the potential existence of low levels of MAp19 heterodimerized with MASP-1/-3/MAp44. MASP-2 and a presumed degradation fragment of MASP-2 (~42 kDa) were also detected upon capture of MASP-2 under associating conditions (lane 1). Although the presumed degradation fragment had a molecular size close to that of MAp44, we could exclude that it was MAp44, because the Ab is specific for MASP-2/MAp19 and because we did not detect this band after pull-down of MASP-1/-3/MAp44 complexes (lanes 3 and 4). The fact that we saw only a very weak band for MASP-2 itself under dissociating conditions when pulling down MASP-2 indicates a rather limited sensitivity of this approach, probably also explaining why we did not detect MASP-2 after pull-down of MASP-1/-3/MAp44.

In summary, the results from pull-down and immunoblotting served as a further indication that heterocomplexes are indeed found in serum.
Co-complexes in serum are titratable and colocalize with their presumed constituents on size-exclusion chromatography

Further analyzing the co-complexes occurring naturally in serum, we first titrated them. As can be seen in Fig. 7A, MAP44–MASP-3, MASP-2–MASP-2/MAp19, and MASP-2–MASP-1 complexes titrated out upon serial dilution of serum. We proceeded to analyze the migration of such complexes on size-exclusion chromatography. As can be seen in Fig. 7B and 7C, MAP44–MASP-3 co-complexes colocalized with MAP44, H-ficolin, and MBL, whereas the MASP-2–MASP-1 co-complexes colocalized with MASP-2, H-ficolin, and L-ficolin. Of note, in Fig. 7C, co-complexes and their constituent MASP/MAps appeared to preferentially colocalize with H-ficolin, less with MBL, and not at all with the higher-oligomeric MBL. However, this may be a consequence of the much higher relative abundance of H-ficolin and L-ficolin compared with MBL and higher-oligomeric forms of MBL, which again means that much more MASP/MAps and co-complexes are associated with H-ficolin and L-ficolin. Nonetheless, our results again supported the nature and stability of these co-complexes.

The levels of different co-complexes vary between sera and are normally distributed

Because the levels of MBL and ficolins on the one hand, and MASPs and MAps on the other hand, vary significantly between individuals (31), it seemed plausible that so should the levels of the various co-complexes. We found the levels of MAP44–MASP-3 and MASP-2–MASP-1 co-complexes in normal human sera to be variable, but they conformed to a normal distribution (both data sets passed Kolmogorov–Smirnov, D’Agostino and Pearson omnibus, and Shapiro–Wilk normality tests) (Fig. 8A, 8B).

We proceeded to examine further the observed MBL-dependent co-complex formation in a simplified recombinant system.

**FIGURE 5.** Co-complexes can be formed with both MBL and L-ficolin. (A) MASP-2 (0.15 μg/ml final concentration) and MAP44 (0.75 μg/ml final concentration) were incubated with 3-fold dilution series (1, 0.25, and 0.063 μg/ml final concentration) of either recombinant MBL or L-ficolin purified from serum and then assayed for co-complex formation. MASP-2–MAP44 (8B5–5F5) co-complexes (black bars, MASP-2 + MAP44) as a function of the concentration of each PRM (x-axis). MASP-2 and PRM (horizontally striped bars), MAP44 and PRM (diagonally striped bars), or PRM in buffer only (white bars) were included as controls. Data are mean with SD of duplicates. (B) As in (A), but for MASP-2–MASP-3 (8B5–5F5) co-complexes (0.15 and 0.26 μg/ml final concentration, respectively). (C) As in (A), but for MAP44–MASP-1 (2D5–rat 3) co-complexes (0.75 and 1.68 μg/ml final concentration, respectively). (D) As in (A), but for MAP44–MASP-3 (2DS-38.12.3) co-complexes (0.75 and 0.26 μg/ml final concentration, respectively).

**FIGURE 6.** Pull-down of co-complexes from serum and analysis by immunoblotting. (A) Pull-down from serum with anti-MASP-2 (8B5) under associating (calcium-containing buffer; lane 1) or dissociating (high salt and EDTA containing buffer; lane 2) conditions. Samples were run under nonreducing conditions and blot developed for MAP44 (R74B). Autocontrast was used to enhance the clarity of this blot. Molecular size markers are indicated on the side. (B) Pull-down from serum with anti-MASP-2 (8B5; lanes 1 and 2) and anti-MASP-1/-3/MAp44 (5F5, lanes 3 and 4) under associating (lanes 1 and 3) or dissociating (lanes 2 and 4) conditions. Samples were run under nonreducing conditions and blot developed for MASP-2/MAp19 (1.3B7).
The formation of co-complexes is highly dependent on the relative levels of the constituents

We performed experiments in which we mixed a constant amount of MASP-2 with MASP-1, MAp44, or MASP-3, or MAp44 with MASP-1 or MASP-3, and then titrated the amount of MBL. As can be seen from Fig. 9A and 9B, the amount of co-complexes detected depended on the concentration of MBL.

MASP-1–MASP-2 co-complex level correlates with C4-deposition capacity

We proceeded to examine whether the degree of complement activation correlated with the amount of MASP-1–MASP-2 co-complex. For the previous experiments involving recombinant proteins, we used active-site serine-to-alanine mutant versions of the MASPs to rule out any interference from their proteolytic activities. For the present experiment, we switched to wild-type MASP-1 and MASP-2, produced in their zymogen states, as we described previously (26).

Keeping the concentrations of MASP-2 and MBL constant, we titrated the amount of MASP-1, measuring in parallel the level of co-complex formed and the amount of C4 deposition on a mannan-coated surface. As can be seen in Fig. 10A, there was a congruence between the two curves. Again, the formation of co-complexes was highly dependent on the relative levels of the constituents, peaking close to the 1:1 ratio of MASP-1/MASP-2. The C4-deposition curve on mannan was shifted slightly toward favoring an excess of MASP-2. Plotting one versus the other indicated a close correlation between the level of C4 deposition and the amount of co-complex formed (Fig. 10B), which was confirmed by Spearman correlation analysis ($r = +0.82$) and a two-tailed $p$ value $<0.0001$.

Based on our observations in the recombinant system, we decided to examine whether the level of MASP-1–MASP-2 co-complexes would influence the degree of lectin pathway activation in serum. The levels of MASP-1–MASP-2 co-complexes were measured in four sera. The sera displayed very low, low, intermediate, or high amounts of co-complexes. As we established in this study, the complexes may be formed with MBL or H-, L-, or M-ficolin. Noting that the level of C4 deposition on mannan is
Catalytically inactive MASP-2 (0.15 μg/ml final) was mixed 1:1 with catalytically inactive MASP-1 (1.68 μg/ml final), MAp44 (0.75 μg/ml final), or catalytically inactive MASP-3 (0.26 μg/ml final) and incubated with recombinant MBL at varying concentrations before assay- ing for MASP-2–MASP-1/-3/MAp44 co-complex formation (capture 8B5–develop 5F5). Data are mean and SD based on four measurements in two independent experiments. Recombinant MAp44 (0.75 μg/ml final concentration) was mixed 1:1 with catalytically inactive MASP-1 (1.68 μg/ml final concentration) or MASP-3 (0.26 μg/ml final concentration), incubated with recombinant MBL at varying concentrations, and assayed for MAp44–MASP-1 co-complex formation (C, capture 2D5–develop rat 3) or MAp44–MASP-3 co-complex formation (D, capture 2D5–develop 38, 12, 3), respectively. Data are mean and SD based on four measurements in two independent experiments.

FIGURE 9. MBL concentration–dependent formation of co-complexes. (A) Catalytically inactive MASP-2 (0.15 μg/ml final) was mixed 1:1 with catalytically inactive MASP-1 (1.68 μg/ml final), MAp44 (0.75 μg/ml final), or catalytically inactive MASP-3 (0.26 μg/ml final) and incubated with recombinant MBL at varying concentrations before assay- ing for MASP-2–MASP-1/-3/MAp44 co-complex formation (capture 8B5–develop 5F5). Data are mean and SD based on four measurements in two independent experiments. (B) Recombinant MAp44 (0.75 μg/ml final concentration) was mixed 1:1 with catalytically inactive MASP-1 (1.68 μg/ml final concentration) or MASP-3 (0.26 μg/ml final concentration), incubated with recombinant MBL at varying concentrations, and assayed for MAp44–MASP-1 co-complex formation (C, capture 2D5–develop rat 3) or MAp44–MASP-3 co-complex formation (D, capture 2D5–develop 38, 12, 3), respectively. Data are mean and SD based on four measurements in two independent experiments.

highly dependent on the concentration of MBL in sera, and that we have no good ligand surface for all four PRMs, the ability of the sera to deposit C4 was analyzed upon affinity capture in microtitr well coated with anti–MASP-1/-3/MAp44 Ab. We pre- cluded any interference from the C1 complex by performing the serum incubation in a high salt–concentration MBL-binding buffer, followed by addition of exogenous purified human C4 for the C4 fragment–deposition step, as previously described (38). As can be seen in Fig. 10C, there was a very good correlation between the amount of co-complex measured and the level of C4 deposition observed in this set-up, indicating that such co- complexes may play an important functional role in serum.

MAp44 inhibits complement activation by virtue of disruption of MASP-1–MASP-2 co-complexes

Our observations regarding the importance of MASP-1–MASP-2 co-complexes raised the novel possibility that MAp44, which we previously found to inhibit lectin pathway activation, does not do so simply by the complete displacement of MASP-2 from MBL or ficolins, rather it disrupts co-complexes. This more sophisticated mode of inhibition would separate MASP-1 from MASP-2, hence preventing transactivation; presumably, it would be more efficient because displacement of either MASP would be efficacious. To test our hypothesis, we titrated MAp44 into a fixed amount of MASP-1, MASP-2, or MBL. We then assayed, in parallel, the degree of C4 deposition, as well as the amounts of MASP-1 and MAp44 bound, all on an anti–MASP-2 (8B5) capture coat. This set-up ensures a constant level of MASP-2, while allowing for direct measurement of MASP-2–MASP-1 co-complex formation by determination of the amount of MASP-1 bound and, simulta-
gene by mutually exclusive splicing, as do MASP-2 and MAp19 from the MASP2 gene. Although MASP-3 is broadly expressed, MASP-1 is expressed exclusively in the liver, and MASP44 is produced mainly in heart and liver (3, 32, 41). MASP-2 and MAp19 are produced in the liver (19). The exact cell types and subsets are not known.

Addressing scenario 2, we demonstrated that the addition of MBL causes the formation of larger heterocomplexes (Figs. 2, 3). Naturally, such co-complex formation would require the existence of two or more binding sites for a MASP or MAp dimer on each MBL molecule. Indeed, previous observations in the literature indicate that this could well be the case (23). Importantly, the MBL-dependent co-complex formation that we observe is independent of ligand-binding activity. The MASP binding sites in MBL and ficolins were suggested to be equivalent (42). We further found in this study that MBL, L-ficolin, and H-ficolin all possessed the capacity to form co-complexes (Fig. 5, Supplemental Fig. 2). This indicates that L-ficolin and H-ficolin purified from human plasma also harbor multiple binding sites for MASPs and MAps. Given the structural and ultrastructural homology of MBL, H-ficolin, L-ficolin, and M-ficolin, we believe that M-ficolin should have the same property. As mentioned previously, when analyzing the existence of co-complexes in serum, we cannot readily discriminate between co-complexes formed by these different PRMs. However, we confirmed the existence of the co-complexes detected in the solid-phase assays by performing pull-down from serum, followed by immunoblotting (Fig. 6). Further supporting the nature and stability of these co-complexes, we found that the co-complexes in serum colocalize with their presumed constituents upon analysis by gel-permeation chromatography (Fig. 7).

We found that the levels of various co-complexes of MASPs and MAps vary from individual to individual (Fig. 8), indicating the possibility of a functional impact. Importantly, the sampling method is critical when analyzing co-complexes, because our data indicate that the EDTA used for preparation of EDTA plasma may cause dissociation of some of the pre-existing complexes, followed by reassociation when the samples are diluted in the calcium-containing sample buffer, in effect scrambling the complexes (Fig. 8). A further inference from this is that the natural level of co-complex is lower than that resulting from a scrambling, indicating that certain complexes are preferentially formed. This could be the result of restricted coexpression in certain cell types or populations (e.g., MASP-2 alone with MBL alone, and so forth, as discussed above).

Considering the well-established requirement for both calcium chelation and high ionic strength to disrupt MBL/MAp and MBL/MAp complexes, it may seem surprising that EDTA alone has such an effect on the serum co-complexes measured in this study. However, our previous work indicated that the interaction of MASPs and MAps with ficolins is rather sensitive to calcium chelation alone. Thus, we observed previously that, in the presence of calcium, most of the MASP-1, MASP-2, and MAp19 in serum emerged on gel-permeation chromatography as large complexes that were not associated with MBL, whereas in the presence of EDTA alone, most of these components formed smaller complexes (33). This was before the characterization of the role of ficolins in the lectin pathway; however, in retrospect, this accounts for the observation at that time that >95% of the total MASPs and MAp19 found in serum were not complexed with MBL. The molar dominance of ficolins over MBL explains the marked effect of EDTA alone, because ficolins would be expected to drive the majority of co-complex formation.

For the in vitro generation of such co-complexes of different MASPs and MAps in a clean system, we find a delicate balance of the constituents. Too little MBL results in little co-complex formation, whereas too much MBL has the same effect (Fig. 9). In the former situation, there is too little MBL to generate significant levels of co-complexes, and/or each MBL is saturated with either MASP/MAp, whereas in the latter scenario, there is so much MBL...
that each has only a single binding site occupied and, hence, no co-complex is formed. In between these two extremes is a maximum, which should reflect a balanced stoichiometry of the concentrations of the two MASPs/MAps and the available binding sites on MBL.

The observed curves fit well with the theoretical considerations underlying co-complex formation of two different binding partners with a third molecule harboring multiple binding sites for these binding partners (Figs. 9, 10). However, this is a highly complex and nontrivial scenario, because the MBL used is polydisperse and, hence, contains MBL oligomers with different numbers of binding sites for MASPs and MAps, and we do not know these numbers. Some MBL oligomers may have only one binding site for MASPs and MAps, whereas others have two or more. Several previous studies made progress toward understanding of the interaction of MASPs with various oligomers of MBL, but the subject remains controversial. Chen and Wallis (23) reported that each MASP dimer contains binding sites for two MBL subunits and that both sites had to be occupied by subunits from a single MBL oligomer to form a stable complex. Thus, they concluded that the smallest functional unit for complement activation consists of MBL dimers bound to MASP-1 or MASP-2 homodimers. According to this model, trimers and tetramers of MBL should be able to form complexes containing up to two MASPs. This scenario fits well with the results from this study. However, Teillet et al. (5) reported that there was no difference in MASP binding between the two predominant MBL forms in serum, trimers, and tetramers and that these forms bound only a single MASP dimer. Nonetheless, Phillips et al. (43) more recently modeled MASP, C1r, and C1s interactions with MBL and C1q, arriving at two or four binding sites/dimer, again suggesting that tetrameric MBL could harbor two MASP dimers. A further detailed analysis of this subject awaits the purification of defined oligomers of MBL. Even less is known about the oligomer distribution of the ficolins and their relative capacity to bind one or more MASP dimers. In this study, we demonstrated that both L-ficolin and H-ficolin purified from serum are able to support the formation of co-complexes.

To our knowledge, we also provide the first indication of a functional role of such co-complexes in activation of the lectin pathway. We demonstrate in a clean system in vitro that the level of co-complex of MASP-1 and MASP-2 correlates well with the degree of C4 deposition on a mannan surface (Fig. 10A, 10B). We further find a correlation between C4 deposition and MASP-2 in complex with MASP-1–3/MAp44 in serum (Fig. 10C). Finally, we demonstrate that MAp44 may inhibit lectin pathway activation, not simply by brute force displacement of MASP-2 from MBL, but also by simple disruption of co-complexes of MASP-2 with MASP-1 (Fig. 11). This elegant mode of inhibition allows for a more potent effect, because the displacement of either MASP-1 or MASP-2 attenuates lectin pathway activation. Of note, in the present system we only considered the effect on C4 deposition, which is directly downstream of MASP-2. Hence, the observed effect upon displacement of MASP-1 is due solely to the importance of MASP-1 in transactivating MASP-2. However, MASP-1 also was found to cleave a significant amount of the C2 required for convertase formation in serum (10, 13). Hence, in a system examining C3 deposition and further downstream points, one would envision the inhibitory role of MAp44 to be even more significant.

In conclusion, for MASP-1 to be able to transactivate MASP-2, the two need to colocalize during activation. Three possible scenarios were presented in the introduction: heterodimers, co-complexes, or cooperation of distinct complexes. In light of the present findings, the first scenario does not seem significant, whereas the second and third scenarios remain viable and are not necessarily mutually exclusive options. We addressed the second option in this study, which conceptually parallels that of the C1 complex, demonstrating that co-complexes are indeed present and functional in human serum and that they may be functional targets of the endogenous natural inhibitor MAp44.

Disclosures
The authors have no financial conflicts of interest.

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