The Role of Mannose-Binding Lectin-Associated Serine Protease-3 in Activation of the Alternative Complement Pathway

Daisuke Iwaki, Kazuko Kanno, Minoru Takahashi, Yuichi Endo, Misao Matsushita and Teizo Fujita

*J Immunol* 2011; 187:3751-3758; Prepublished online 24 August 2011; doi: 10.4049/jimmunol.1100280

http://www.jimmunol.org/content/187/7/3751

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/08/24/jimmunol.1100280.DC1

References

This article cites 29 articles, 18 of which you can access for free at:

http://www.jimmunol.org/content/187/7/3751.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Role of Mannose-Binding Lectin-Associated Serine Protease-3 in Activation of the Alternative Complement Pathway

Daisuke Iwaki,* Kazuko Kanno,* Minoru Takahashi,* Yuichi Endo,* Misao Matsushita,† and Teizo Fujita*

Mannose-binding lectin (MBL)-associated serine proteases (MASPs) are responsible for activation of the lectin complement pathway. Three types of MASPs (MASP-1, MASP-2, and MASP-3) are complexed with MBL and ficolins in serum. Although MASP-1 and MASP-2 are known to contribute to complement activation, the function of MASP-3 remains unclear. In this study, we investigated the mechanism of MASP-3 activation and its substrate using the recombinant mouse MASP-3 (rMASP-3) and several different types of MASP-deficient mice. A proenzyme rMASP-3 was obtained that was not autoactivated during preparation. The recombinant enzyme was activated by incubation with Staphylococcus aureus in the presence of MBL-A, but not MBL-C. In vivo studies revealed the phagocytic activities of MASP-1/3–deficient mice and all MASPs (MASP-1/2/3)–deficient mice against S. aureus and bacterial clearance in these mice were lower than those in wild-type and MASP-2–deficient mice. Sera from all MASPs-deficient mice showed significantly lower C3 deposition activity on the bacteria compared with that of wild-type serum, and addition of rMASP-3 to the deficient serum restored C3 deposition. The low C3 deposition in sera from all MASPs-deficient mice was probably caused by the low level factor B activation that was ameliorated by the addition of rMASP-3. Furthermore, rMASP-3 directly activated factors B and D in vitro. These results suggested that MASP-3 complexed with MBL is converted to an active form by incubation with bacterial targets, and that activated MASP-3 triggered the initial activation step of the alternative complement pathway. The Journal of Immunology, 2011, 187: 3751–3758.
MASP-1 and MASP-3 are generated from the same gene by alternative splicing, consisting of the same H chain and distinct L chains (5). The function of MASP-3 is still unknown, including its activation mechanism and physiological substrates. In this study, we report the molecular basis of MASP-3 activation, the involvement of MASP-3 in the alternative pathway activation, and the possible substrates of MASP-3.

Materials and Methods

**Mice**

MASP-1/3-deficient (M1/M3 KO) and MASP-2-deficient (M2 KO) mice in a C57BL/6J background were prepared as described previously (8, 10). To obtain homozygous MASP-1/2/3-deficient (all MASPs KO) mice, male M1/M3 KO mice were crossed with female M2 KO mice and their heterozygous offspring were subsequently intercrossed. To generate homozygous all MASPs/C4-deficient (all MASPs/C4 KO) mice, all MASPs KO mice were crossed with C4-deficient mice in the C57BL/6J background (The Jackson Laboratory). In all experiments, 8-wk-old mice were used according to the guidelines for animal experimentation of Fukushima Medical University.

**Proteins**

Mouse rMBL-A and mouse rMBL-C were purchased from R&D Systems. Human C3 was from Quidel and the protein was subjected to freeze-thaw cycles to obtain C3(H2O) (11). Recombinant proenzyme of mouse Df (pro-Df) containing a five-residue activation peptide at its N terminus was prepared as described previously (9). Human MASP-1 and human Bf were purified from the serum (12, 13). Mouse rMASP-3 with a C-terminal V5-His tag was prepared using the Drosophila expression system (Invitrogen) (14). Approximately 1 mg purified rMASP-3 was obtained from 1200 ml culture supernatant.

**Bacteria**

*Staphylococcus aureus* (15) was cultivated overnight at 37°C in Luria-Bertani broth and then diluted 1:50 in fresh broth. After incubation at 37°C for 3 h, bacteria at mid-log phase were washed with PBS. The concentration of resuspended cells was adjusted by measuring the absorption at 660 nm (OD 0.5 = 1 × 10^6 CFU/ml). Heat-killed bacteria were prepared by heating the bacteria suspension at 70°C for 1 h. Labeling of heat-killed *S. aureus* with FITC was performed as follows. The bacteria (1 × 10^7) were suspended in 1 ml 0.1 M sodium carbonate buffer (pH 9.0) containing 0.1 mg/ml FITC (Sigma-Aldrich) and were incubated with rotation in the dark at room temperature for 1 h. They were then washed with HBSS (Sigma-Aldrich) and resuspended in the same buffer (1 × 10^7/ml).

**SDS-PAGE and immunoblot analysis**

Samples were electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions and proteins were transferred to polyvinylidene difluoride membranes. Proteins were then detected with HRP-conjugated antibodies using an LAS-3000 image analyzer (Fujifilm).

**Analysis of mouse rMASP-3 activation**

rMASP-3 (2 or 3 µg) and mouse serum (20 µl) were incubated together in a total volume of 40 µl in TBS-Ca^2+ (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 5 mM CaCl_2) on ice overnight. This led to complex formation of MASP-3 with MBL/ficolin in the serum. The mixture was then added to 500 µl TBS-Ca^2+/BSA containing heat-killed *S. aureus* (2 × 10^5) on ice overnight. The mixture was added to 500 µl TBS-Ca^2+/BSA containing heat-killed *S. aureus* (2 × 10^5) and incubated with rotation at 37°C for 30 min. The detection of MASP-3 activation was performed as described above.

rMASP-3 activation by MASP-1 was analyzed as follows. rMASP-3 (2 µg) and all MASPs KO serum (20 µl) were incubated together with or without active human MASP-1 (2 µg) in a total volume of 40 µl in TBS-Ca^2+ on ice overnight. The sample was incubated with heat-killed *S. aureus* (2 × 10^5) suspended in 500 µl TBS-Ca^2+/BSA at 4°C for 30 min, then further incubated at 37°C for 10 min with rotation. After incubation, detection of rMASP-3 activation was performed as described above. In another experiment, rMASP-3 (0.2 µg) was incubated alone or with active human MASP-1 (0.2 µg) in a total volume of 10 µl TBS-Ca^2+ containing 0.02% (w/v) BSA at 37°C for 5–60 min. After incubation, the reaction mixture was subjected to the immunoblot analysis to detect direct cleavage of rMASP-3 by MASP-1.

**Binding of mouse rMASP-3 to mouse MBLs**

Equal molar amounts (8.6 pmol each) of rMBL-A, rMBL-C, or BSA in 50 µl PBS were placed in microtiter wells (Nunc MaxiSorp) and incubated at 4°C overnight. After blocking the wells with HEPES-Ca^2+/BSA (10 mM HEPES [pH 7.4], 0.15 M NaCl, 5 mM CaCl_2, 5% [w/v] BSA), rMASP-3 was 2-fold serially diluted with HEPES-Ca^2+/BSA in the wells and incubated at 4°C for 3 h. The wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween), and HRP-conjugated anti-His tag Ab was added for each well. After washing with PBS, the plates were washed with PBS-Tween and then 3,3',5,5'-tetramethylbenzidine solution was added to each well. After developing, 1 M H_3PO_4 was added and the absorbance was measured at 450 nm.

**Analysis of C3 deposition on the bacterial cell surface**

C3 deposition on the bacterial cell surface was analyzed in a flow cytometer. All MASPs KO serum (10 µl) was incubated with or without mouse rMASP-3 in a total volume of 20 µl TBS-Ca^2+ buffer at 37°C overnight. The mixture was added to 80 µl HBSS containing heat-killed *S. aureus* (1 × 10^8) and further incubated with rotation at 37°C for 1.5, 5, or 30 min. After centrifugation (12,000 × g, 3 min), pellets were washed with ice-cold HBSS and incubated with anti-mouse C3 Ab (HyCult Biotechnology, product no. HM1065) in PBS containing 0.1% (w/v) BSA (PBS/BSA), with rotation at 4°C for 30 min. After washing with ice-cold PBS/BSA, the pellets were incubated with FITC-conjugated anti-rat IgGs Ab (Dako, product no. F0234) in PBS/BSA with rotation at 4°C for 30 min. After washing with ice-cold PBS, the pellets were fixed with 1% (w/v) paraformaldehyde in PBS and analyzed using the FACScalibur and CellQuest software (BD Biosciences).

**In vitro phagocytosis of bacteria**

Mouse peritoneal macrophages were prepared as follows. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% Brewer thioglycolate medium (Sigma-Aldrich) into male C57BL/6J mice. Peritoneal exudate cells were harvested 48 h after injection by peritoneal lavage with ice-cold HBSS. After washing, the cells were resuspended in ice-cold HBSS and used immediately for the following in vitro phagocytosis assay.

Various amounts of mouse rMASP-3 and 10 µl all MASPs KO serum were incubated together in a total volume of 20 µl TBS-Ca^2+ on ice overnight. The mixture was added to 80 µl HBSS containing FITC-labeled *S. aureus* (1 × 10^8) and further incubated with rotation in the dark at 37°C for 30 min. After centrifugation (12,000 × g, 3 min), pellets were washed and resuspended in 50 µl ice-cold HBSS. The suspension was mixed with 50 µl HBSS containing the thioglycolate-elicited peritoneal macrophages (2 × 10^6) from normal mice, and then incubated with rotation in the dark at 37°C for 30 min. After centrifugation (400 × g, 10 min), pellets were washed with TBS containing 1% (w/v) paraformaldehyde. The cells were then resuspended with trypan blue to quench extracellular fluorescence. The fluorescence intensity of phagocytosed bacteria in the cells was measured by flow cytometry and the mean fluorescence intensity per cell was determined.

**In vivo phagocytosis of bacteria**

Male mice were injected i.p. with FITC-labeled *S. aureus* (2 × 10^7) in 200 µl HBSS. One hour after injection, peritoneal cells were harvested from their abdominal cavities as described above. The cells were washed with ice-cold PBS and fixed with paraformaldehyde, and extracellular fluorescence was quenched using trypan blue. The flow cytometry phagocytosis assay was performed as described above for the in vitro phagocytosis of bacteria.

**Bacterial loads in the organs of infected mice**

Male mice were injected i.p. with a nonlethal dose (2 × 10^7 CFU) of live *S. aureus* suspended in 200 µl sterile saline. Three days after injection, the...
mice were anesthetized and exsanguinated, and their organs were collected. The organs were washed with sterile saline, weighed, and homogenized in 2 ml sterile water. Dilutions of the homogenates were streaked onto Luria-Bertani agar plates and cultured at 37°C for 24 h. Colony numbers on these plates were counted and the results were expressed as CFU/g (wet weight) of organ.

**Analysis of Bf activation**

Mouse serum (1 μl) was incubated with or without mouse rMASP-3 (2 μg) in a total volume of 10 μl TBS-Ca²⁺ on ice overnight. The samples were further incubated with or without heat-killed *S. aureus* (3 × 10⁷) in TBS-Ca²⁺ containing 5 mM MgCl₂ (TBS-Ca²⁺/Mg²⁺) with rotation at 37°C for 30 min (total volume 20 μl). After incubation, 4 μl 6× reducing SDS-PAGE sample buffer was added to the samples and incubated at 80°C for 10 min. After centrifugation, the supernatant was subjected to SDS-PAGE and immunoblotting with an anti-Bf Ab to detect the released Bf from Bf. In this reconstitution assay for Bf activation, the samples containing various combinations of rMBL-A (1 μg), mouse RASP3 (2 μg), proDf (0.2 μg), human C3(H₂O) (1.5 μg), and human Bf (0.2 μg) were pre-incubated in TBS-Ca²⁺ on ice overnight. The samples were further incubated with heat-killed *S. aureus* (3 × 10⁷) in TBS-Ca²⁺/Mg²⁺ with rotation at 37°C for 3 h (total volume 20 μl). Detection of Bf activation was performed as described above.

**Df activation by mouse rMASP-3**

Cleavage of Df by rMASP-3 was analyzed as described previously (9). In brief, proDf (0.1 μg) was incubated alone or with rMASP-3 in a proenzyme form (2 μg) in TBS-Ca²⁺/Mg²⁺ at 37°C for 1 h (total volume 40 μl). As a control, proDf was incubated with 1 or 2 μg active mouse RASP-1 (8) instead of rMASP-3. After incubation, samples were treated with N-glycosidase F and subjected to SDS-PAGE and immunoblotting with an anti-Df Ab.

**Statistical analysis**

StatView 5.0 software (SAS Institute) was used for statistical analysis.

**Results**

**Characteristics of mouse rMASP-3 and its activation**

rMASP-3 was expressed in Drosophila S2 cells and purified from the culture supernatant using a Ni-NTA agarose column. SDS-PAGE analysis of the recombinant protein revealed a single band of 105 kDa under reducing conditions (Fig. 1A, lane 1). The data suggested that the recombinant protein was a proenzyme, similar to recombinant human MASP-3 (5, 16, 17).

We then tried to find substances to induce rMASP-3 activation. rMASP-3 was added to wild-type mouse serum and incubated with mannan-agarose, GlcNAc-agarose, or heat-killed *S. aureus* at 37°C for 3 h. After incubation with the bacteria, the L chain of activated rMASP-3 was clearly detected (Fig. 1A, lane 3), whereas incubation with the agarose conjugates showed low activation levels (Fig. 1A, lanes 5 and 7). Thus, we revealed that heat-killed *S. aureus* is an effective activator of rMASP-3. The activation induced by the bacteria was time-dependent (1–30 min) (Supplemental Fig. 1). We also tried to reproduce MASP-3 activation by incubation with heat-killed *S. aureus* and combinations of the recombinant proteins, such as rMASP-3 and rMBLS. When rMASP-3 and rMBL-A were incubated together with the bacteria at 37°C for 30 min, rMASP-3 was obviously activated (Fig. 1B, lane 3). When rMBL-C was used instead of rMBL-A, the activation was weak (Fig. 1B, lane 4) and rMASP-3 was not activated in the absence of rMBLS (Fig. 1B, lane 5). Next, we investigated why rMBL-A, but not rMBL-C, activates rMASP-3. SDS-PAGE analysis showed that both rMBL-A and rMBL-C exhibited oligomeric structures similar to their native proteins (Supplemental Fig. 2). However, the binding of rMASP-3 to rMBL-C was much lower than to rMBL-A (Fig. 1C).

Next, we examined rMASP-3 activation in the MASP-deficient sera. The mixture of rMASP-3 and mouse serum was incubated with the bacteria at 4°C for 30 min and then at 37°C for 10 min. As shown in Fig. 1, in a reconstitution assay for Bf activation, the samples containing various combinations of rMBL-A (1 μg), mouse RASP3 (2 μg), proDf (0.2 μg), human C3(H₂O) (1.5 μg), and human Bf (0.2 μg) were pre-incubated in TBS-Ca²⁺ on ice overnight. The samples were further incubated with heat-killed *S. aureus* (3 × 10⁷) in TBS-Ca²⁺/Mg²⁺ with rotation at 37°C for 3 h (total volume 20 μl). Detection of Bf activation was performed as described above.

**FIGURE 1.** Activation of rMASP-3. A, rMASP-3 activation is induced by heat-killed *S. aureus*. rMASP-3 was added to wild-type serum and incubated with heat-killed *S. aureus*, mannan-agarose, or GlcNAc-agarose at 37°C for 3 h. The activation of rMASP-3 was analyzed by immunoblotting to detect the L chain of activated rMASP-3. Lane 1, rMASP-3 (1 μg) alone was subjected to reducing SDS-PAGE and proteins in the gel were stained with Coomassie brilliant blue R250. Lane 2, rMASP-3 (0.05 μg) alone without incubation was subjected to reducing SDS-PAGE and immunoblotting. B, rMBL-A is necessary for the rMASP-3 activation induced by heat-killed *S. aureus*. The mixture of rMASP-3 and rMBL-A or rMBL-C was incubated with heat-killed *S. aureus* at 37°C for 30 min and the L chain of activated rMASP-3 was detected by immunoblotting. Lane 1, rMASP-3 (0.1 μg) alone without incubation was subjected to reducing SDS-PAGE and immunoblotting. C, Binding of rMASP-3 to rMBLS. rMASP-3 was added to microtiter wells coated with equal molar amounts of rMBL-A, rMBL-C, or BSA and incubated at 4°C for 3 h. The binding of rMASP-3 was detected by HRP-conjugated anti-His tag Ab. The data shown are the means ± SE of three experiments.
shown in Fig. 2A, rMASP-3 activation was lowest in all MASPs KO serum, and the other two types of deficient sera also showed lower activation than that of the wild-type serum. The band intensity of L chain of activated rMASP-3 in all MASPs KO serum was 4% of the wild-type serum, and the intensities in M2 KO and M1/M3 KO sera were 61 and 37%, respectively. The activation in all MASPs KO serum was increased by addition of MASP-1 (Fig. 2B, left panel, lane 3), and rMASP-3 was directly cleaved by MASP-1 in a time-dependent manner (Fig. 2B, right panel). These data suggested that MASP-1 has the ability to activate MASP-3.

C3 opsonization of bacteria

C3 deposition on the bacterial cell surface was analyzed by flow cytometry by incubation of wild-type serum, all MASPs KO serum, and all MASPs KO serum containing rMASP-3 with heat-killed S. aureus. The deficient mouse serum showed significantly lower C3 deposition activity compared with wild-type mouse serum, and this activity was restored by addition of rMASP-3 to the serum (Fig. 3A). After 5 min incubation with the bacteria, the deficient serum containing rMASP-3 showed a higher level of C3 deposition than that of the deficient serum alone and almost the same level as wild-type mouse serum after 30 min incubation. We then examined the ability of the deficient mouse serum to opsonize bacteria (Fig. 3B). FITC-labeled S. aureus was incubated with the deficient serum containing various amounts of rMASP-3 and then further incubated with peritoneal macrophages. The fluorescence intensity of bacteria in the cells was increased by the addition of rMASP-3. The mean fluorescence intensity of the deficient serum was much lower than that of the wild-type serum (42% of wild-type serum), and the intensity was dose-dependently increased by the addition of rMASP-3, reaching 90% of that of the wild-type serum at a dose of 1 μg/ml rMASP-3. Thus, it was determined that rMASP-3 had the ability to promote C3 opsonization in serum.

Activation of Bf and Df by mouse rMASP-3

As previous studies have indicated (5, 16), rMASP-3 was unable to directly cleave C3 or C4 (Supplemental Fig. 3). MASP-3-mediated C3 opsonization may therefore not be caused by activation of the lectin pathway. We next examined whether MASP-3 is involved in Bf activation, a major component of the alternative pathway. After incubation with heat-killed S. aureus, Bf activation in serum was analyzed by immunoblotting to detect fragment Ba (Fig. 4A). Bf activation in M2 KO serum was detected at almost the same level as in wild-type mouse serum. However, M1/M3 KO and all MASPs KO sera exhibited low levels of Bf activation, and this activation was restored by the addition of rMASP-3 to these sera. The restoration was dose- and time-dependent and Bf activation was not further increased after >30 min incubation (Supplemental Fig. 4). All MASPs/C4 KO serum also exhibited a low level of Bf activation and this activation was restored by the addition of rMASP-3 (Fig. 4B). The levels of restored activation in all MASPs KO and all MASPs/C4 KO sera were similar to each other, indicating that the activation is independent of C4. The Bf

![Figure 2](http://www.jimmunol.org/Downloaded_from/http://www.jimmunol.org.org)  
**FIGURE 2.** MASP-1 and MASP-2 participate in rMASP-3 activation. A. Deficiencies of MASP-1 and MASP-2 in serum decrease rMASP-3 activation. Samples containing rMASP-3 and mouse serum were preincubated with heat-killed S. aureus at 4°C for 30 min and further incubated at 37°C for 10 min. The L chain of activated rMASP-3 was detected by immunoblotting. The experiment was performed twice with similar results. The data are representative of one experiment (left panel). Each band intensity of L chain in the MASP-deficient sera was compared with the wild-type (right panel). The intensity in the wild-type serum is presented as 100. The data shown are the mean relative intensities ± SE of two experiments. B, rMASP-3 is activated by MASP-1. rMASP-3 with or without active human MASP-1 was added to all MASPs KO serum and preincubated with heat-killed S. aureus at 4°C for 30 min, then further incubated at 37°C for 10 min (left panel). rMASP-3 was incubated alone or with active human MASP-1 at 37°C for 5–60 min (right panel). After these incubations, the L chain of activated rMASP-3 was detected by immunoblotting.
and C3\( (H_2O) \) (Fig. 4a).

A single band was detected in the experiment using human Bf (Fig. 5, lane 4) and mouse Bf (similar results were reported in Ref. 18), whereas there was no statistically significant difference between M2 KO and wild-type mice (Fig. 6B). These data suggested that the in vivo bacterial phagocytic and clearance efficiencies of M1/M3 KO and all MASPs KO mice are inferior to those of wild-type and M2 KO mice.

**Discussion**

In previous studies, recombinant MASP-1 and MASP-2 were autoactivated during purification and were obtained as activated enzymes (8, 14, 19, 20), whereas recombinant MASP-3 was purified as a proenzyme without autoactivation (5, 16, 17). Because little was known regarding MASP-3 activation, there are only a few reports on its proteolytic activity. In a previous study (16), recombinant human MASP-3 protein was obtained by incubating the proenzyme at 4°C for several weeks. The activated protein cleaved a dipeptide thioester substrate, but it was considered that activation was caused by a contaminating protease from the cell culture supernatant. Another report indicated that the recombinant human MASP-3 catalytic domain cleaved an insulin-like growth factor-binding protein, as well as synthetic peptides (21). Thus, there is still insufficient information to understand the physiological role of MASP-3.

In this study, mouse rMASP-3 was obtained as a single-chain proenzyme and was therefore a good tool to clarify its activation mechanism. Binding of the MBL/ficolin-MASP complexes to GlcNAc- or mannan-agarose activated MASP-1 and MASP-2 (3, 22). In contrast, rMASP-3 activation was virtually undetectable after incubation with these agarose conjugates. We found that heat-killed *S. aureus* was an effective inducer for MASP-3 activation. Because the Bf cleavage was increased by the addition of proDf (Fig. 4C, lane 4), the addition of proDf was observed in the absence of rMASP-3 (Fig. 4C, lanes 5 and 6).

Because the Bf cleavage was increased by the addition of proDf, MASP-3 may have the ability to activate Df as well as Bf. We examined Df activation by rMASP-3. When proDf was incubated alone or with rMASP-3, the molecular weight of Df incubated with rMASP-3 (Fig. 5, lane 4) was only slightly lower than that of proDf incubated alone (Fig. 5, lanes 1 and 5), and the same result was obtained after incubation with rMASP-1 (Fig. 5, lanes 2 and 3). These data indicate that rMASP-3, as well as rMASP-1, can directly cleave the activation peptide from proDf.

**Decreased in vivo bacterial phagocytic and clearance activities of M1/M3 KO and all MASPs KO mice**

We examined in vivo phagocytosis of bacteria by mouse resident peritoneal cells. One hour after i.p. injection of FITC-labeled *S. aureus*, peritoneal cells were harvested and the mean fluorescence intensities of cells that had phagocytosed bacteria were measured by flow cytometry. The mean fluorescence intensities in M1/M3 KO and all MASPs KO mice were significantly lower than those of wild-type mice (59 and 57% of wild-type mice, respectively), whereas there was no statistically significant difference between M2 KO and wild-type mice (Fig. 6A). Moreover, we examined the ability of mice to clear bacteria from infected organs. Bacterial loads in different organs (spleen, kidney, liver, and lung) of mice were determined at day 3 after i.p. infection with *S. aureus*. The bacterial loads in M1/M3 KO and all MASPs KO mice were higher than in wild-type and M2 KO mice in all organs, and they showed statistically significant differences compared with wild-type mice (Fig. 6B). These data suggested that the in vivo bacterial phagocytic and clearance efficiencies of M1/M3 KO and all MASPs KO mice are inferior to those of wild-type and M2 KO mice.

**Activation caused by rMASP-3 was not detected in the absence of bacteria.** Two bands of fragment Ba were seen in Fig. 4A and 4B, probably owing to the heterogeneity of the sugar moiety within mouse Bf (similar results were reported in Ref. 18), whereas a single band was detected in the experiment using human Bf (Fig. 4C). We next tried to reconstitute the Bf activation caused by MASP-3 with combinations of purified proteins and heat-killed *S. aureus*. Bf was activated in the presence of rMASP-3, rMBL-A, and C3(H_2O) (Fig. 4C, lane 3). Cleavage was further increased by the addition of proDf (Fig. 4C, lane 4). In contrast, no cleavage was observed in the absence of rMASP-3 (Fig. 4C, lanes 5 and 6).
found that zymosan from *Saccharomyces cerevisiae* was another ligand that effectively induced MASP-3 activation (data not shown). The reconstitution assay revealed that MBL was essential for MASP-3 activation and the ability of rMBL-A was much higher than that of rMBL-C. The low ability of rMBL-C may be due to its low activity to form a complex with MASP-3. A similar observation had previously been reported that mouse MBL-C exhibited a lower C4 deposition activity than did mouse MBL-A (23, 24). rMASP-3 also bound to recombinant mouse ficolin A and ficolin B, but these ficolin/rMASP-3 complexes had no ability to activate rMASP-3 (data not shown).

We next investigated the involvement of MASP-3 in complement activation. All MASPs KO serum showed low C3 deposition on *S. aureus*. The addition of rMASP-3 to the deficient serum increased the level of C3 deposition on the bacteria, taking 30 min to reach the same level as wild-type serum. The delay in C3 deposition was due to its low ability to activate MASP-3. The ability of all MASPs KO serum was the lowest among the three types of deficient sera, and the other two deficient sera also showed lower ability than that of wild-type serum. MASP-1 and MASP-2 therefore might be required for quick activation of MASP-3. Because the activation of rMASP-3 in M1/M3 KO serum was lower than in M2 KO serum, MASP-1 might be more important than MASP-2 for MASP-3 activation. The activated form of MASP-3 was found in MBL/MASPs complexes isolated from human plasma (5), and this might be due to active MASP-1 in the complexes, because rMASP-3 was activated by MASP-1 in this study. MASP-1 seems to have the ability to activate MASP-3 as well as MASP-2 (8). The addition of rMASP-3 also restored the ability of all MASPs KO serum to opsonize bacteria, and the phagocytosis level was increased up to 90% that of wild-type serum with 1 μg/ml rMASP-3. This concentration is reasonable considering the mean serum level of human MASP-3 (6.4 mg/l) (17). It is therefore likely that MASP-3 contributes to complement activation in vivo.

The results of C3 deposition suggested that MASP-3 is involved in the C3 opsonization of bacteria. However, we observed neither direct C3 cleavage nor C4 activation by rMASP-3. Additionally, it was previously observed that MASP-3 was not able to cleave C2 (16). These results indicated that the MASP-3–mediated C3 opsonization is not caused by activation of the lectin pathway. Therefore, we next investigated the effect of MASP-3 on activation of the alternative pathway. The sera from all MASPs KO and all MASPs/C4 KO mice exhibited low levels of Bf activation, as seen in M1/M3 KO mice (9), and the activation in these sera was restored by the addition of rMASP-3. These data suggested that MASP-3 is involved in activation of the alternative pathway. Because the presence of C4 did not influence Bf activation by rMASP-3, the classical pathway is probably not involved in this activation. In the in vitro reconstitution assay for Bf cleavage, Bf was cleaved in the presence of rMASP-3, rMBL-A, and C3(H2O), and human Bf were incubated with heat-killed *S. aureus* at 37°C for 3 h. Fragment Ba was detected by immunoblotting.
with C1 inhibitor (16) and MASP-3 may therefore be more effective than MASP-1 in activating Df in plasma.

The C3 opsonization shown in Fig. 3 may be due to Bf activation induced by MASP-3 rather than to direct C3 activation by MASP-3. A putative model for MASP-3-mediated complement activation is shown in Fig. 7. MASP-3 appears to possess the ability to activate both Bf and Df; however, it is not clear which activation plays a more important part in the physiological role of MASP-3. To clarify this matter, all MASPs/Df-deficient mice will need to be generated.

In contrast to MASP-1 and MASP-2, there has been little research into the function of MASP-3. In this study, we found that proenzyme MASP-3 complexed with MBL was activated by bacteria, and that activated MASP-3 induced activation of the alternative pathway, leading to C3 opsonization of bacteria. Although previous studies suggested that MASP-3 inhibited MASP-2–dependent C4 deposition (5, 17), and similar results were observed in our experiments with wild-type and M1/M3 KO mice (see Supplemental Fig. 3A), we propose that activation of the alternative pathway may be a more physiological function of MASP-3. However, we do not know to what extent the MASP-3–mediated pathway contributes to complement activation. This is probably not a major pathway of complement activation, but it might be able to boost the alternative pathway or act as a backup pathway in the case of deficiencies of complement components, such as the MBL-dependent C2 bypass pathway (26). Furthermore, our in vivo study with MASP-deficient mice revealed that the bacterial phagocytic and clearance activities of M1/M3 KO and all MASPs KO mice were significantly lower than those of wild-type and M2 KO mice. Although MASP-2 is thought to play a key role in activation of the lectin pathway, these data suggested that MASP-1 and/or MASP-3 may be more important than MASP-2 in the phagocytosis and clearance of bacteria. Recently, mutations in the MASP-3 gene have been reported to be a cause of the Carnevale, Mingarelli, Malpuech and Michels syndromes (the so-called 3MC syndrome), a disorder that includes craniofacial defects (27). MASP-3 may also play an important role in embryonic development.

The pathway investigated in this study might be a vestige of the ancestral complement system. Complement is one of the most ancient host defense systems. Some primitive vertebrates and invertebrates also possess complement components, and they are thought to compose ancient complement activation pathways without Ab involvement (28). Ascidians, which belong to the

![Figure 6](image_url)

**FIGURE 6.** M1/M3 KO and all MASPs KO mice show decreased bacterial phagocytic and clearance activities in vivo. A, In vivo phagocytosis of *S. aureus* by mouse resident peritoneal cells. Mice were injected i.p. with FITC-labeled *S. aureus* and peritoneal cells were harvested from their abdominal cavities 1 h after injection. The mean fluorescence intensity of cells that had phagocytosed bacteria was measured by flow cytometry. The data shown are the means ± SE of the number of animals indicated below each bar. *p < 0.05 compared with wild-type mice as analyzed by the unpaired Student t test. B, Bacterial loads in organs of mice infected with *S. aureus*. Mice were injected i.p. with live *S. aureus* and bacterial loads in organs were determined 3 d after injection as described in Materials and Methods. The data shown are the means ± SE of 10 animals for each group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with wild-type mice as analyzed by the Mann–Whitney U test.

![Figure 7](image_url)

**FIGURE 7.** Putative MASP-3–mediated pathway of complement activation. MBL is composed of trimeric subunits and each MASP forms a homodimer. Binding of MBL to carbohydrates on the bacterial surface induces activation of MASP-3 in the MBL complex. Activated MASP-3 cleaves C3(H2O)-bound Bf. The resulting C3bBb complex is the initial C3 convertase. Bacterial surface-bound C3b acts as an opsonin or associates with Bf to generate more C3 convertase (C3bBb). Proenzyme MASP-3 can activate Df and MASP-1 activates MASP-3.
subphylum Urochordata, have an ancient lectin-based complement system (29). Homologs of MASP and Bf have been found in these primitive animals, whereas Df homologs have never been detected. Our proposed pathway, in which the MBL/MASP complex directly activates the alternative pathway, might provide insight into the evolution of the complement system.

Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental figure legends**

**FIGURE S1.** Activation of mouse rMASP-3 is time-dependently increased. Mouse rMASP-3 (2 μg) and wild-type serum (20 μl) were incubated together in a total volume of 40 μl in TBS-Ca$^{2+}$ on ice overnight. The mixture was added to 500 μl of TBS-Ca$^{2+}$/BSA containing heat-killed *S. aureus* ($2 \times 10^8$), pre-incubated with rotation at 4°C for 30 min, and then incubated at 37°C for 1–30 min. After incubation, the pellets were washed with TBS-Ca$^{2+}$ and incubated in reducing SDS-PAGE sample buffer at 80°C for 10 min. After centrifugation, the supernatants were subjected to SDS-PAGE and immunoblotting with HRP-conjugated anti-His tag antibody to detect the L chain of activated mouse rMASP-3. BG, the same procedure was performed in the absence of both rMASP-3 and mouse serum as a background. PC, 0.05 μg of rMASP-3 alone without incubation was subjected to reducing SDS-PAGE and immunoblotting.

**FIGURE S2.** Mouse rMBL-A and mouse rMBL-C exhibit oligomeric structures like their native proteins. A, rMBL-A and rMBL-C (1 μg of each) were subjected to SDS-PAGE on a 10% gel under reducing conditions and the gel was stained with SYBR Green. B, rMBL-A, rMBL-C, and serum proteins that bound to mannan-agarose, were subjected to SDS-PAGE on a 5% gel under non-reducing conditions and immunoblotting with anti-mouse MBL-A or anti-mouse MBL-C antibodies (HyCult Biotechnology).

**FIGURE S3.** Mouse rMASP-3 neither directly cleaves C3 nor activates C4. A, C4 deposition on solid-phase *S. aureus*. Various amounts of rMASP-3 and mouse serum (0.5 μl) were pre-incubated together in a total volume of 20 μl of TBS-Ca$^{2+}$ on ice overnight. Then, samples were diluted with TBS-Ca$^{2+}$ containing 0.1% (w/v) BSA up to 100 μl. The diluted samples were added to microtiter wells with heat-killed *S. aureus* and incubated at room temperature for 30 min. After washing the wells, human C4 was added to each well and incubated on ice for 30 min. C4 deposition in the wells was measured using HRP-conjugated anti-human C4 polyclonal antibody (Biogenesis). B, Cleavage of C3. Mouse rMASP-3 (2 μg), mouse rMBL-A (1 μg), and human C3 (2 μg) were pre-incubated together in a total volume of 20 μl of TBS-Ca$^{2+}$, Mg$^{2+}$ on ice.
overnight. The mixture was further incubated with heat-killed *S. aureus* \((3 \times 10^7)\) in TBS-Ca\(^{2+}\), Mg\(^{2+}\) (total volume 20 μl) with rotation at 37°C for 3 h. After the incubation, 4 μl of 6 × reducing SDS-PAGE sample buffer was added to the sample and incubated at 80°C for 10 min. After centrifugation, supernatants were subjected to reducing SDS-PAGE on a 10% gel and immunoblotting with an anti-C3a antibody (R&D Systems). As a positive control, C3 was incubated with active human MASP-1 (*lane 5*).

**FIGURE S4.** Bf activation in all MASPs KO serum is dose- and time-dependently restored by mouse rMASP-3. (*A*) Dose-dependent Bf activation by rMASP-3. Mouse serum (1 μl) and various amounts of rMASP-3 were pre-incubated together in a total volume of 10 μl of TBS-Ca\(^{2+}\) on ice overnight. The samples were further incubated with or without heat-killed *S. aureus* \((3 \times 10^7)\) in TBS-Ca\(^{2+}\), Mg\(^{2+}\) with rotation at 37°C for 30 min (total volume 20 μl). After incubation, 4 μl of 6 × reducing SDS-PAGE sample buffer was added to the samples and incubated at 80°C for 10 min. After centrifugation, the supernatant was subjected to SDS-PAGE and immunoblotting with an anti-Bf antibody to detect the released Ba from Bf. (*B*) Time-dependent Bf activation by rMASP-3. Mouse serum (1 μl) and rMASP-3 (1 μg) were pre-incubated together in a total volume of 10 μl of TBS-Ca\(^{2+}\) on ice overnight. The samples were further incubated with heat-killed *S. aureus* \((3 \times 10^7)\) in TBS-Ca\(^{2+}\), Mg\(^{2+}\) with rotation at 37°C for 0–120 min (total volume 20 μl). The detection of Bf activation was performed as described above.
Fig. S2
Fig. S3

A

C4 deposition (A450)

rMASP-3 (μg)

WT serum
M1/M3 KO serum
all MASP's KO serum

B

kDa

1: C3
2: rMASP-3 + rMBL-A + C3 + S. aureus
3: C3 + S. aureus
4: S. aureus
5: MASP-1 + C3

C3α
A

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
serum & WT & all MASP3 KO & - \\
\hline
rMASP-3 (μg/ml) & 0 & 0 & 0.5 & 2.5 & 5 & 25 & 50 & 100 & 0 \\
S. aureus & - & + & - & + & - & + & - & + & - \\
\hline
\end{tabular}
\end{table}

B

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
serum & all MASP3 KO & WT & - \\
\hline
time (min) & 0 & 5 & 10 & 20 & 30 & 60 & 120 & 120 & - \\
\hline
\end{tabular}
\end{table}

Fig. S4