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Mannose-Binding Lectin (MBL)-Associated Serine Protease (MASP)-1 Contributes to Activation of the Lectin Complement Pathway

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The complement system plays an important role in innate immunity. In the lectin complement pathway, mannos-binding lectin (MBL) and ficolins act as recognition molecules, and MBL-associated serine protease (MASP) is a key enzyme. It has been suggested that MASP-2 is responsible for the activation of C4. Other serine proteases (MASP-1 and MASP-3) are also associated with MBL or ficolins; however, their functions are still controversial. In this study, a MASP-1- and MASP-3-deficient mouse model (MASP1−/−/H11546) was generated by a gene targeting strategy to investigate the roles of MASP-1 and MASP-3 in the lectin pathway. Serum derived from MASP1−/−/H11546 mice showed significantly lower activity of both C4 and C3 deposition on mannan-agarose, and this low activity was restored by the addition of recombinant MASP-1. MASP-1/3-deficient serum showed a significant delay for activation of MASP-2 compared with normal serum. Reconstitution of recombinant MASP-1 in MASP-1/3-deficient serum was able to promote the activation of MASP-2. From these results, we propose that MASP-1 contributes to the activation of the lectin pathway, probably through the activation of MASP-2. The Journal of Immunology, 2008, 180: 6132–6138.

In the lectin pathway, mannos-binding lectin (MBL)3 or ficolins recognize carbohydrates (such as mannos and N-acetylgalactosamine) on microorganisms (5, 6). Activation of the pathway is triggered by serine proteases that are associated with MBL or ficolins, termed MASP (MBL-associated serine proteases) (7). MASP are similar to C1r and C1s in the classical pathway, and have highly conserved domain structures, including C1r/C1s/Uegf/bone morphogenetic protein 1, epidermal growth factor-like, C1r/C1s/Uegf/bone morphogenetic protein 2, short consensus repeat 1, short consensus repeat 2, and serine protease domains. Three kinds of MASP, MASP-1 (7), MASP-2 (8), and MASP-3 (9), and a truncated form of MASP-2, sMAP (10, 11), are present in MBL and ficolin complexes. Some in vitro evidence has suggested that MASP-2 alone is sufficient to trigger complement activation. First, only MASP-2 has proteolytic activity toward C4 and C2, similar to C1s in the classical pathway, although MASP-1 is able to cleave C2 (8, 12). Second, recombinant MASP-2 is easily activated by itself during purification (13), indicating that autoactivation of MASP-2 might occur in the lectin pathway cascade. However, the role of MASP-1 in the lectin pathway is still controversial; MASP-1 is capable of cleaving C3 directly (9, 14, 15), resulting in activation of the alternative pathway (14). These results suggest that there might be another route, independent of MASP-2, for the activation of the complement system (14). However, it has not been confirmed whether cleavage of C3 by MASP-1 would actually involve activation of the lectin pathway in vivo, as MASP-1 activity is very low (16). The function of MASP-3 is not yet clear. MASP3 mRNA

Abbreviations used in this paper: MBL, mannose-binding lectin; MASP, MBL-associated serine protease; ES, embryonic stem; sMAP, small MBL-associated protein; rMASP-1n, recombinant native type of MASP-1; rMASP-1i, recombinant inactive MASP-1 mutant; rMASP-1K, recombinant MASP-1 mutant modified by replacing the arginine129 with lysine; rMASP-2i, recombinant inactive MASP-2 mutant.

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is transcribed by an alternative splicing/polyadenylation mechanism from the MASP1/3 gene, in which the regions encoding the serine protease domains of MASP-3 and MASP-1 are tandemly located (9).

In mouse, the MBL-MASP complex was first described as a Ra-reactive factor that binds specifically to Ra chemotype strains of Salmonella (17). Murine MASP-1 (18) and MASP-2 (19) have already been cloned and characterized. We previously described murine MASP3 cDNA from the livers of C57BL/6J mice (20). In the present study, we generated MASP-1 and MASP-3 double-deficient mice using a standard gene targeting method. This knock-out mouse showed insufficient abilities to activate C3, C4, and C5.

Materials and Methods

Generation of MASP1/3-deficient mice

Genomic DNAs, including the murine MASP1/3 gene, were isolated from a 129sv phage genomic library (Stratagene). The targeting vector was constructed as illustrated in Fig. 1A and transfected into 11 cells by electroporation. After selection with 200 μg/ml G418 (Invitrogen), embryonic stem (ES) clones were picked up, and their genotypes were analyzed by Southern blot analysis of genomic DNA, including part of the first intron. The targeting vector was constructed by replacing the arginine429 with lysine (rMASP-1K) have been expressed and purified in insect cells. Native type of recombinant MASP-1 was defined as rMASP-1n. Mutant type of recombinant MASP-1 was defined as rMASP-1i in which the arginine429 was replaced with lysine. In addition, we used recombinant inactive MASP-1 mutant (rMASP-1i) in which the serine627 was replaced with alanine.

Two recombinants, rMASP-1n and rMASP-1i were generated as follows. The cDNA encoding the entire coding region of the MASP1 gene was amplified by RT-PCR. A sequence encoding 6× His was added at the 3′-end of the cDNA instead of the native stop codon. The cDNA was ligated into pVL1393 vector (BD Biosciences), to generate rMASP-1n. To construct rMASP-1i, the codon for the serine residue at amino acid position 627, essential for the active center of MASP-1, was replaced with an alanine site-directed mutagenesis based on PCR. Both plasmid constructs were transfected into Sf21 cells with BacPAK6 viral DNA (Bsu36I digest) using Bactefect reagent (BD Biosciences). After incubation for 3 days at 27°C, recombinant virus was harvested from culture supernatants. Virus plaques were made by infection of virus into an agarose-fixed monolayer of Sf21 cells. Four plaques were picked up from each construct. These virus stocks were further amplified by infection into Sf21 cells. For expression of recombinant proteins, Sf21 cells were routinely cultured in 500-ml Erlenmeyer flasks containing 200 ml of S900II serum-free medium (Invitrogen). For large scale preparation, 300 ml of Sf21 cells (0.5×10^{6}/ml) were infected with recombinant baculovirus by 5–10 multiplicity of infection. To purify recombinant proteins, culture supernatant was concentrated by an Amicon stirred cell with YM-30 filter (Millipore) until protein concentration was estimated by BCA protein assay (Pierce).

Expression and purification of recombinant murine MASP-2i

Recombinant murine MASP-2i, in which the serine residue at the active center of the protease domain was replaced with an alanine, was expressed in Drosophila Schneider 2 cells and purified by a nickel column as described (21).
Polyclonal Abs against murine MASP-1 and MASP-2

Two kinds of polypeptide corresponding to the regional sequences of the L chain of MASP-1 (Glu584 to Asn680) and the H chain of MASP-2, including the sMAP region (Thr86 to Leu248), were expressed as recombinant protein- 
teins using a pET21a vector (Novagen) in Escherichia coli. The products were purified from a gel after SDS-PAGE by electro-elution (Bio-Rad). Polyclonal Abs were prepared by immunizing rabbits with the purified polypeptides as Ags. These Abs react with the denatured protein in SDS-
PAGE. To establish a polyclonal Ab that recognizes native MASP-1, a 
rabbit was immunized with mASP-1.

Detection of MASP-1 in murine sera on mannan-coated plates

Maxi-Sorp plates (Nunc) were coated with 10 μl of 10 μg/ml muran (M3640; Sigma-Aldrich) in sodium carbonate buffer (pH 9.6) soaked over-
night. Blocking was done by adding 1% BSA in TBS/5 mM CaCl2 (TBS/ 
Ca) buffer. After washing with TBS/Ca containing 0.05% Tween 20 (TBS/ 
Ca/tw), serially diluted murine serum in TBS/Ca/tw was added to the wells. 
After incubation for 30 min at 37°C, the wells were washed four times with 
TBS/Ca/tw. Each well was incubated with polyclonal anti-mouse MASP-1 
for 30 min at room temperature, and then washed four times with TBS/ 
Ca/tw. The wells were further incubated with HRP-conjugated anti-rabbit 
Ig for 30 min. Enzymatic color development was achieved by 3,3,5,5-
tetramethylbenzidine-Microwell Peroxidase Substrate system (KPL) and the 
reaction was stopped by adding 1M H3PO4. Color was detected by the absorbance at 450 nm.

Western blotting of MBL-MASP complexes eluted from mannan-agarose

Mouse serum was diluted with TBS/Ca buffer. The diluted serum was 
incubated with 20 μl of muran-agarose (Sigma-Aldrich). After washing 
the agarose four times with TBS/Ca/tw, the proteins captured on the aga-
rose were eluted with 50 μl of 1X SDS-PAGE loading buffer with or 
without 2-ME. Eluted samples were separated by SDS-PAGE followed by 
blotting to polyvinylidene fluoride membrane (Immobilon P; Millipore) 
in a semidyrid system (Bio-Rad). The blotted membranes were then subjected to 
Western blotting. Briefly, the membrane was incubated with 
anti-MASP-1 or anti-MASP-2 Abs and then with HRP-conjugated anti-
rabbit IgG (DakoCytomation). Proteins were detected using the ECL Plus 
Western blotting detection system (GE Healthcare Biosciences) and visu-
alized in a LAS3000 (Fuji Film).

C4-deposition on mannan-coated microtiter plates

MaxiSorp surface of FluoloNunc plates (Nunc) were coated with 100 μl of 10 
μg/ml mannan in sodium carbonate buffer (pH 9.6) soaked overnight. Blocking was done by adding 1% BSA in TBS buffer. Serially diluted 
murine serum in GVB buffer containing 1% BSA (GVB/BSA) was added to 
mannan-coated wells. After incubation for the indicated periods, the wells 
were washed four times with TBS/Ca/tw buffer. Each well was in-
cubated with diluted purified human C4, at a final concentration of 5 
μg/ml in GVB/BSA buffer at 37°C for 30 min. MASP-1 captured on wells was detected by rabbit anti-
MASP1 and MASP-3. RT-PCR for murine MASP3 mRNA. Forward primers were common to 
both MASP1 and MASP3 transcripts, and reverse primers were specific for the 
L chain of MASP3. RT-PCR was nested using a primer set of 1F/1R for the 
first PCR and another set of 1F/2R for the second PCR (1415 bp). Template 
was total RNA derived from each murine liver. To evaluate the relative quanti-
ties of mRNAs, Gapdh was amplified (729 bp).

Results

Generation of MASP1/3 knockout mouse

To assess the roles of MASP-1 and MASP-3 in vivo, we estab-
lished a gene targeted mouse that lacks both MASP-1 and 
MASP-3. The MASP1/3 gene has 10 exons that encode the H chain 
common to both MASP1 and MASP3. A targeting vector was con- 
structed to replace the second exon with a neomycin resistant gene 
 cassette (Fig. 1A). Heterozy-
ous mice (Fig. 1B) and, furthermore, back-
crossed with C57BL/6J for more than five generations to eliminate the 129/Sv genetic background.
MASP1/3−/− mice (from 4 to 10 wk old) were significantly smaller than control mice (p < 0.01, Student t test), although they showed no abnormality in either macroscopic appearance or microscopic analysis (data not shown). The average body weights of males at 6 wk of age were 20.8 ± 1.97 g (+/+), 21.1 ± 1.34 g (+/−), and 16.8 ± 1.66 g (−/−) (p = 0.0004, compared with +/+ ) and those of females were 17.9 ± 1.16 g (+/+), 17.5 ± 2.38 g (+/−), and 14.8 ± 1.73 g (−/−) (p < 0.0001, compared with +/+ ). The frequency of MASP1/3−/− mice developed by intercrossing of MASP1/3−/− was slightly lower (+/+ : +/− : −/− = 114: 223: 63) than the expected frequency. Although MASP1/3−/− mice were fertile, the pups resulting from an intercross of MASP1/3−/− mice were more vulnerable. A possible reason is that nursing MASP-1- and MASP-3-deficient mothers are too small to provide sufficient milk. Two strains, derived from two independently targeted ES clones, showed the same body weight phenotype and similar frequencies of homozygous pups. This excludes the possibility that another gene locus is additionally disrupted. These data suggest that MASP-1 and/or MASP-3 might be involved, in part, in development and growth.

MASP-1 and MASP-3 are completely absent in MASP1/3−/− mice

To investigate whether MASP-1 is entirely absent in MASP1/3−/− mice, the amount of MASP-1 in mouse serum was estimated by an ELISA system using an anti-mouse MASP-1 Ab. As shown in Fig. 2A, no MASP-1 was detected by this method in the serum of MASP1/3−/− mice, whereas MASP-1 in the sera of MASP1/3+/+ and MASP1/3−/− mice was captured in a dose-dependent manner. In another experiment, MBL-MASP complexes in mouse serum were pulled down with mannan-agarose. By Western blotting of the complexes with anti-mouse MASP-1 under nonreducing conditions, both the active form and the proenzyme form of MASP-1 were found in MASP1/3-expressing mice (MASP1/3+/+ and MASP1/3−/−) (Fig. 2B, lanes 1 and 2, respectively). However, no detectable band derived from MASP-1 was observed in MASP1/3−/− mice (lane 3). Several additional bands (100, 140, and 200 kDa) were also detected in the serum of MASP1/3−/−. They might be MASP-3 itself or complexes with serine protease inhibitors, such as C1 esterase inhibitor or α2-macroglobulin, although they have not yet been identified. These results confirmed that MASP-1 protein was completely absent from MASP1/3−/− mice.

To assess whether or not MASP-3 is present in MASP1/3−/− mice, RT-PCR of murine MASP3 was conducted, because our attempt failed to generate a high enough titer of Ab against the L chain of MASP-3. Using liver cDNA from wild-type mouse as a template, a 1415 bp cDNA fragment spanning both the H and L chain coding regions of MASP3 was specifically amplified; however, no such product could be amplified from the liver cDNA of MASP1/3−/− mice (Fig. 2C). These results suggest that not only MASP-1, but also MASP-3, is absent in MASP1/3−/− mice.

FIGURE 3. C4 deposition activity of murine serum on mannan-coated microtiter wells. C4 deposition assay by murine serum in mannan-coated microtiter wells was measured as described in Materials and Methods. A, ●, MASP1/3+/+ mouse; ○, MASP1/3−/− mouse. Fluorescence Intensity of each point shows the average for sera from three mice as relative fluorescence unit (RFU). B, Kinetics of C4 deposition by murine sera in mannan-coated wells. A total of 5 μl of serum was reacted in each point. ●, MASP1/3+/+ mouse; ○, MASP1/3−/− mouse. rMASP-1K (200 ng) was incubated with MASP1/3−/− serum (∆). Fluorescence intensity of each point shows the average for sera from three mice as RFU.

FIGURE 4. Activation of MASP-2 by mouse serum. A, Individual mouse serum derived from MASP1/3+/+ (lanes 1 and 2) and MASP1/3−/− (lanes 3 and 4) was incubated on ice with mannan-agarose in a buffer containing 1 μg/ml aprotinin to prevent activation of MASP-2. Bound MASP-2 was run in SDS-PAGE under reducing conditions and detected by using an anti-MASP-2 H chain Ab, which recognizes both MASP-2 and sMAP. C, Effect of rMASP-1 on MASP-2 activation. Sera from MASP1/3−/− (lanes 1, 3, and 5) and MASP1/3+/+ mice (lanes 2, 4, and 6) were incubated with mannan-agarose for 10 (lanes 1 and 2), 20 (lanes 3 and 4), and 45 min (lanes 5 and 6), and then subjected to Western blotting using an anti-MASP-2 H chain Ab, which recognizes both MASP-2 and sMAP.
To assess the activity of the lectin pathway in mice with low MASP-2 activation, coated plates increased in a dose-dependent manner (Fig. 3A). Using sera from MASP1/3−/− mice, the amount of C4-deposition on mannan-coated plates increased in a dose-dependent manner (Fig. 3A). In contrast, C4-deposition from the sera of MASP1/3−/− mice was significantly lower than that in MASP1/3+/− mice. Kinetics plots of C4-deposition using 5 µl of serum also showed a very low activity in MASP1/3−/− mice (Fig. 3B). These results support the possibility that MASP-1 or MASP-3 is involved in the activation of the lectin complement pathway. To determine whether lack of MASP-1 contributes to the reduced activity of complement activation in MASP1/3−/− mice, recombinant mouse MASP-1K was added to the serum. rMASP-1K was modified from native-type of MASP-1 by replacing the arginine residue at the reactive site P1 for activation of MASP-1 with lysine. The same strategy was conducted when recombinant MASP-2 was expressed by Chen and Wallis (22). Their mutant MASP-2K reduced the rate of its auto-activation during synthesis and purification. We have attempted to produce recombinant native-type of mouse MASP-1 by baculovirus expression system, but very poor protein could be purified. Instead of the native type of MASP-1, a large amount of rMASP-1K could be purified from a high five cell line that was stably transfected with an expression vector. The C4-deposition activity of MASP1/3−/− mouse serum was restored by the reconstitution of rMASP-1K (Fig. 3B).

In the lectin pathway, it has been well known that MASP-2 cleaves C4 and C2. Next, we asked whether MASP-2 was activated in the absence of MASP-1. MBL-MASP complex was captured by mannan-agarose from mouse serum diluted with TBS/Ca buffer, and subsequently subjected to Western blotting using an Ab recognizing the H chain of MASP-2. Aprotinin was able to prevent the activation of MASP-2 completely in MBL complexes derived from sera of MASP1/3+/− and MASP1/3−/− (Fig. 4A). The level of proenzyme form of MASP-2 (88 kDa) in serum of MASP1/3−/− was comparable with that in serum of MASP1/3+/−, expecting that the concentrations of MASP-2 in both sera were not different. When serum of MASP1/3+/− mice was incubated in the absence of aprotinin, a band of 62 kDa corresponding active form of MASP-2 was detected, in addition to an 88 kDa band corresponding to the proenzyme form of MASP-2 (Fig. 4B). The bands of ~20 kDa seen in all lanes were the murine counterpart of human sMAP, a truncated form of MASP-2. In MBL-MASP complex from MASP1/3+/− mice, the active form of MASP-2 was observed following 10, 20, and 45 min of incubation with mannan-agarose (Fig. 4B, lanes 2, 4, and 6). In contrast, MASP-1- and MASP-3-deficient mice revealed no active form of MASP-2 following up to 20 min of incubation with mannan-agarose (lanes 1 and 3). MASP-2 was converted to the activated form after 30 min; however, the amount of active MASP-2 in MASP1/3−/− mice was lower than that in MASP1/3+/− mice. Addition of rMASP-1n into the sera of MASP1/3−/− mice restored the MASP-2 activation (Fig. 4C).

Cleavage of recombinant inactive MASP-2 mutant (rMASP-2I) by rMASP-1K

To assess whether MASP-1 cleaves MASP-2 directly, we incubated a recombinant mutant of MASP-2, rMASP-2I, with rMASP-1K. Previous studies have shown that rMASP-2 was easily auto-activated during its preparation (13). Therefore, we used rMASP-2I as a ligand. This mutant was not cleaved by itself because it lacks protease activity. As shown in Fig. 5, rMASP-1K could not cleave rMASP-2I directly. In contrast, rMASP-1i could not cleave rMASP-2I, even if the concentration of rMASP-1i was more than 2-fold higher than that of rMASP-1K, indicating the specific activity of rMASP-1K.

MASP1/3−/− mice show low C3 cleavage ability

To assess C3 activation by the lectin pathway in mouse serum, diluted serum was incubated with mannan-coated plates, and deposited endogenous C3 was determined using an anti-C3c Ab. C3
deposition from the sera of MASP1/3−/− mice was much lower than C3 deposition from the sera of MASP1/3+/+ mice (Fig. 6A). The kinetics of C3 activation by 2 μl of serum was very slow in MASP1/3−/− mice (Fig. 6B). By adding rMASP-1K into the serum of MASP1/3−/− mice, the C3-deposition activity was restored.

We also examined C3 activation by yeast-derived-zymosan particles. When zymosan particles were incubated with normal murine serum, the significant C3 deposition on the particles was observed for 1-min incubation (Fig. 7A). This activity was almost inhibited by adding 0.1 M mannose. Further incubation (5 min) allowed the C3 deposition on the particles by normal murine serum even if adding mannose (Fig. 7B). These results suggested that the lectin pathway plays an important role in the early event for complement activation on zymosan surface, although alternative pathway might be mainly involved in the late complement activation. In MASP1/3−/− serum, there was no C3 deposition on zymosan particles for 1-min incubation (Fig. 7C). rMASP-1n, but not rMASP-1i, could increase the C3 deposition activity in serum of MASP1/3−/− for 1-min incubation (Fig. 7D).

Discussion

We have generated mice deficient in MASP-1 and MASP-3 by gene targeting, and have used these mice to investigate the role of MASP-1 in the activation of the lectin complement pathway. Among MASP-1, MASP-2, and MASP-3, only MASP-2 has C4 cleavage activity (8, 12). A number of studies in vitro supported the possibility that MASP-2 alone is sufficient to activate complement in the absence of MASP-1 and MASP-3. Surprisingly, however, serum derived from MASP1/3−/− has a decreased ability to activate C4 on mannan-coated microtiter wells. No activation of MASP-2 was observed in MASP-1- and MASP-3-deficient serum, when serum was incubated with mannan-agarose for 10 min, whereas activation of MASP-2 was observed in MASP-1- and MASP-3-containing serum (Fig. 4). rMASP-1K restored C4 and MASP-2 activation in MASP-1- and MASP-3-deficient serum (Figs. 3 and 4). These results support the possibility that MASP-1 also contributes to the activation of the lectin pathway at the step of MASP-2 activation.

The precise mechanism by which MASP-1 affects the activation of MASP-2 is not clear. One possible explanation is that MASP-1 cleaves MASP-2 directly. To verify this possibility, we incubated a mutant of rMASP-2i with rMASP-1K. As shown in Fig. 5, it was confirmed that rMASP-1K possesses activity to cleave rMASP-2i in vitro. Another possibility is that MASP-1 enhances the autoactivation of MASP-2. In any case, it has been previously reported that the complex of MASP-1 contains MBL and sMAP, but not MASP-2, using an immunoprecipitation assay with an anti-MASP-1 Ab (23). So far, there is no evidence that MASP-1 and MASP-2 associate with each other in an oligomer of MBL. In peripheral blood, MBL may form individual complexes with MASP-1, MASP-2, or MASP-3, respectively. When these MBL/MASPs complexes bind to carbohydrates on microorganisms, it is possible that in the vicinity of this binding, MBL/MASP-1 may interact with MBL/MASP-2.

MASP-2 was activated even in the absence of MASP-1 and MASP-3, when the serum was incubated with mannan for >30 min (Fig. 4). It has been reported that rMASP-2 is easily autoactivated and rMASP-2 alone associated with MBL is sufficient for adding 0.1 M mannose. C. Comparison of C3 deposition activity between serum of C57BL/6 (opened histogram) and that of MASP1/3−/− (filled histogram). D. MASP1/3−/− serum (grayed histogram) was reconstituted with 100 ng of rMASP-1n (solid line) and 300 ng of rMASP-1i (mutant type) (dot line). Incubation time was 1 min.
the activation of C4 (13). However, we would like to point out the fact that MASP-2 circulates as a zymogen in peripheral blood, suggesting that some unknown mechanism prevents autoactivation of MASP-2. A possible explanation is that a physiological conformational change in MBL that binds to MASP-2 or some inhibitors (C1r or C1s) may prevent the autoactivation of MASP-2. When MBL recognizes a carbohydrate structure, conformational changes in MBL structure may alter the stability of MASP-2, as reported by Wallis (24). Under physiological conditions, MASP-1 may affect the structure or stability of the MBL-MASP-2 complex, thereby resulting in activation of MASP-2.

We also investigated the deposition of C3 from murine serum onto mannan. All complement pathways contribute C3 cleavage to form C3b and C3a that have important roles in the proceeding complement cascades. In MASP-1- and MASP-3-deficient serum, a significantly lower amount of C3 deposition was observed than that in MASP-1- and MASP-3-containing serum. No significant difference of concentrations of C3 and C4 between wild-type and MASP-1/3-deficient sera was observed (data not shown). MASP-1- and MASP-3-deficient serum has decreased activity to form a C3 convertase, C4b2a, supporting the possibility that C3 deposition from MASP-1- and MASP-3-deficient serum was also lower. From several reports, it is clear that MASP-1 can cleave C2 (12, 22). In addition to MASP-2, therefore, MASP-1 activates C2 and also plays an important role in the formation of the C3 convertase, C4b2a. The other possible explanation is that MASP-1 directly activates C3, as reported previously in human, thereby resulting in activation of MASP-2.

In conclusion, we generated MASP-1- and MASP-3-deficient mice, and found that MASP-1 plays a pivotal role in the activation of the lectin pathway, probably through activation of MASP-2.

Disclosures
The authors have no financial conflict of interest.

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