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Cutting Edge: Role of MASP-3 in the Physiological Activation of Factor D of the Alternative Complement Pathway

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The complement system, a part of the innate immune system, can be activated via three different pathways. In the alternative pathway, a factor D (FD) plays essential roles in both the initiation and the amplification loop and circulates as an active form. Mannose-binding lectin–associated serine proteases (MASPs) are key enzymes of the lectin pathway, and MASP-1 and/or MASP-3 are reported to be involved in the activation of FD. In the current study, we generated mice monospecifically deficient for MASP-1 or MASP-3 and found that the sera of the MASP-1–deficient mice lacked lectin pathway activity, but those of the MASP-3–deficient mice lacked alternative pathway activity with a zymogen FD. Furthermore, the results indicate that MASP-3 but not MASP-1 activates the zymogen FD under physiological conditions and MASP-3 circulates predominantly as an active form. Therefore, our study illustrates that, in mice, MASP-3 orchestrates the overall complement reaction through the activation of FD. The Journal of Immunology, 2019, 203: 1411–1416.

The complement system plays important roles in innate immunity, maintaining biological homeostasis and acting as immune surveillance (1). The complement system can be activated via three different pathways; the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (2). Activation of the CP and LP are initiated when pattern recognition molecules (PRMs) (i.e., C1q in the CP; mannose-binding lectin (MBL), ficolins, and collectins in the LP) in a complex with serine proteases bind to ligands.

PRMs of the LP form a complex with MBL-associated serine proteases (MASPs) (2). Once PRMs bind to their activators or ligands, the serine proteases complexed therewith are activated and cleave C4 and C2 to form a C3 convertase, C4b2a. Unlike the CP and LP, activation of the AP is initiated by spontaneous hydrolysis of C3 at low levels. Once C3(H2O) binds to complement factor B (FB), complement factor D (FD), circulating as an active form cleaves FB to form an initial C3 convertase, C3(H2O)Bb. C3(H2O)Bb cleaves C3 to C3a and C3b; the latter covalently binds to microbial surfaces and binds FB, which will be cleaved by FD to form the AP C3 convertase, C3bBb. Both the C3 convertases (i.e., C4b2a for the CP and LP, and C3bBb for the AP) cleave C3 to C3a and C3b; the latter binds FB, which is again cleaved by FD to form the additional C3bBb, generating large amounts of C3b via the amplification loop. Therefore, FD plays essential roles in the initiation of the AP and the subsequent amplification loop that significantly contributes to immunological responses elicited by all complement activation pathways (1).

MASP-1 and MASP-3 are produced mainly in the liver by alternative splicing from the common Masp1 gene, with the result that they have a common H chain and distinct L chains (2). The L chain consists of the serine protease domain, transcribed from either MASP-1–specific split exons or an MASP-3–specific single exon. Previously, we generated a MASP-1/3–deficient mouse by targeting the common exon of Masp1 (3, 4). These mice showed a lack of AP activity with a zymogen FD (pro-FD) in addition to a lack of LP activity, indicating that MASP-1 and/or MASP-3 are required for activation of the AP. To elucidate the individual roles of MASP-1 and/or MASP-3 in physiological amplification of the AP, we generated a MASP-3–specific single exon. Previously, we generated a MASP-1/3–deficient mouse by targeting the common exon of Masp1 (3, 4). These mice showed a lack of AP activity with a zymogen FD (pro-FD) in addition to a lack of LP activity, indicating that MASP-1 and/or MASP-3 are required for activation of the AP. To elucidate the individual roles of
MASP-1 and MASP-3 in the activation of the LP and of the AP via the activation of FD, we generated mice monospecifically deficient for MASP-1 or MASP-3. We found that MASP-1 and MASP-3 play independent roles in the physiological activation of the LP and AP, respectively.

Materials and Methods
Generation of mice monospecifically deficient for MASP-1 or MASP-3

Mice monospecifically deficient for MASP-1 or MASP-3 were generated using the CRISPR/Cas9 system. Guide RNAs (gRNAs) for the deletion of MASP-1–specific exons (gRNA no. 1, 5'-TACCCATTGAGGTTCACGACG-3' and gRNA no. 2, 5'-GGATGTTTCTAAGGGCCTACC-3') or for the deletion of a MASP-3–specific exon (gRNA no. 3, 5'-TGGCTAAGTGCTGTTTGGACG-3' and gRNA no. 4, 5'-CGATTTGCAACTATGTCGTG-3') were designed using the CRISPRdirect Web software (5). The annealed oligonucleotides were inserted into the BbsI restriction site in the pSpCas9(2BB)-2A-Puro (PX459) V2.0 (Addgene, Watertown, MA). These plasmids were microinjected into zygotes isolated from C57BL/6J mice, then, the injected zygotes were transferred to pseudopregnant female mice. The pups were backcrossed with C57BL/6J mice (also used as wild-type [WT] mice; CLEA Japan, Tokyo, Japan) to yield heterozygous F1 mutant mice, followed by inbreeding to yield heterozygous F2 (Masp1+/−/Masp3+/−) or homozygous (Masp1−/−/Masp3−/−) mutant mice. Genotyping PCR was performed using their tail genomic DNA with specific primers as follows: for genotyping of the MASP-1–specific exons, m1F (5'-GGATGTTTCTAAGGGCCTACC-3') and m1R-1 (5'-GTCCTGGGGGCTTACAGTC-3') and m1R-2 (5'-AACACAGTTTTCTACAGTTG-3') were used to amplify 1275- and 935-bp fragments for the WT and mutant alleles, respectively. For genotyping of the MASP-3–specific exon, m3F (5'-CCGGCTCTGCCATGTTT-3') and m3R-1 (5'-GGATGTTTCTAAGGGCCTACC-3') and m3R-2 (5'-CACAGTGGAGAAATACGAGC-3') were used to amplify 588- and 395-bp fragments for the WT and mutant alleles, respectively. DNA sequencing was performed to confirm the editing of the genome with deletion of targeted exon(s) by Macrogen (Tokyo, Japan).

MASP-2–deficient (Masp2−/−) and MASP-1/3–deficient mice (Masp1−/−/Masp3−/−) have previously been described. All animal experiments that included housing, breeding, and use of the mice were reviewed and approved by the Animal Experiments Committee of Fukushima Medical University (approval no. 29032) and conducted in accordance with the guidelines for the care and use of laboratory animals established by the Committee.

Real-time RT-PCR

Total RNAs were extracted from mouse liver using a mirVana Mini Isolation Kit (GE Healthcare, Buckinghamshire, U.K.) and used for synthesis of first-strand cDNA using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). Real-time PCR was performed using the synthesized first-strand cDNAs. Fast SYBR Green Master Mix (Thermofisher Scientific, Waltham, MA), and the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Primer sets used for real-time PCR are as follows: 5'-AGTTGCTCAGAGAGAAGCTGTCG-3’ and 5'-AGGACGGTGCTGAACCCAGT-3’ for MASP-1; 5’-AGTTGCTCAGAGAAGCTGTCG-3’ and 5’-ACCCTGATCTGCCATC-3’ for MASP-3; and 5’-TACCCATTGAGGTTCACGACG-3’ and 5’-CTCTCCAATCCATTGCTGC-3’ for Masp1 gene. β-actin cDNA was amplified using specific primers (5’-CTTGGAGAAGAGGCGTTGA-3’ and 5’-ATCCCAACCACATCACACCC-3’) as an internal control for normalizing mRNA levels among the samples.

Ab preparation

The anti-mouse MASP-1 L chain polyclonal Ab was raised in the MASP-1–deficient mice. The anti-mouse MASP-3 L chain polyclonal Ab was raised in a rabbit. A mouse MASP-1 C-terminal peptide (NKDWQRITGVRIN) and a mouse MASP-3 C-terminal peptide (CLWEEMNSPRYDLQVER) were synthesized by Scrumb (Tokyo, Japan), and Immuno-Biological Laboratories (Gunma, Japan), respectively.

ELISA

Serum levels of MASP-1 and MASP-3 in a complex with MBL were measured by ELISA using microplates coated with 10 μg/well mannan (Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium carbonate buffer (pH 9.6). Mouse serum was diluted at 10% with TBS (pH 7.4) containing 5 mM CaCl2, and the captured MASP-1 or MASP-3 was detected with mouse anti-mouse MASP-1 L chain Ab or rabbit anti-mouse MASP-3 L chain Ab and appropriate HRP-conjugated secondary Abs. Serum levels of C3 (7), ficolin-A (8), total FD, and pro-FD (4), were measured by ELISA as described previously. Serum levels of C4 (MyBioSource, San Diego, CA), MBL-A (Hycult Biotech, Plymouth Meeting, PA), and MBL-C (Hycult) were measured using ELISA kits according to the manufacturers’ instructions.

C4 and C3 deposition assays and hemolytic assay

LP activity was measured by a C4 deposition assay as previously described (3). AP activity was measured by C3 deposition assays using microplates coated with zymosan, mannan, or LPSs derived from Escherichia coli, Salmonella typhimurium, and Pseudomonas aeruginosa (Sigma-Aldrich) in 0.1 M sodium carbonate buffer (pH 9.6) by overnight incubation at 4˚C. C3 deposition on the microplates was assayed as described previously (4). C3 deposition on zymosan was also assayed by flow cytometry as described previously (3). C3 deposition levels were expressed as mean fluorescence intensity obtained by BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ). The hemolytic assay against rabbit erythrocyte was performed as described previously (4).

Western blotting

Western blotting of MASP-3 was performed using mouse sera, plasma, and serum MBL–MASP complex purified with mannan-agarose (Sigma-Aldrich). Serum and plasma samples were prepared in the presence of a protease inhibitor mixture (Sigma-Aldrich) and pretreated with Proteome Pure P2 Mouse Serum Protein Immunodepletion Resin (R&D Systems, Minneapolis, MN). Western blotting of FD was performed as described previously (4). After SDS-PAGE, target proteins were analyzed by Western blotting using rabbit anti-mouse MASP-3 L chain Ab or rabbit anti-mouse FD Ab, followed by incubation with HRP-conjugated swine anti-rabbit Ig (Abiko, Glostrup, Denmark).

Statistical analysis

Statistical analyses were performed by Dunnett multiple comparison test or two-way ANOVA using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA). Differences with a p value < 0.05 were considered statistically significant.

Results and Discussion

Generation of mice monospecifically deficient for MASP-1 or MASP-3 using the CRISPR/Cas9 system

We generated mice monospecifically deficient for MASP-1 (Maspl−/−) or MASP-3 (Maspl−/−) by deletion of the Masp1 gene exons 13–18 or an exon 12 encoding the L chain of MASP-1 or MASP-3, respectively (Fig. 1A). Deletion of targeted exon(s) was confirmed by PCR analysis of mouse tail genomic DNA (Fig. 1B) and sequencing of the PCR products (Supplemental Fig. 1A). Selective deficiency of MASP-1 or MASP-3 in mice induced by genome editing was confirmed by measuring their hepatic mRNA levels and serum levels of MASP3 in a complex with MBL (Supplemental Fig. 1B, 1C). Of interest, hepatic mRNA levels of MASP-1 in the Masp3−/− mice were significantly higher than those in the WT mice (p < 0.0001), whereas serum levels of MASP-3 were significantly higher in the Maspl−/− mice than in the WT mice (p < 0.0001). In addition, there were no significant differences in hepatic mRNA levels of MApp4, a splice variant of Masp1 gene lacking a serine protease domain (9). We further measured the serum levels of complement C3, C4, MBL-A, MBL-C, and ficolin-A, and there were no significant differences between the four groups (Supplemental Fig. 1D). These results indicate that the impact of the absence of MASP-1 and/or MASP-3 on serum levels of C3, C4, MBL-A, MBL-C, and ficolin-A is minimal.

Independent roles of MASP-1 and MASP-3 in the activation of the complement pathways

To define the roles of MASP-1 and MASP-3 in complement activation, we evaluated the LP activity in sera from the mice deficient for MASP-1 or MASP-3 by the C4 deposition assay.
Sera from the MASP-1–deficient mice showed little to no C4 deposition, whereas sera from the WT and MASP-3–deficient mice showed obvious C4 deposition with significant differences between the groups ($p = 0.0027$) (Fig. 2A). These results indicate that MASP-1 plays an essential role in the physiological LP activation and is consistent with the previous results (3).

We next evaluated the AP activity in sera from the mice. Sera from the MASP-3–deficient mice failed to deposit C3 on microplates coated with zymosan, mannan, or LPSs, whereas sera from the WT and MASP-1–deficient mice showed C3 deposition (Fig. 2B). Also, kinetic analysis of the C3 deposition on zymosan particles by flow cytometry showed little to no C3 deposition on zymosan in sera from the MASP-3–deficient mice (Fig. 2C, Supplemental Fig. 2A). By contrast, sera from the WT and MASP-1–deficient mice showed apparent C3 deposition on zymosan time dependently. In addition, a rabbit erythrocyte hemolytic assay demonstrated no hemolysis in sera from the MASP-3–deficient mice (Fig. 2D). Under physiological conditions, therefore, it is clear that MASP-3 plays an essential role in the AP activation, whereas MASP-1 is dispensable for the AP activation in mice. Our

**FIGURE 1.** Generation of mice monospecifically deficient for MASP-1 or MASP-3. (A) A scheme of mouse *Masp1* gene that consists of exons 1–11 encoding the common H chain between MASP-1 and MASP-3, an exon 12 encoding the MASP-3 L chain, and exons 13–18 encoding the MASP-1 L chain. Thin vertical arrows indicate the location of two sets of gRNAs targeting introns for deletion of MASP-1– or MASP-3–specific exon(s). Bold horizontal arrows indicate the location of the primers used for genotyping. (B) Genotyping of Masp1–/– (left panel) or Masp3–/– mice (right panel) using mouse tail genomic DNA. M, molecular size marker; +/-, WT; +/-, heterozygous mutant; –/–, homozygous mutant.

**FIGURE 2.** MASP-1 contributes to the LP activation, whereas MASP-3 contributes to the AP activation. (A) C4 deposition activity of mouse serum on mannan-coated microtiter wells ($n = 3–5$). (B) C3 deposition activity of mouse serum on microtiter wells coated with mannan, mannan, or LPSs from three different microorganisms ($n = 3–6$). (C) Kinetic analysis of C3 deposition activity of mouse serum on zymosan particles by flow cytometry ($n = 3$). (D) Serum hemolytic activity using rabbit erythrocytes ($n = 3$). (E) The activation state of circulating FD analyzed by Western blotting. Data are represented as means ± SEM. Asterisks represent statistical differences against the values for the WT mice at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Dunnett multiple comparisons at each point of age and serum concentration for (C) and (D), respectively.
results differ from previous in vitro studies that have shown MASP-1 to be essential for LPS-induced but not for zymosan-induced AP activation (10). In this study, sera from the MASP-1–deficient mice did not show altered LPS-induced AP activity compared with that from the WT mice (Fig. 2B). Thus, it is unlikely that MASP-1 plays a physiological role in AP activation induced by LPS.

FD circulates predominantly as an active form and plays an essential role in the activation of the AP (11). We previously reported that FD circulated as a pro-FD in MASP-1/3–deficient mice that lack AP activity (4). However, it remained uncertain whether MASP-1, MASP-3, or both are required for the activation of FD. In this study, FD was detected predominantly as a pro-FD in the sera of the MASP-3–deficient mice, although it was detected as an active form, which lacks five N-terminal amino acids of pro-FD (4), in the sera of the WT and MASP-1–deficient mice (Fig. 2E, Supplemental Fig. 2B). Although both MASP-1 and MASP-3 can cleave pro-FD in in vitro experiments (4, 12, 13), our in vivo results clearly indicate that MASP-3 is essential for the activation of FD. This study, using mice monospecifically deficient for MASP-1 or MASP-3, is, to our knowledge, the first to report the independent roles of MASP-1 and MASP-3 in the physiological activation of the LP and AP via FD activation.

MASP-3 circulated as an active form regardless of the role of MASP-1

MASP-1 circulates as an inactive or proenzyme form, and it turns into an active form when the PRM/MASP-1 complex binds to carbohydrates (2). Recently, Orozsán et al. (14) demonstrated that MASP-3 circulates as an active form in humans and proposed a mechanism for MASP-3 activation by MASP-1. However, it is unknown whether MASP-3 circulates as an active form in the absence of MASP-1 or how MASP-3 is activated in vivo. To elucidate the activation state of circulating MASP-3 and its interaction with MASP-1, we analyzed the activation state of MASP-3 by Western blotting using mouse MASP-3 L chain–specific Ab.

Strikingly, MASP-3 was detected predominantly as an active form in the sera of the MASP-1–deficient and MASP-2–deficient mice as well as in the sera of the WT mice (Fig. 3A, Supplemental Fig. 3). We also analyzed MASP-3 in plasma because MASP-1 and MASP-2 in a complex with MBL can potentially be activated by blood coagulation (15). Similarly, MASP-3 was detected as an active form in plasma of the WT and MASP-1–deficient mice. These data indicate that MASP-3 circulates predominantly as an active form in WT and MASP-1–deficient mice (Fig. 3B). We further analyzed the activation state of serum MASP-3 in a complex with MBL in the circulation. As shown in Fig. 3C, MASP-3, which was collected with mannan-agarose from WT and MASP-1–deficient sera in the presence of protease inhibitors, was also activated. Taken together, these data indicate that MASP-3, including that in a complex with MBL, circulates as an active form.

Our previous studies showed mice deficient for MASP-1/3 had a pro-FD in the circulation and lacked AP activity in addition to lacking LP activity (3, 4). Although it has been demonstrated that MASP-1 has broad catalytic activity against components of the AP, including C3, FD, and MASP-3 (2, 4), our studies evaluating AP activity in MASP-1–deficient sera did not show attenuation of AP activity but, rather, enhanced AP activity compared with that of WT sera, likely because of elevated MASP-3 levels in the sera of the MASP-1–deficient mice (Supplemental Fig. 1C). In addition, no alteration in the morphology of circulating FD was observed in the MASP-1–deficient mice. In contrast, MASP-3–deficient sera showed a lack of AP activity with pro-FD. As summarized in Fig. 4, MASP-1 and MASP-3 are required for the activation of MASP-2 and FD, respectively. Thus, in vivo, MASP-1 and MASP-3 play independent roles in the activation of the LP and AP.

The complement system plays an important role in host defense. However, it is also involved in the development of numerous inflammatory disorders, including autoimmune diseases. We previously reported the essential roles of MASP-1 and/or MASP-3 in the development of murine models of Ab-induced rheumatoid arthritis or systemic lupus erythematosus (lupus) (7, 16). Although the activation of the complement system in both models seems to be initiated via the CP, the data demonstrated significant contributions of the LP and/or AP to the development of immune complex–mediated arthritis or glomerulonephritis. The reduction in glomerular injury observed in MASP-1/3–deficient lupus-prone MRL/lpr mice is more pronounced than in MRL/lpr mice deficient for FD or FB, suggesting that there is an additive beneficial effect of the absence of the LP activity in addition to the AP activity to protect from glomerular injury. Recent studies suggest a novel role of MASP-1 in the blood coagulation system (17), a disorder which can also be involved in inflammatory vascular diseases, such as lupus. Further studies
using MASP-1 or MASP-3–deficient murine models of rheumatoid arthritis and lupus are required to clarify individual roles of MASP-1 and MASP-3 in the development of the diseases.

Another important finding is that MASP-3 circulates predominantly as an active form in mice, which is consistent with that in humans (14). MASP-3 and FD are unique and exceptional complement serine proteases, in that they circulate in an active form. In contrast, all serine proteases of the CP (i.e., C1r and C1s) and LP (i.e., MASP-1 and MASP-2) circulate as proenzymes orzymogens. The CP and LP serine proteases are activated when PRMs in a complex with them bind to the ligand. In addition, the C1-inhibitor, which exposes the reactive site loop mimicking the substrate specificity of the targeting serine proteases, regulates activation of the serine proteases of the CP and LP. The C1 inhibitor also regulates the activation of the AP by interacting with C3b to inhibit binding of FB to C3b (18). However, neither the C1 inhibitor nor any other complement regulatory protein regulates or interacts with MASP-3 (19) and FD (18), thus allowing them to be activated in the circulation. From these findings, we hypothesize that MASP-3 and FD continue to be active forms as they play an important role in biological homeostasis beyond the complement system. Indeed, we observed significantly reduced body weight in the MASP-3–deficient mice compared with the WT littermates, whereas there was no significant difference in body weight between the WT and MASP-1–deficient mice (Supplemental Fig. 4). In addition, gene mutations in an MASP-3–specific exon, but not in MASP-1–specific exons, are reported in patients with 3MC syndrome, which is characterized by unusual facial features, developmental delay, intellectual disability, hearing loss, and slow growth after birth (20). In contrast, FD, also known as adipin, is mainly synthesized in adipocytes. It was recently reported that FD has a beneficial role in maintaining pancreatic β-cell function and may prevent the development of type 2 diabetes (21).

In conclusion, we generated mice that were monospecifically deficient for MASP-1 or MASP-3 and demonstrated that MASP-3 is essentially required for the physiological activation of FD. Therefore, our study illustrates that MASP-3 plays a pivotal role in the AP via the activation of FD (Fig. 4). In addition, we demonstrated that MASP-3 circulated in an active form. However, it remains unknown how MASP-3 is physiologically activated in the circulation. Further investigations to elucidate the activation mechanism of MASP-3 and its role in the activation of the AP in humans are needed to complete the overall picture of the complement system.

Disclosures
The authors have no financial conflicts of interest.

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