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MASP3 Deficiency in Mice Reduces but Does Not Abrogate Alternative Pathway Complement Activity Due to Intrinsic Profactor D Activity

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Complement factor D (FD) is a rate-limiting enzyme of the alternative pathway (AP). Recent studies have suggested that it is synthesized as an inactive precursor and that its conversion to enzymatically active FD is catalyzed by mannann-binding lectin-associated serine protease 3 (MASP3). However, whether MASP3 is essential for AP complement activity remains uncertain. It has been shown that Masp1/3 gene knockout did not prevent AP complement overactivation in a factor H–knockout mouse, and a human patient lacking MASP3 still retained AP complement activity. In this study, we have assessed AP complement activity in a Masp3-knockout mouse generated by CRISPR/Cas9 editing of the Masp1/3 gene. We confirmed specific Masp3 gene inactivation by showing intact MASP1 protein expression and absence of mature FD in the mutant mice. Using several assays, including LPS- and zymosan-induced C3b deposition and rabbit RBC lysis tests, we detected plasma concentration–dependent AP complement activity in Masp3 gene–inactivated mice. Thus, although not measurable in 5% plasma, significant AP complement activity was detected in 20–50% plasma of Masp3 gene–inactivated mice. Furthermore, whereas FD gene deletion provided more than 90% protection of CD55/Crry-deficient RBCs from AP complement-mediated extravascular hemolysis, Masp3 gene deletion only provided 30% protection in the same study. We also found pro-FD to possess intrinsic catalytic activity, albeit at a much lower level than mature FD. Our data suggest that MASP3 deficiency reduces but does not abrogate AP complement activity and that this is explained by intrinsic pro-FD activity, which can be physiologically relevant in vivo. The Journal of Immunology, 2023, 210: 1543–1551.

The complement system is composed of a group of plasma proteins that can be activated in a cascade of reactions to produce effectors for host defense (1, 2). Many of the complement proteins are dormant inactive proteases that are activated either by autocatalysis through conformational change or by cleavage with upstream proteases (1, 2). Complement activation occurs via three pathways: the classical (CP), lectin (LP), and alternative (AP) pathways (1, 2). C1r, C1s, and C2 are key proteases of the CP that are triggered by immune complexes (1, 2). Mannose-binding lectin–associated serine proteases (MASPs) are proteases of the LP that is triggered by pattern recognition molecules such as mannose-binding lectin and ficolins (3). The constitutively active AP is driven by two proteases, factor B (FB) and factor D (FD), with FD being upstream of FB and a rate-limiting enzyme (4–6). Many of the proteases of the complement system, including C1s, MASPs, FB, and FD, are targets of antimicrobiol agents and are not required for human defense (7–9).

Three MASP enzymes in the lectin pathway have been identified: MASP1, MASP2, and MASP3 (3). MASP2 is encoded by the Masp2 gene (10, 11), whereas MASP1 and MASP3 are derived from a single Masp1/3 gene via alternative splicing (12). Until recently, the relationship among the three MASP enzymes and their role in LP activation were not clear (13). Although studies in mice and humans clearly showed that MASP2 is critical for LP complement activity (14, 15), gene knockout of Masp1/3, which eliminated the production of both MASP1 and MASP3, also abrogated LP activity (13). Surprisingly, Masp1/3 gene inactivation additionally inhibited AP complement activity in mice (16). On one hand, subsequent experiments based on exon-specific Masp1/3 gene mutation in mice, leading to selective MASP1 or MASP3 enzyme deficiency, established that MASP1, working upstream of MASP2, but not MASP3, is required for LP complement activation. On the other hand, selective Masp3 gene inactivation, like nonspecific Masp1/3 gene knockout but unlike selective Masp1 gene inactivation, impaired AP complement activity (17).

It is now understood that the mechanism by which MASP3 regulates AP complement activity is through its role in FD maturation by cleaving off 5 aa at the N-terminus of an FD precursor referred to as “pro-FD” (16). Historically, FD has been thought to be constitutively active and is regarded as the only protease of the complement pathways that does not require activation (4). That MASP3 plays a key role in the maturation of FD has renewed our knowledge of FD biosynthesis and function and places MASP3 in the AP complement cascade (16). However, several uncertainties remain, chief among them being the degree to which AP complement activity is dependent on MASP3. In both the original report of a Masp1/3
gene knockout mouse and a subsequent paper on an Masp3 single-knockout mouse, AP complement activation was described to be abrogated (16, 17). However, a separate study of the same Masp3/3-knockout mouse and an Masp1/3-factor H double-knockout mouse revealed high AP complement activity in both mouse strains, as assessed by a rabbit RBC lysis test and C3 glomerulopathy phenotyping, respectively (18). Furthermore, it has been reported that a human patient with 3MC (Mingarelli, Malpeuch,Michels, and Carnevale syndromes) who was deficient in MASP3 still retained significant AP complement activity (19, 20). In the present study, we have evaluated AP complement activity in a new Masp3-specific knockout mouse generated by CRISPR/Cas9. We found that MASP3 deficiency reduced but did not abrogate AP complement activity, and this can be explained by intrinsic pro-FD activity in the absence of MASP3. Our data clarify the uncertainties regarding the role of MASP3 in AP complement activation and have implications for therapeutic target selection in the treatment of AP complement-mediated diseases.

Materials and Methods

Generation of Masp3-knockout mice

MASP3 mRNA and MASP1 mRNA are transcribed from the same Masp1/3 gene via alternative splicing (12). In order to generate a Masp3-specific knockout mouse, we disrupted the mRNA expression of Masp3, which plays a critical role in the lectin pathway, we used the CRISPR/Cas9 system (23) of a commercial cDNA plasmid (Transomic, BC106945) by editing using 5′-ACACCTGGTGAAAGAACCTATCCGTC-3′ (forward-1) and 5′-CCGGAAATTCGACACTGCAGCTCGTTACTC-3′ (forward-2) and 5′-CCGGATCTTCCCTCAGGATGTTGAGCTCT-3′ (reverse primer). The cDNA was subcloned into the pCAGGS vector with a C-terminal His tag and, recombinant protein was expressed in HEK293 cells by cDNA transfection with polyethyleneimine. Recombinant FD was purified from culture medium using Ni-nitrolic acid His Tag resin (Qiagen, 30210).

Generation of recombinant mouse FD

Mouse mature FD cDNA was amplified by nested PCR using mouse FD cDNA plasmid (Sino Biologicals, MG50539-M) as a template with 5′-GGGCGGTGTTAGTGCCAGAATCTTGTTGCGAGGACC-3′ (forward-1), 5′-CCGGAAATTCGACACTGCAGCTCGTTACTC-3′ (forward-2) and 5′-CCGGATCTTCCCTCAGGATGTTGAGCTCT-3′ (reverse primer) primers. The cDNA was cloned into the pCAGGS vector with a C-terminal His tag, and recombinant protein was expressed in HEK293 cells by cDNA transfection with polyethyleneimine. Recombinant FD was purified from culture medium using Ni-nitrolic acid His Tag resin (Qiagen, 30210).

Generation of anti-mouse MASP3 and anti-mouse FD Abs

Rabbit anti-mouse MASP3 and anti-mouse FD polyclonal Abs were generated by Cocalico Biologicals (Stevens, PA) using purified recombinant mouse MASP3 or mouse FD protein as an immunogen (450 μg/rabbit in five immunizations). Total IgG from antisera was purified by the caprylic acid method (25). From total IgG Abs, MASP3 or FD reacting IgGs were enriched by affinity column chromatography using Sepharose beads coupled with recombinant mouse MASP3 or FD.

LPS assay

Microtiter plates (Nunc Maxisorb, 442404) were coated with LPS (Salmonella typhosa LPS; Sigma-Aldrich) (2 μg/well) in PBS for 1 h at 37°C. After the plate wells were washed with PBST (PBS and 0.05% Tween) three times, wells were treated with a blocking buffer (1% BSA in PBS) for 1 h at room temperature (RT). Mouse plasma (50 μg/ml bivalirudin final), collected using bivalirudin (Hospira, Lake Forest, IL; 50 μg/ml) as an anticoagulant and diluted to the desired percentage with Mg2+2-EGTA GVB+ (gelatin veronal buffer) buffer, was then added to the plate wells (50 μl/well). Plasma samples diluted in the same way but containing EDTA (10 mM) were used as negative controls of AP complement activation. AP complement activation in the plate was allowed to proceed for 1 h at 37°C. After being washed three times with PBST, plate wells were incubated with an HRP-conjugated goat anti-mouse C3 polyclonal Ab (MP Biologicals, 0855557; 1:4000 diluted in blocking buffer) for 1 h at RT. The plate was washed three times with PBST and deloading with HRP substrate (100 μl 1-step Ultra TMB, Thermo Fisher Scientific). After 5 min, the reaction was stopped with 50 μl of 2N H2SO4, and the plate was read at 450 nm in a microplate reader. In some experiments, the tested mouse plasma was pretreated with sodium polyanethole sulfonate (SPECS; Sigma-Aldrich, P2008; 150 μg/ml) before being added to the plate. SPECS was used to inhibit AP and complement activities.

Rabbit CBC lysis assay

Mouse serum was diluted to 10% with GVB++ buffer, then mixed and incubated with Ab-sensitized chicken RBC (Rockland Immunochemicals, R401-0050; 2.5 × 10⁶ cells per reaction, final volume 50 μl at 37°C for 30 min. Ab-sensitized chicken RBCs were prepared by incubating the cells with a rabbit anti-chicken RBC Ab (150 μg/ml; Rockland Immunochemicals, 103-41390) on ice for 1 h. The percentage of lysis was calculated by normalizing the cell-free hemoglobin OD value to that of a completely lysed RBC sample through hypotonic lysis in water. To check the effect of SPECS on chicken RBC lysis, SPECS was used with different concentrations of SPECS before hemolytic assays.

Rabbit CBC lysis assay

The rabbit RBC lysis assay was performed with mouse bivalirudin plasma diluted to 20% or 50% in GVB-Mg2+-EGTA (10 mM) buffer. Plasma was mixed with 5 μl of rabbit RBC suspension (5 × 10⁶/ml; Rockland Immunochemicals, R403-0100) in a final volume of 50 μl and incubated at 37°C for 30 min. In the mouse FD mAb blocking assay, plasma was pretreated with 500 μg/ml mouse anti-mouse FD mAb (clone 14F11-3, generated in-house) for 30 min at 4°C before incubating with rabbit RBCs. The reaction was stopped with 100 μl of cold 10 mM EDTA in PBS. Cells were centrifuged at 1500 rpm for 5 min at 4°C. The OD of the collected supernatant was measured at 405 nm.

Mannan LP assay

Microtiter plates (Nunc Maxisorb, 442404) were coated with 50 μl/well mannan (Sigma-Aldrich, 20 μg/ml) in sodium bicarbonate buffer, pH 9.0, overnight at 4°C. After the plate wells were washed with PBST three times,
the wells were treated with a blocking buffer (1% BSA in PBS) for 1 h at RT. Mouse bivalirudin plasma, diluted to the desired percentage with GVB buffer containing SPS (150 μg/ml final), was added to the plate wells (50 μl/well). Samples diluted in the same way but containing EDTA (10 mM) were used as negative controls of complement activation. Complement activation in the plate wells was conducted for 1 h at 37°C. After being washed three times with PBST, plate wells were incubated with an HRP-conjugated goat anti-mouse C3 polyclonal Ab (1:4000 diluted in blocking washed three times with PBST, plate wells were incubated with an HRP-conjugated goat anti-mouse C3 polyclonal Ab (1:4000 diluted in blocking washed three times with PBST, plate wells were incubated with an HRP-conjugated goat anti-mouse C3 polyclonal Ab (1:4000 diluted in blocking washed three times with PBST). The plate was washed three times with PBST and developed with HRP substrate (100 mM buffer) for 1 h at RT. The plate was washed three times with PBST and developed with HRP substrate (100 μl 1-step Ultra TMB, Thermo Fisher Scientific). After 5 min, the reaction was stopped with 50 μl of 2N H2SO4, and the plate was read at 450 nm in a microplate reader.

**OVA/anti-OVA immune complex CP assay**

OVA/anti-OVA immune complexes for complement activation were prepared as previously described (26). Briefly, 10% mouse bivalirudin plasma was diluted with GVB buffer, and 50 μl was added to each well of ELISA plates prefixed with OVA/anti-OVA immune complexes. The plates were incubated at 37°C for 1 h followed by detection of plate-bound activated C3 using HRP-conjugated goat anti-mouse C3 polyclonal Ab.

**Zymosan AP assay**

The zymosan-induced AP complement activation assay was performed as described previously (26). Briefly, 2.5 ml zymosan (2.5 mg/ml) was mixed and incubated with 50 μl 20% bivalirudin plasma diluted in Mg2+-EGTA GVB2 for 15 min at 37°C, and C3 deposition was detected by FITC-conjugated goat anti-mouse C3 (MP Biologicals, 0855500; 1:250 dilution) and analyzed by FACS.

**Extravascular hemolysis (EVH) test**

The EVH test was performed as previously described (27). Briefly, CD55−/− Crry−/−C3−/− mouse erythrocytes (from 150 μl blood for each recipient mouse) were labeled ex vivo with CFSE (Molecular Probes) in 1 ml PBS containing 5 mM CFSE. The labeling reaction was carried out at RT for 5 min, and then cells were washed several times in PBS. CFSE-labeled CD55−/− Crry−/− C3−/− erythrocytes were transfused i.v. (retro-orbital route) into WT, Masp3-knockout, or FD-knockout mice. Blood was collected from the tail vein at 5 min and various subsequent time points as indicated, and the percentage of labeled erythrocytes present was calculated after FACS analysis and normalized to values at 5 min in each mouse.

**Western blot analysis**

For detection of MASp3, mouse EDTA plasma (2 μl) was resolved on 4–12% ExpressPlus gradient polyacrylamide gel (GenScript, M41212) under nonreducing conditions and transferred to polyvinylidene difluoride membranes. MASp3 was detected by sequential incubation with purified rabbit anti-mouse MASp3 IgG (1 μg/ml), HRP-conjugated goat anti-rabbit IgG (1:4000 dilution; Sigma-Aldrich), and an ECL detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). For detection of mouse FD and pro-FD, 2 μl bivalirudin plasma was first deglycosylated with thePNGase F kit from New England Biolabs (P0704S), and proteins were resolved on a 4–20% ExpressPlus gradient polyacrylamide gel (GenScript, M42012) under reducing conditions. FD was detected by using rabbit polyclonal anti-mouse

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**FIGURE 1.** Generation and characterization of Masp3 gene knockout mice. (A) Schematic representation of the mouse Maspl/3 gene locus and Masp3 gene targeting strategy. Gray boxes indicate the location of exons. The locations of CRNA1 and CRNA3 targeting sites and sequences are indicated by open arrowheads and underlines, respectively. Locations of PCR primers (F1 and R1) used for mouse genotyping are shown by solid arrows. The nucleotide sequences of the two targeting sites and the recombinant sequence after partial exon 12 deletion (470 bp) are also shown. (B) Representative result of PCR genotyping of WT (+/+), heterozygous (+/−), and homozygous (−/−) Masp3 gene knockout mice. The WT allele is represented by a 756-bp band, whereas the mutant allele is represented by a 252-bp band. (C) Western blot analysis showing a rabbit anti-mouse MASP3 Ab recognized both recombinant MASp3 (lane 1) and MASp3p1 (6627A) (lane 2) as they share the same H chain. (D) Western blot analysis showing a rabbit anti-MASP3 polyclonal Ab showing MASP3 protein was absent in homozygous Masp3 knockout mice (−/−) and severely reduced in heterozygous Masp3-knockout mice (+/−) compared with WT mice (+/+) (E). In contrast, MASp1 protein levels were similar in all three genotypes of mice (representative of four experiments). (F) Western blot analysis of mouse plasma (2 μl/lane) using a rabbit anti-MASP3 polyclonal Ab showing FD protein in Masp3−/− mice to have a slightly higher m.w., suggesting it was pro-FD. Note there was no trace of mature FD in Masp3−/− mice. Plasma samples were deglycosylated for 2 h before being analyzed on 4–20% gradient polyacrylamide gel under reducing conditions (representative of three independent experiments). Data in (B–D) are representative of at least two independent experiments.
FD IgGs (2 µg/ml) generated in-house as the primary Ab and the same procedures as in MASP3 detection.

**Functional testing of mouse FD in normal human serum (NHS)**

To test if mouse FD can replace human FD and support AP complement activity in NHS (Complement Technology, Inc.), an LPS-induced AP complement activation assay was performed using 10% NHS in which human FD was blocked with a neutralizing mAb (AFD, generated in-house as a recombinant human IgG4 mAb according to published sequences [28, 29; 10 µg/ml final concentration]) and recombinant mouse FD (generated in-house and C-terminal His-tagged [S. Sato, unpublished observations]) added to various concentrations. C3b deposition was detected by HRP-conjugated goat anti-human C3 polyclonal Ab (MP Biologicals, 0855237).

**Assessment of conversion of recombinant pro-FD to FD in vitro by recombinant MASP3**

To test the conversion of recombinant pro-FD to mature FD by recombinant MASP3, 2 µg of recombinant pro-FD with an N-terminal 6xHis-tag was incubated with 1 µg recombinant MASP3 in 20 µl PBS at 37°C for different lengths of time. The samples were then subjected to deglycosylation treatment using the PNGase kit (New England Biolabs, P0704S) before Western blot detection using an anti-6xHis-tag mAb (R&D Systems, MAB050H) for the presence of absence of the 6xHis-tag in pro-FD and mature FD, respectively.

**Factor B cleavage assay**

Different amounts of pro-FD or MASP3-treated pro-FD (mature FD) were incubated with 2 µg human FB (Complement Tech, A135) and 2.5 µg human C3b (Complement Tech, A113) in a total volume of 25 µl PBS containing 5 mM MgCl₂ at 37°C for 1 h. FB cleavage by pro-FD or mature FD was assessed by running the reaction mixtures on a 4–12% ExpressPlus gradient polyacrylamide gel under reducing conditions and staining the gel with Coomassie blue. The degree of FB cleavage was measured by densitometry of Bb band intensity using the LI-COR Odyssey Fc system (LI-COR Biosciences).

**Results**

MASP3 and MASP1 mRNAs are transcribed by alternative splicing from a single Masp1/3 gene (12). To generate a Masp3-specific knockout (Masps⁻/⁻) mouse without disturbing Masp1 gene expression, we used CRISPR/Cas9 Ribonucleoprotein-mediated gene targeting to delete exon 12 of the Masp1/3 gene (Fig. 1A). Exon 12 is specific to Masp3 and encodes the serine protease domain of MASP3 (12). Two CrRNAs were designed to excise a 470-bp fragment from exon 12 (Fig. 1A). The expected deletion of 470 bp from exon 12 was also confirmed by sequencing of the mutant allele (Fig. 1A). To help confirm MASP3 deficiency in the mutant mice, we recombinantly expressed mouse MASP3 as a His-tagged protein and used it to generate a polyclonal rabbit anti-mouse MASP3 Ab (Fig. 1C, 1D). Because MASP3 and MASP1 share a common H chain, the rabbit anti-mouse MASP3 Ab recognized both recombinant MASP3 and recombinant MASP1, which was expressed as His-tagged protein with an S627A mutation in the protease domain (23) (Fig. 1C, 1D). Using this Ab, we performed Western blot analysis of mouse plasma and confirmed that MASP3 protein was completely absent in Masps⁻/⁻ mice, whereas MASP1 protein was present at normal levels (Fig. 1E). These data demonstrated selective inactivation of Masp3 without affecting Masp1 gene expression in Masps⁻/⁻ mice. We also analyzed the effect of MASP3 deficiency on FD maturation. As shown in Fig. 1F, Western

![FIGURE 2. Assessment of lectin and CP complement activity in Masps⁻/⁻ mouse plasma. (A) SPS dose dependently inhibited mouse CP complement activity in a chicken RBC (cRBC) hemolytic assay. Percentage of cRBC lysis was normalized to a cRBC sample completely lysed by hypotonic shock in double-distilled water. (B) SPS at 150 µg/ml also effectively inhibited mouse AP complement activity in an LPS-induced C3b deposition ELISA. (C) Assessment of LP complement activity in a mannan-based C3b deposition ELISA in the presence of 150 µg/ml SPS. The result showed normal LP activity in Masps⁻/⁻ mice (MaspsKO). (D) Assessment of CP complement activity in an OVA/anti-OVA immune complex–based C3b deposition ELISA. The result showed reduced CP activity in Masps⁻/⁻ mice (MaspsKO), likely reflecting impaired amplification by the alternative pathway. (A–D) All used 10% plasma from WT, Masps⁻/⁻ (MaspsKO), C5-knockout (C5KO), C4-knockout (C4KO), FB-knockout (fBKO), and C3-knockout (C3KO) mice. WT plasma with EDTA added and C5KO, C4KO, fBKO, and C3KO mouse plasma were used as negative controls. Results in (A) and (B) are representative of two independent experiments each, and each bar represents the average of duplicate assays. Results shown in (C) and (D) are mean values with SD of triplicate assays and representative of at least two independent experiments. *p = 0.0104, **p = 0.0074.](image-url)
 blot analysis of deglycosylated plasma samples detected FD in Masp3<sup>−/−</sup> mice to have a slightly higher m.w. than FD in WT mice, consistent with lack of processing of pro-FD to mature FD in the absence of MASP3. These results collectively showed that the Masp3 gene was specifically and completely inactivated in the mutant mice and that no MASP3 protein and mature FD were present in them.

To assess the impact of MASP3 deficiency on complement activity, we performed pathway-specific in vitro complement activation assays using 10% bivalirudin anticoagulated plasma from Masp3<sup>−/−</sup> mice. Because mannan can activate CP and AP complement, to assay mannan-induced LP complement activity specifically, we tested and used a known chemical inhibitor of human CP and AP complement, SPS (30), in WT mouse plasma. As shown in Fig. 2A, we found that SPS dose-dependently inhibited mouse CP complement in a chicken RBC lysis assay, achieving complete inhibition at 150 μg/ml. At this concentration, SPS also efficiently blocked LPS-induced mouse AP complement activation (Fig. 2B). We next tested mannan-induced LP complement activity in Masp3<sup>−/−</sup> mice in the presence of SPS. Fig. 2C shows that Masp3<sup>−/−</sup> mice retained normal LP complement activity, suggesting that MASP3 is not required for LP complement activation. We also tested CP complement activity using an OVA/anti-OVA immune complex–based ELISA (in GVB and without SPS) and found it to be partially impaired in Masp3<sup>−/−</sup> mice (Fig. 2D), likely due to reduced contribution from AP amplification as we have demonstrated previously with properdin- and FB-deficient mice (26).

An assay of LPS-induced AP complement activation in 10% plasma of Masp3<sup>−/−</sup> mice showed severely impaired activity compared with WT mice (Fig. 3A), but the degree of reduction varied between different experiments (data not shown). The impairment in AP complement activation could be corrected by the addition of recombinant mouse MASP3 (Fig. 3A). To more definitively establish if MASP3 deficiency abrogated AP complement activity, we performed an LPS-induced complement activation assay using different concentrations of plasma. As shown in Fig. 3B–3D, we found partial AP complement activity in 20% and 50% but not 5% Masp3<sup>−/−</sup> mouse plasma. This was in clear contrast to FD-knockout mouse plasma, which showed no AP complement activity at any concentrations. These data suggested that AP complement activity in Masp3<sup>−/−</sup> mice was substantially reduced but not abrogated as in FD-knockout mice.

To verify the above conclusion, we measured AP complement activity in Masp3<sup>−/−</sup> mice using several other in vitro and in vivo assays. Fig. 4A and 4B show the results of zymosan-induced AP complement activation in 20% plasma, using as a readout C3b deposition of zymosan particles measured by FACS. As expected, no complement activation occurred on zymosan with FD-knockout mouse plasma. In contrast, significant C3b opsonization was detected on zymosan incubated with Masp3<sup>−/−</sup> mouse plasma, although it was much less than that detected on zymosan incubated with WT mouse plasma. We detected a similar plasma concentration–dependent AP complement activity in Masp3<sup>−/−</sup> mice in the rabbit RBC hemolytic assay. Although varied and generally lower than WT activity was detected in 20% Masp3<sup>−/−</sup> mouse plasma, 50% Masp3<sup>−/−</sup> mouse plasma was almost as active as WT mouse plasma in lysing rabbit RBCs (Fig. 5A, 5B). Finally, we used an in vivo assay, involving transfusion and EVH of CFSE-labeled CD55/Crry-deficient RBCs (27), to assess AP complement activity in Masp3<sup>−/−</sup> mice. In this assay, when RBCs harvested from CD55/Crry/C3 triple-knockout mice are transfused into complement-sufficient recipient mice, they are susceptible to, and rapidly eliminated by, AP complement-mediated phagocytosis (31). Fig. 4C shows that although CD55/Crry/C3 knockout mouse RBCs were largely protected from elimination over a 3-d period after transfusion into FD-knockout recipient mice, ~70% of such transfused cells were eliminated in Masp3<sup>−/−</sup> recipient mice, an outcome that was closer to that observed in WT recipients than in FD-knockout recipients.

Partial AP complement activity in Masp3<sup>−/−</sup> mice may arise from an FD bypass mechanism via other protease(s) that can cleave FB or from intrinsic activity of pro-FD. To distinguish these two hypotheses, we used a function-blocking anti-mouse FD mAb (14F11-3) developed in-house (Fig. 5C) to investigate if the partial AP complement activity in Masp3<sup>−/−</sup> mice could be blocked by FD neutralization. Fig. 5D shows that at 500 μg/ml, mAb 14F11-3 completely blocked rabbit RBC lysis by 50% plasma of WT or Masp3<sup>−/−</sup> mice. This result suggested that the partial AP complement activity in Masp3<sup>−/−</sup> mice was dependent on pro-FD and not on other nonspecific proteases. To establish directly that...
mouse pro-FD has intrinsic FB-cleaving activity to support AP complement activation, we performed an in vitro FB cleavage assay by mixing human FB with human C3b and mouse pro-FD or pro-FD pretreated with recombinant mouse MASP3. We used human FB and C3b due to their easy availability as commercial reagents and after confirming that mouse FD can substitute for human FD to support AP complement activation in NHS (Fig. 6A). We also confirmed by Western blot analysis that mouse pro-FD was effectively converted to mature FD, as indicated by the loss of N-terminal His-tag, after incubating with mouse MASP3 (Fig. 6B, 6C). In this FB cleavage assay, we found that both pro-FD and mature FD were able to cleave FB, providing direct evidence that pro-FD has intrinsic enzymatic activity toward C3b-complexed FB. It was clear, however, that mature FD had higher activity than pro-FD, especially when present at a lower concentration in the reaction (Fig. 6D, 6E).

**Discussion**

One of the surprising findings in the study of MASP enzymes was a previously unrecognized role of MASP3 in converting pro-FD to mature FD by cleaving 5 aa at the N-terminus. This discovery was originally made in an Masp1/3 gene knockout mouse that lacked both MASP1 and MASP3 proteins and had impaired LP and AP complement activity (13, 16). Subsequent studies of Masp1- and Masp3-specific knockout mice established that MASP1 but not MASP3 was required for LP, and, conversely, MASP3 but not MASP1 was implicated in AP complement activity (17). In these studies, both Masp1/3 gene knockout and Masp3-specific knockout mice were described to be lacking AP complement activity (16, 17), leading to the conclusions that pro-FD is nonfunctional and that its conversion to catalytically active FD by MASP3 is critical to AP complement. This concept has led to an effort to target MASP3 as a therapeutic approach to treat AP complement-mediated human diseases (7).

The role of MASP3 in AP complement activation became somewhat controversial as other contradictory evidence emerged (18–20). First, a human patient with 3MC syndrome whose blood lacked both MASP1 and MASP3 proteins was found to have fully functional AP complement activity (19, 20). This raised the question whether the finding made in mice was species specific and cast doubt on a critical role of MASP3 in human AP complement activation. Second, an independent study by a different research group of the same Masp1/3 gene knockout mouse showed close to normal AP complement activity in 30% serum using a rabbit RBC hemolytic assay, directly contradicting.
Plasma was completely blocked by mAb 14F11-3, suggesting that the AP activity detected in activity assays (16, 17), and this may explain why no activity was detected in 50% plasma of Masp3−/− mice. Each symbol represents data of plasma from an individual mouse. WT (n = 5), Masp3KO (n = 11), and fDKO (n = 4). (C) Demonstration of dose-dependent inhibition of LPS-induced AP complement activity in 10% WT mouse plasma by a function-blocking mouse anti-mouse FD mAb (clone 14F11-3). At 50 or 100 μg/ml, mAb 14F11-3 completely inhibited mouse AP activity. EDTA serum was used as a positive control for complement inhibition. Values are the average of duplicate assays using pooled plasma from three or four mice. (D) Effect of anti-FD mAb 14F11-3 (500 μg/ml) on rabbit RBC lysis by 50% WT (n = 4) or Masp3−/− (Masp3KO; n = 4) mouse plasma. The data show that AP complement activity in WT and Masp3−/− mouse plasma was completely blocked by mAb 14F11-3, suggesting that the AP activity detected in Masp3−/− mice was dependent on pro-FD and not on other nonspecific protease(s). FD-knockout (fDKO) mouse plasma was used as a negative control, which produced no hemolysis.

The realization of a role of MASP3 in FD maturation and AP complement activation has stimulated interest in therapeutically targeting human MASP3 for AP complement-mediated diseases (7). Our results presented in this study, if replicated in the human complement system, as has been implied by a recent study (32), suggest that...
such an approach is unlikely to inhibit AP complement completely. Although a partial inhibition of AP activity afforded by MASP3 inhibitors may produce clinical benefit, as illustrated by a murine arthritis model in Masp1/3-knockout mice and mice with Masp3 knockdown with siRNA (33, 34), it may not achieve clinical efficacy in other diseases where pro-FD activity is sufficient to cause full or partial disease pathology. The latter scenario finds examples in the murine model of C3 glomerulopathy as reported by Ruseva et al. (18) and in the murine EVH model described in this study with relevance to AP complement-driven human renal diseases and paroxysmal nocturnal hemoglobinuria, respectively.

Disclosures
The authors have no financial conflicts of interest.

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