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Mechanisms of Mannose-Binding Lectin-Associated Serine Proteases-1/3 Activation of the Alternative Pathway of Complement

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Abstract

Mannose-binding lectin-associated serine proteases-1/3 (MASP-1/3) are essential in activating the alternative pathway (AP) of complement through cleaving pro-factor D (pro-Df) into mature Df. MASP are believed to require binding to mannose binding lectins (MBL) or ficolins (FCN) to carry out their biological activities. Murine sera have been reported to contain MBL-A, MBL-C, and FCN-A, but not FCN-B that exists endogenously in monocytes and is thought not to bind MASP-1. We examined some possible mechanisms whereby MASP-1/3 might activate the AP. Collagen antibody-induced arthritis, a murine model of inflammatory arthritis dependent on the AP, was unchanged in mice lacking MBL-A, MBL-C, and FCN-A (*MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice) in comparison to wild-type mice. The in vitro induction of the AP by adherent mAb to collagen II was intact using sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice. Furthermore, sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice lacked pro-Df and possessed only mature Df. Gel filtration of sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice showed the presence of MASP-1 protein in fractions containing proteins smaller than the migration of MBL-A and MBL-C in sera from *C4*$^{-/-}$ mice, suggesting possible binding of MASP-1 to an unknown protein. Lastly, we show that FCN-B was present in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$mice and that it was bound to MASP-1. We conclude that MASP-1 does not require binding to MBL-A, MBL-C, or FCN-A to activate the AP. MASP-1 may cleave pro-Df into mature Df through binding to FCN-B or to an unknown protein, or may function as an unbound soluble protein.

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Keywords

Complement activation pathways; Collagen antibody induced arthritis; Inflammation; MBL; MASP-1; Ficolin-B

1. Introduction

The complement system is part of the innate immune system and plays important roles in host resistance to infection. However, the complement system may mediate tissue damage in autoimmune and inflammatory diseases that involve many different organs. Three complement activation pathways have been described in the fluid phase and on cell surfaces: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP) (Ricklin et al., 2010). All 3 pathways result in formation of C3 convertases and C5 convertases, leading to the release of the biologically active fragments C3a and C5a and to generation of the membrane attack complex.

The CP is initiated by IgG or IgM engagement of the C1 complex consisting of C1q, C1s, and C1r. This is followed by the local cleavage of C4 and C2 leading to formation of the CP C3 convertase C4b2a. The LP is activated by the binding of a complex of mannan binding lectin (MBL), ficolin (FCN), and MBL-associated proteases (MASPs) to carbohydrates and acetylated residues on cell surfaces (Fujita et al., 2004; Thiel, 2007). C2 and C4 are then cleaved, generating the CP C3 convertase. The AP is activated by low-grade spontaneous hydrolysis of C3 in plasma to form C3(H_2O) with generation of C3b and interaction with factor B to form a C3bB complex. Complement factor D (Df) then cleaves factor B into fragments Ba and Bb with formation of the AP C3 convertase C3bBb (Harboe and Mollnes, 2008; Pangburn et al., 1981). C3b generated by the CP and AP convertases binds covalently to cell surfaces. The AP may greatly enhance the local generation of C3b, initially formed by any of the 3 complement activation pathways, by a process of amplification.

The serine proteases associated with MBL or FCN vary considerably in structure and function. MASP-1 and MASP-3 areformed from one gene by alternative splicing. Similarly, MASP-2, and its truncated form MBL-associated protease of 19 kDa (MAp19), are formed by alternative splicing from a different single gene (Takahashi et al., 1999). The MASPs circulate as biologically inactive pro-enzymes with MASP-1 and MASP-2 activated from zymogen forms by autocatalysis (Ambrus et al., 2003). MBL enhances MASP-2 activity after binding of the MBL/MASP-2 complex to a cell surface substrate by the MBL moiety. MBL then increases the rate of MASP-2 autocatalysis leading to cleavage of nearby covalently attached C4 and C2. It is not known whether MASP-1 requires a similar process of MBL- and substrate-dependent activation in vivo. However, recombinant MASP-1K is able to cleave pro-factor D (pro-Df) into mature factor D in the fluid phase in vitro in the absence of serum as a source of MBL or FCN (Takahashi et al., 2010).

MASP-1 has evolutionary and structural differences from the other early complement proteases, and may function as a promiscuous protease (Dobo et al., 2009). MASP-1 may augment initiation of the LP through direct cleavage of C2, but not of C4, and by promoting the activation of MASP-2 (Matsushita et al., 2000; Moller-Kristensen et al., 2007; Rossi et al., 2001; Takahashi et al., 2008). MASP-1 weakly cleaves C3(H_2O), but this activity is probably not biologically relevant (Hajela et al., 2002; Matsushita et al., 2000). However, MASP-1 exhibits activity on substrates outside of the complement system including fibrinogen, factor XIII (transglutaminase), and protease-activated receptor 4 (PAR4) (Hajela et al., 2002; Megyeri et al., 2009). In addition, MASP-1 exhibits thrombin-like properties in vitro by cleaving synthetic substrates after an Arg or Lys residue and being

inhibited by antithrombin (Megyeri et al., 2009; Takahashi et al., 1999; Takahashi et al., 2010). Although most MASP-1 may be present in plasma in complex with MBL or FCN, some unbound or free MASP-1 may also exist (Takahashi et al., 2010; Terai et al., 1997; Thiel et al., 2000). The major, if not sole, function of MASP-2 is to cleave C2 and C4, leading to formation of the CP C3 convertase. The function of MASP-3 is largely unknown; MASP-3 is present primarily in complex with FCN-3 where it down-regulates FCN-3-mediated activation of the LP (Skjoedt et al., 2010).

The generation of mice genetically deficient in the MASP-1/3 gene has led to new information about the unique role of MASP-1/3 in the AP. No activation of the AP in vitro was present in sera from *MASP1/3*$^{-/-}$ mice, with a lack of cleavage of factor B into fragments Ba and Bb. The circulating Df in these mice was present solely in the zymogen form (pro-Df) (Takahashi et al., 2010). Additional studies show that collagen antibody-induced arthritis (CAIA), an experimental model of inflammatory arthritis that is dependent on the AP, is markedly inhibited in *MASP1/3*$^{-/-}$ mice (Banda et al., 2010a; Banda et al., 2007; Banda et al., 2010b; Banda et al., 2006). The addition of recombinant human Df restored the ability of sera from *MASP1/3*$^{-/-}$ mice to generate C3 deposition and C5a generation in vitro by the AP after stimulation by adherent anti-collagen II (CII) mAb (Banda et al., 2010b).

The structure of FCN and MBL are similar with each possessing an N-terminal collagenlike domain (Fujita et al., 2004). MBL possess a C-terminal carbohydrate recognition domain and FCN have a C-terminal fibrinogen-like domain responsible for carbohydrate binding. Humans express one form of MBL whereas mice exhibit two forms, MBL-A and MBL-C. MBL are primarily synthesized in the liver and are found in the circulation. Humans express 3 forms of FCN: FCN-1 or M-ficolin, FCN-2 or L-ficolin, and FCN-3, or H-ficolin (Endo et al., 2007). However, mice only possess 2 forms of FCN, FCN-A and FCN-B. The serum types of FCN, such as human FCN-2 and FCN-3 and murine FCN-A, are synthesized in the liver and are present in the circulation. However, human FCN-1 and murine FCN-B are thought to be absent in serum but to be present both in secretory granules and on the surface of monocytes and macrophages (Endo et al., 2007; Runza et al., 2008). Human FCN-1 binds both MASP-1 and MASP-2 with cleavage of C4 by the bound MASP-2; human MASP-1 also binds to human FCN-1 but a function has not been described (Liu et al., 2005). Although mouse FCN-A binds MASP-2 and MAp19 with activation of the LP, mouse FCN-B binds to neither protease (Endo et al., 2005).

The objectives of these studies were to examine CAIA in mice lacking MBL-A, MBL-C, and FCN-A, and to determine the possible mechanisms of MASP-1 cleavage of pro-Df into mature Df resulting in activation of the AP.

2. Materials and Methods

2.1. Mice

Eight to 10-week-old homozygous *MBL*$^{-/-}$*/FCN A*$^{-/-}$ C57BL/6 male mice were used for this study of Arthrogen-induced CAIA. *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice lacking MBL-A, MBL-C, and FCN-A, or mice lacking only FCN-A, were obtained from Dr. K. Takahashi. The *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice were generated by Drs. Stahl and Takahashi by cross breeding *MBL A/C*$^{-/-}$ mice with *FCN A*$^{-/-}$ mice obtained from Dr. Fujita. The identity of *the MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was determined by RT-PCR on DNA obtained from tail cuttings. Studies on these mice have not previously been described. Sera for *in vitro* studies were obtained from *C3*$^{-/-}$, *C4*$^{-/-}$, *Bf*$^{-/-}$, *Df*$^{-/-}$, and *MASP1/3*$^{-/-}$ mice. Our laboratory has maintained colonies of *C3*$^{-/-}$, *C4*$^{-/-}$, *Bf*$^{-/-}$, *Df*$^{-/-}$, and *MASP1/3*$^{-/-}$ C57BL/6 homozygous mice with the F10 progeny used for this study. Age-matched and sex-matched C57BL/6 mice were used as

wild type (WT) controls (Jackson Laboratories). All animals were kept in a barrier animal facility with a climate-controlled environment having 12-h light/dark cycles. Filter top cages were used with 3 mice in each cage. During the course of this study, all experimental mice were fed breeder's chow provided by the Center for Laboratory Animal Care, University of Colorado School of Medicine.

2.2. Induction of collagen antibody-induced arthritis

CAIA was induced in *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice by using a cocktail of 4 mAb to bovine CII (Arthrogen-CIA, Chondrex) suspended in sterile Dulbecco's PBS. Age and sex-matched WT C57BL/6 mice were used as controls for these studies. All 4 mAb (3 IgG2a and 1 IgG2b) in this cocktail recognize conserved epitopes within the CB11 fragment, whose recognition sequences are shared by CII in many species. All mice received i.p. injections of 8 mg/mouse of Arthrogen on day 0 and 50 μg/mouse of LPS from E. coli strain 0111B4 on day 3 to synchronize the development of arthritis. All mice started to develop arthritis at day 4 and were sacrified at day 10, including 3 age-matched *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice that were not treated with Arthrogen-CIA.

2.3. Examination for clinical disease activity

The prevalence of disease and severity of clinical disease activity (CDA) in all groups of *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice were determined every day by a trained individual blinded to the experimental treatment group. The CDA score is based on a 3 point scale per paw: 0 = normal joint; 1 = slight inflammation and redness; 2 = severe erythema and swelling affecting the entire paw with inhibition of use; and 3 = deformed paw or joint with ankylosis, joint rigidity and loss of function. The total CDA score is based on all 4 paws with a maximum score of 12 for each mouse.

2.4. Histopathology and C3 Deposition

Both fore limbs and the right hind limb, with knee joint, ankle and paw, from WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice with CAIA at day 10 were fixed in 10% neutral-buffered formalin (NBF) to examine for histopathological changes and C3 deposition. Histopathology with scores for inflammation, pannus, cartilage and bone damage was assessed by using Toluidine-blue (T-blue) according to our published criteria (Banda et al., 2006). C3 deposition was assessed by using a primary polyclonal goat anti-mouse C3 Ab (dilution 1:10,000) (ICN Pharmaceuticals, Costa Mesa, CA) and detected by goat anti-HRP polymer kit per manufacturer's instructions (Biocare Medical, Concord, CA). Visualization of reactivity against C3 protein was carried out using 3', 3' diaminobenzidine solution substrate (DakoCytomation, Carpinteria, CA) that reacts with HRP and produces a brown color stain.

2.5. Levels of complement components in the sera from complement-deficient and WT mice

Absolute serum levels of C1q, C4, C3, factor B, factor D and MASP-1 proteins were measured in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$, *FCN A*$^{-/-}$, and WT mice using standard ELISA protocols, according to our published methods (Banda et al., 2007). The absolute levels of MASP-1 protein in sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice could not be detected with adequate sensitivity due to the nonavailability of anti-MASP-1 Ab that can detect MASP-1 without the use of mannan on ELISA plates.

2.6. C3 deposition and C5a generation in vitro

We examined C3 deposition and C5a generation induced by adherent IgG *in vitro* and mediated by various murine sera. Sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$, *FCN A*$^{-/-}$, *MASP1/3*$^{-/-}$, *C3*$^{-/-}$, and WT mice were used in this experiment. To assure specific activation of the AP

only, sera were diluted 1:10 in calcium-deficient buffer with 5 mM $MgCl_2$ and 10 mM ethylene glycol tetraacetic acid (EGTA). The diluted sera were then added to 96-well Costar ELISA plates pre-coated with anti-CII mAb (Arthrogen, 2.5 ug/well) and incubated at 37° C for 1 h. In some experiments a specific mAb to factor B (clone 1379) was used to examine for specificity of the AP (Thurman et al., 2005). The levels of C3 deposition on the plate and of C5a generation in the supernatant were measured by ELISA (Banda et al., 2007). The reaction was stopped with 2N H_2SO_4 and absorbance read at 450 nm, correcting for background at 550 nm.

2.7. Western blot analysis of sera for detecting Df and pro-Df

To examine for the presence of mature Df and pro-Df, sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$, *MASP1/3*$^{-/-}$, *Df*$^{-/-}$, and WT mice were electrophoresed on a 10% NuPAGE Bis-Tris gel under reducing conditions with 1x MOPS buffer. Serum samples were diluted 1:24 in 1x SDS buffer. After transfer, the PVDF membrane was blocked with 5% milk in 1xPBS/0.5% Tween 20 solution for 2 h. The blots were then incubated for 24 h at 4° C with goat anti-mouse Df Ab (dilution 1:200) (Santa Cruz Biotechnology). This Ab reacts with both pro-Df and mature Df. Rabbit anti-goat HRP was used as the secondary Ab (dilution 1:2000) (Cappel), as described above. The blots were washed 3x for 10 min each in 1xPBS/0.5% Tween 20 solution. The blots were then developed for 3 min by using a 1:1 mixture of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

To detect pro-Df, a primary rabbit polyclonal Ab (1:5000) was used that reacts only with the 5-residue N-terminal peptide (QPRGR) that is present on pro-Df and absent from active murine Df (developed by Dr. M. Takahashi). The secondary Ab was HRP-conjugated goat anti-rabbit IgG (1:2,000, Hycult Biotechnology). The blot was developed as described above.

2.8. Gel filtration chromatography and Western blot analysis of MASP-1/3 proteins in the sera of MBL$^{-/-}$/FCN A$^{-/-}$ mice

Gel filtration chromatography followed by SDS-PAGE and Western blot analysis on selected fractions were used to examine for MASP-1/3 proteins in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice. A pre-packed Superdex™ 6 10/300 GL gel filtration column (GE Healthcare) was equilibrated with 20 mM Tris/Cl (pH 7.4) and 150 mM NaCl. Sera (0.2 ml) from *MASP1/3*$^{-/-}$, *C4*$^{-/-}$, *and MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice were loaded onto the column; sera from *MASP-1/3*$^{-/-}$ and *C4*$^{-/-}$ mice were used as negative and positive controls, respectively. Elution fractions of 1 ml each were collected. The MW of proteins in each fraction were estimated using standards including bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B12 (1.3 kDa); these standards eluted at peak volumes of 13.61, 16.82, 18.63, 19.80 and 22.51 ml, respectively. A 10% SDS-PAGE gel under reducing conditions was used to further separate eluted fractions between 12 and 15 ml. The presence of MASP-1/3 proteins in these four fractions was examined for using Western blot analysis. The primary antibody used to detect MASP-1/3 proteins was a polyclonal rabbit Ab to mouse MASP-1/3 (dilution 1:500) (H260, Santa Cruz Biotechnology). MASP-1/3 proteins present in the Western blot analyses performed under reducing conditions were ~ 81 kDa, as previously reported (Takahashi et al., 2008).

2.9. Western blot analysis for detecting FCN-A and FCN-B

Selective adsorption of FCN-A and FCN-B from serum of WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was performed using N-acetyl-D-glucosamine-agarose beads (GlcNAc) (Sigma). Briefly, 100 μl of serum (undiluted for FCN-B and 1:100 dilution for FCN-A) was mixed with 100 μl of binding buffer (20 mM Tris, 500 mM NaCl, 5 mM $CaCl_2$) and 50 μl of a bead/binding

buffer suspension. The mix was incubated overnight at 4°C on a rocker. Beads were washed with binding buffer x3 and incubated for 10 min at 80°C with NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent (Invitrogen). Samples were electrophoresed on a NuPAGE 10% Bis-Tris Gel in 1x MOPS SDS running buffer (Invitrogen) and then transferred to a PVDF membrane. FCN-A and FCN-B were detected using 1:20,000 and 1:5,000 dilutions of rabbit anti-mouse Ab, respectively (contributed by Dr. Y. Endo). The anti-FCN-A Ab cross-reacted slightly with FCN-B. A 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit Ab adsorbed against heavy and light chains of human, mouse and rat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as the secondary Ab for detection of both FCN-A and FCN-B. For detection of MASP-1, the primary Ab (rabbit Ab to murine MASP-1) was produced by Dr. M. Takahashi (Takahashi et al., 2008) and the secondary Ab was a 1:10,000 dilution of goat anti-rabbit IgG (Jackson Laboratories). Extensive efforts to detect MASP-1/3 in serum from *MBL*$^{-/-}$*/FCN A*$^{-/-}$mice using sandwich ELISAs with capture Ab to FCN-B or MASP-1 were not successful due to high levels of background and the lack of antibodies adequate for capture in ELISAs. Also, the concentration of FCN-B in murine sera is very low at ~200 ng/ml (Dr. Y. Endo, unpublished data).

2.10. Statistical analyses

P-values were calculated by using Student's t test. The data in all graphs, histograms and tables have been shown as the mean ± SEM with $p < 0.05$ considered significant. An unpaired two-tailed t-test was used to analyze these data. Preliminary analyses using a null hypothesis for w-statistics indicated that the data were normally distributed.

3. Results

3.1. CAIA in MBL$^{-/-}$/FCN A$^{-/-}$ mice

Clinical disease activity (CDA) and prevalence of arthritis in WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was examined daily through day 10. The prevalence of disease both in WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was 100% at day 10 (Fig. 1A). The CDA in WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice at day 10 was 8.0 ± 1.26 and 9.0 ± 1.26, respectively (Fig. 1B). There was no significant difference ($p < 0.80$) in the CDA between WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice at any day through day 10 when the mice were sacrificed. No decrease in CDA was seen in mice lacking both MBL-A and MBL-C (Banda et al., 2007) or lacking only FCN-A (data not shown). Thus, in contrast to the results obtained with *MASP-1/3*$^{-/-}$ mice where CAIA was largely inhibited (Banda et al., 2010b), the absence of MBL-A, MBL-C, and FCN-A had no influence on disease.

3.2. Histopathology and C3 deposition in WT and MBL$^{-/-}$FCN A$^{-/-}$ mice with CAIA

All WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice with CAIA were sacrificed at day 10. Both fore limbs and right hind limb (knee, ankle and paw) were fixed in 10% NBF for analysis of histopathology and C3 deposition. All joint mean scores (AJM) for inflammation ($p < 0.44$), pannus ($p < 0.56$), cartilage damage ($p < 0.27$), and bone damage ($p < 0.13$) were not significantly different between *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice (Fig. 2A). C3 deposition was examined in the knee joints of WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice. Again, no significant differences were seen in the levels of C3 deposition in synovium ($p < 0.58$) as well as on the surface of cartilage ($p < 0.95$) in the knee joints between WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice (Fig. 2B). These results are consistent with unchanged CDA scores in *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice in comparison with WT.

3.3. Levels of complement protein in mouse sera

To ensure that no confounding results were produced by unexpected changes in the levels of other essential complement activation pathway proteins, the levels of C1q, C3, C4, factor B, factor D (both pro-Df and mature forms), and MASP-1 were measured by ELISA in the sera from WT, *MBL*$^{-/-}$*/FCN A*$^{-/-}$, and *FCN A*$^{-/-}$ mice. There were no significant differences in the absolute levels of all these components among sera from WT, *MBL*$^{-/-}$*/FCN A*$^{-/-}$ or *FCN A*$^{-/-}$ mice (Table 1).

3.4. Sera from MBL$^{-/-}$/FCN A$^{-/-}$ mice exhibit a fully functional AP after induction by adherent IgG

We next examined activation of the AP in vitro by adherent mAb to CII (Banda et al., 2006). Sera from WT, *C3*$^{-/-}$, *MASP1/3*$^{-/-}$, *MBL*$^{-/-}$*/FCN A*$^{-/-}$, and *FCN A*$^{-/-}$ mice were used with calcium-deficient buffer containing 5 mM $MgCl_2$ and 10 mM EGTA, conditions where only the AP is active. High levels of C3 deposition were observed using sera from WT, *MBL*$^{-/-}$*/FCN A*$^{-/-}$, and *FCN A*$^{-/-}$ mice (Fig. 3A). The C3 deposition using sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was specific for the AP as it was completely inhibited by a mAb to factor B (Thurman et al., 2005) (Fig. 3B). High levels of C5a generation were also observed using sera from WT, *MBL*$^{-/-}$*/FCN A*$^{-/-}$, and *FCN A*$^{-/-}$ mice (Fig. 3C). Again, the generation of C5a using sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was specific for the AP as it was completely inhibited by a mAb to factor B (Fig. 3D). No C3 deposition or C5a generation induced by the adherent mAb to CII were observed using sera from *C3*$^{-/-}$ or *MASP1/3*$^{-/-}$ mice. Thus, the AP can be robustly activated in vitro by adherent mAb to CII in the absence of MBL-A, MBL-C, and FCN-A.

3.5. Only mature Df is present in sera from MBL$^{-/-}$/FCN A$^{-/-}$ mice

It has been demonstrated that pro-Df is the only circulating form of Df present in the circulation of *MASP1/3*$^{-/-}$ mice (Takahashi et al., 2008) and these mice are resistant to CAIA (Banda et al., 2010b). Sera from *Df*$^{-/-}$, *MASP1/3*$^{-/-}$, *MBL*$^{-/-}$*/FCN A*$^{-/-}$, and WT mice were examined for the presence of pro-Df and mature DF using Western blot analysis. These experiments used a polyclonal Ab that recognizes both pro-Df and mature Df and a mAb specific for pro-Df. Sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice demonstrated a broad band of 40–44kDa that theoretically could contain both forms of Df (Fig. 4A). However, using a specific mAb, pro-Df was not detected in the sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice but was only observed in the sera from *MASP1/3*$^{-/-}$ mice (Fig. 4B), as previously observed (Banda et al., 2010b; Takahashi et al., 2010).

These results indicate that mature Df, but not pro-Df, is present in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice. Thus, these mice possess the ability to cleave pro-Df into mature Df in the absence of MBL-A, MBL-C, and FCN-A. In addition, this mature Df is, or has the potential to become, biologically active as sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice generated the factor B cleavage products Ba and Bb after incubation on adherent IgG mAb to CII (data not shown).

3.6. MASP-1/3 proteins are present in the sera of MBL$^{-/-}$/FCN A$^{-/-}$ mice

We examined for the presence of MASP-1/3 proteins in murine sera deficient in MBL-A, MBL-C, and FCN-A. Sera from *MASP1/3*$^{-/-}$, *C4*$^{-/-}$ and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice were separated by gel filtration chromatography. Four different fractions were selected for SDS-PAGE and Western blot analysis with the elution volumes of 12, 13, 14, and 15 ml. Examination of WT serum showed the majority of MBL-A in WT serum to be present in fractions 13 and 14 with MBL-C found in fractions 12 and 13 and FCN polymers in the size range of MBL-A (data not shown). The serum from a *C4*$^{-/-}$ mouse, which possesses all components of the lectin pathway, showed the presence of MASP-1/3 proteins (81 kDa

bands) in fractions 12–15 from gel filtration chromatography, but primarily in fractions 12 and 13, after SDS-PAGE under reducing conditions and Western blot analyses (Fig. 5). MASP-1/3 proteins were also present in the serum of a *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mouse but primarily in fraction 15, suggesting possible binding to an unknown protein at a slightly smaller MW than MBL-A and MBL-C (~200 – 300 kDa). All of the elution fractions from the serum of a *MASP-1/3*$^{-/-}$ mouse showed no detectable MASP-1/3 proteins, suggesting specificity of the bands in the sera of *C4*$^{-/-}$ and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice. Although this gel filtration chromatography separation was performed in the absence of calcium, identical results were obtained in the presence of calcium (data not shown). The observation that MASP-1 was found in the same fractions from gel filtration chromatography as were MBL-A and MBL-C, using a serum from *C4*$^{-/-}$ mice, suggests that possible complexes of these proteins were not dissociated in the absence of calcium.

These results indicate that in the serum of a *C4*$^{-/-}$ mouse, MASP-1/3 proteins are present primarily in the gel filtration fractions containing MBL-A and MBL-C, suggesting an association. However, MASP-1/3 proteins in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice are present in possible complex with an unknown protein of smaller MW than MBL-A or MBL-C. This protein has not been further characterized.

3.7. FCN-B is present in sera from MBL$^{-/-}$/FCN A$^{-/-}$ mice

We examined for the presence of FCN-B in mouse sera. Selective adsorption of FCN-A and FCN-B from sera of WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was performed using GlcNAc beads. The beads containing adsorbed FCN-A and FCN-B, along with other proteins bound to the FCN-A and FCN-B, were examined by SDS-PAGE and Western blot analysis. FCN-B was present in the proteins adsorbed by GlcNAc beads from the sera of both WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice without CAIA (Fig. 6A). Sera from WT or *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice with CAIA showed increased levels of FCN-B (70% increase in WT and 7% in *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice). FCN-A was abundantly present in the proteins adsorbed from WT serum but was absent in the sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice without CAIA (Fig. 6B). MASP-1 protein was present in the adsorbed material obtained from the sera of both WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice without CAIA (Fig. 6C). MASP-1 protein was also present in the serum from a *Df*$^{-/-}$ mouse, lacking any AP activity (Fig. 6D). Similar experiments with mannose-coated beads showed binding of MASP-1 to MBL-C (data not shown); adequate Ab to MBL-A were not available for this experiment. These results clearly show that, in addition to FCN-A, FCN-B is present in murine sera. Furthermore, both FCN-A and FCN-B in murine sera appear to have the ability to bind MASP-1.

4. Discussion

The studies described herein have examined some possible mechanisms whereby MASP-1 cleaves pro-Df into mature Df to activate the AP. This activity of MASP-1 does not require the presence of MBL-A, MBL-C, or FCN-A as CAIA, an experimental animal model of inflammatory arthritis dependent on the AP and MASP-1, was not ameliorated in mice lacking these lectins. Furthermore, serum from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice was fully capable of activating the AP in vitro after induction by adherent IgG mAb to CII in the absence of calcium, which is required for binding of MASP to MBL or FCN. Serum from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice contained mature Df, and lacked any detectable pro-Df, indicating that cleavage of pro-Df in vivo could occur in the absence of MBL-A, MBL-C, and FCN-A. FCN-B was present in the sera of *MBL* $^{-/-}$*/FCN A*$^{-/-}$ mice and FCN-B adsorbed from these sera demonstrated associated MASP-1. Thus, FCN-B may provide a binding molecule for MASP-1 in cleavage of pro-Df and activation of the AP. In addition, MASP-1 was present in sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice possibly bound to an unknown protein of smaller MW than MBL-A, MBL-C, or FCN. Lastly, MASP-1 not bound to any protein may be active in

cleaving pro-Df into Df. However, MASP-1 is secreted as an inactive zymogen and activated MASP-1 is immediately neutralized by C1 esterase inhibitor and by a-2-macroglobin (Takahashi et al., 2010). Thus, it is likely that, in the presence or absence of FCN-B, MASP-1 activation and the subsequent cleavage of pro-Df to mature Df occur locally in tissues.

MBL and FCN bind to specific carbohydrates on bacterial surfaces with initiation of the LP occurring through the activity of bound MASP-2 on C2 and C4 fixed to the complex. Whereas MBL bind to mannose, fucose, and GlcNAc, FCN bind specifically to GlcNAc residues. MASP-1 may amplify initiation of the LP through cleavage and activation of MASP-2. However, MASP-1 exhibits broad protease activity and its major roles in biology may not be in activation of the LP. The recent demonstration that MASP-1 is necessary for activation of the AP *in vivo* and *in vitro* through cleavage of pro-Df into mature Df provides a significant new function for this serine protease, particularly given the important role for the AP in many human diseases (Banda et al., 2010b; Takahashi et al., 2010; Thurman and Holers, 2006).

In determining the mechanism of MASP-1 activation of the AP, it is necessary to review the current biology of Df. The existence of pro-Df was originally described in 1974 and was later shown to be identical to adipsin, expressed at high levels in adipose tissue (Cook et al., 1985; Fearon et al., 1974; Rosen et al., 1989; White et al., 1992). Pro-Df can also be synthesized by synovial macrophages (de Ceulaer et al., 1980). It has remained unclear whether the activation peptide of pro-Df is cleaved by a trypsin-like enzyme inside the cell or during secretion, or pro-Df is secreted intact from synthesizing cells then subsequently cleaved (Yamauchi et al., 1994). The results of recent studies show that pro-Df is secreted by differentiated murine 3T3-L1 adipocytes, suggesting that mature Df can be present outside of the cell, either in the microenvironment and/or in the circulation (Takahashi et al., 2010). Mature Df in the circulation is thought to be inactive, then activated after binding to its substrate, C3bB (Forneris et al., 2010).

Unlike MASP-2, the activation of MASP-1 from a proenzyme may not require the binding of a lectin/MASP complex to a substrate. MASP-1 is known to readily autocatalyze, providing a possible activation mechanism in the absence of a binding protein (Ambrus et al., 2003). The presence of calcium is required for MASP homodimer formation in solution (Thielens et al., 2001; Wallis and Dodd, 2000) and for the binding of human MASP-1 or MASP-2 to immobilized human MBL (Thielens et al., 2001) or FCN-2 (Cseh et al., 2002). Although calcium is also required in the binding of MBL to carbohydrate ligands, the role of calcium in the binding of FCN to GlcNAc remains unclear (Endo et al., 2007; Runza et al., 2008). We observed that sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice mediated adherent IgG-initiated activation of the AP *in vitro* in the absence of calcium. This observation suggests that MBL may not be required in IgG initiation of the AP, although other lectins may be involved.

IgM has been shown to activate the LP through binding of MBL to *N*-linked glycans on the IgM molecule containing mannose and GlcNAc in a calcium-dependent manner (Arnold et al., 2005; McMullen et al., 2006). However, the presence of antigen bound to the IgM blocks this interaction (Arnold et al., 2005). Activation of the LP by polymeric IgA also requires calcium with MBL binding possibly to *N*-linked glycans containing mannose (Roos et al., 2001). IgA, but not IgM, also activates the AP in the absence of calcium, although the responsible molecular determinants on the IgA molecule are unknown (Hiemstra et al., 1988). IgG lacking terminal sialic acid and galactose (G0 IgG) initiates the LP, but G0 IgG is actually more potent in activation of the CP and AP (Banda et al., 2008). However, initiation of the AP, but not of the CP, by adherent IgG was prevented by removal of all *N*-glycans (Banda et al., 2008). Potential mechanisms for *N*-glycans in IgG influencing

initiation of the AP, in addition to the possible role of FCN-B, include binding of $(C3b)_2$ to IgG, binding of properdin to C3b, binding of factor B to C3b, or binding of factor H to C3b (Banda et al., 2008). Alternatively, the possibility exists that MASP-1 may bind directly to the IgG-C3b complex in tissues or may be activated by binding to substrate pro-Df in the complex of IgG, C3b, and factor B.

We described the presence of FCN-B in murine sera with an increase in concentration after the induction of CAIA. FCN-B is present at low levels in sera (data not shown) with concentration enhanced by adsorption on GlcNAc beads. However, the presence of FCN-B in murine serum was first observed by investigators in Japan (Y. Endo et al., manuscript in preparation). We also observed that MASP-1 was present with FCN-B on the GlcNAC beads, suggesting an association of these two proteins in the sera. The described failure of murine MASP-2 to bind to FCN-B, but not rat MASP-1 or MASP-2, was ascribed to a single amino acid change in the collagenous domain of FCN-B that blocked MASP binding (Endo et al., 2005; Girija et al., 2011). However, these experiments did not examine binding of murine MASP-1 to FCN-B and used recombinant proteins that may not have been active (Endo et al., 2005; Girija et al., 2011). Recent experiments have shown that murine MASP-1 binds to recombinant FCN-B (Y. Endo et al., manuscript in preparation).

In a recent publication MASP-1/3 in human serum was observed to co-purify with collectin 11 (CL-K1) by mannose-Sepharose affinity chromatography (Hansen et al., 2010). An association between MASP-1/3 and collectin 11 was shown by ELISA, although activation of the complement system was not examined. The presence of collectin 11 in human serum was originally described by (Keshi et al., 2006) although unlike FCN this collectin did not bind to GlcNAc. Collectin 11 mRNA and protein were subsequently found in various organs in mice, but its presence in murine serum was not described (Motomura et al., 2008). Recombinant collectin 11 migrates with a molecular mass of 34 kDa although it appears to form 200 kDa disulfide-linked dimers of trimeric subunits (Hansen et al., 2010). Gel filtration chromatography of human serum indicates the presence of collectin 11 in oligomers of 440 kDa and larger than 950 kDa, suggesting possible complex formation with other serum proteins (Hansen et al., 2010) . We have not been able to explore whether the unknown MASP-1 binding protein of ~200 – 300 kDa described in Fig. 5 could be collectin 11 because of the lack of availability of Ab to murine collectin 11 and of mice lacking collectin 11 or FCN-B. However, because it does not bind GlcNAC, the possible presence of collectin 11 in murine serum could not explain the apparent interaction between MASP-1 and FCN-B observed by adsorption on GlcNAC beads in Fig. 6.

Conclusions

The results of our studies suggest some possible mechanisms whereby MASP-1 may cleave pro-Df into mature Df to activate the AP. This is based on the fact that *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice were susceptible to CAIA, and the latter disease is dependent on the AP of complement. Importantly FCN B and MASP-1 proteins were present in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice and FCN-B can bind to MASP-1 to activate the AP of complement. Further clarification of this question awaits the development of mice genetically deficient in all 4 lectins, MBL-A, MBL-C, FCN-A, and FCN-B, as well as identification of the possible unknown MASP-1 binding protein in serum suggested by our studies. Further experiments will also be necessary to determine the involved cells in specific tissues, their secreted products, and the interactions leading to local MASP-1 activation of the AP.

HIGHTLIGHTS

- *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice were fully susceptible to collagen antibody induced arthritis.
- *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice have intact alternative pathway of the complement.
- MASP-1 protein does not require binding to MBL A, MBL C and ficolin A to cleave pro-Df.
- FCN-B and MASP-1 are present in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice and FCN-B can bind to MASP-1.

Abbreviations

AP	alternative pathway
CAIA	collagen antibody-induced arthritis
CP	classical pathway
EGTA	ethylene glycol tetraacetic acid
FCN	ficolin(s)
Df	factor D
IC	immune complex
LP	lectin pathway
MBL	mannose binding lectin(s)
MASP-1, 2 and 3	mannose-binding lectin-associated serine proteases 1, 2 and 3
MAp19 or sMAP	mannose-binding lectin-associated protease of 19 kDa
NBF	neutral buffered formalin
pro-Df	pro-factor D
RA	rheumatoid arthritis
T-blue	Toluidine blue

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References

Ambrus G, Gal P, Kojima M, Szilagyi K, Balczer J, Antal J, Graf L, Laich A, Moffatt BE, Schwaeble W, Sim RB, Zavodszky P. Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and −2: a study on recombinant catalytic fragments. J Immunol. 2003; 170:1374–1382. [PubMed: 12538697]

Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA, Rudd PM, Sim RB. Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. J Biol Chem. 2005; 280:29080–29087. [PubMed: 15955802]

Banda NK, Levitt B, Wood AK, Takahashi K, Stahl GL, Holers VM, Arend WP. Complement activation pathways in murine immune complex-induced arthritis and in C3a and C5a generation in vitro. Clin Exp Immunol. 2010a; 159:100–108. [PubMed: 19843088]

Banda NK, Takahashi K, Wood AK, Holers VM, Arend WP. Pathogenic complement activation in collagen antibody-induced arthritis in mice requires amplification by the alternative pathway. J Immunol. 2007; 179:4101–4109. [PubMed: 17785849]

Banda NK, Takahashi M, Levitt B, Glogowska M, Nicholas J, Takahashi K, Stahl GL, Fujita T, Arend WP, Holers VM. Essential role of complement mannose-binding lectin-associated serine proteases-1/3 in the murine collagen antibody-induced model of inflammatory arthritis. J Immunol. 2010b; 185:5598–5606. [PubMed: 20870940]

Banda NK, Thurman JM, Kraus D, Wood A, Carroll MC, Arend WP, Holers VM. Alternative complement pathway activation is essential for inflammation and joint destruction in the passive transfer model of collagen-induced arthritis. J Immunol. 2006; 177:1904–1912. [PubMed: 16849503]

Banda NK, Wood AK, Takahashi K, Levitt B, Rudd PM, Royle L, Abrahams JL, Stahl GL, Holers VM, Arend WP. Initiation of the alternative pathway of murine complement by immune complexes is dependent on N-glycans in IgG antibodies. Arthritis Rheum. 2008; 58:3081–3089. [PubMed: 18821684]

Cook KS, Groves DL, Min HY, Spiegelman BM. A developmentally regulated mRNA from 3T3 adipocytes encodes a novel serine protease homologue. Proc Natl Acad Sci U S A. 1985; 82:6480–6484. [PubMed: 3901003]

Cseh S, Vera L, Matsushita M, Fujita T, Arlaud GJ, Thielens NM. Characterization of the interaction between L-ficolin/p35 and mannan-binding lectin-associated serine proteases-1 and −2. J Immunol. 2002; 169:5735–5743. [PubMed: 12421953]

de Ceulaer C, Papazoglou S, Whaley K. Increased biosynthesis of complement components by cultured monocytes, synovial fluid macrophages and skynovial membrane cells from patients with rheumatoid arthritis. Immunology. 1980; 41:37–43. [PubMed: 6903558]

Dobo J, Harmat V, Beinrohr L, Sebestyen E, Zavodszky P, Gal P. MASP-1, a promiscuous complement protease: structure of its catalytic region reveals the basis of its broad specificity. J Immunol. 2009; 183:1207–1214. [PubMed: 19564340]

Endo Y, Matsushita M, Fujita T. Role of ficolin in innate immunity and its molecular basis. Immunobiology. 2007; 212:371–379. [PubMed: 17544822]

Endo Y, Nakazawa N, Liu Y, Iwaki D, Takahashi M, Fujita T, Nakata M, Matsushita M. Carbohydrate-binding specificities of mouse ficolin A, a splicing variant of ficolin A and ficolin B and their complex formation with MASP-2 and sMAP. Immunogenetics. 2005; 57:837–844. [PubMed: 16328467]

Fearon DT, Austen KF, Ruddy S. Properdin factor D: characterization of its active site and isolation of the precursor form. J Exp Med. 1974; 139:355–366. [PubMed: 4855753]

Forneris F, Ricklin D, Wu J, Tzekou A, Wallace RS, Lambris JD, Gros P. Structures of C3b in complex with factors B and D give insight into complement convertase formation. Science. 2010; 330:1816–1820. [PubMed: 21205667]

Fujita T, Matsushita M, Endo Y. The lectin-complement pathway--its role in innate immunity and evolution. Immunol Rev. 2004; 198:185–202. [PubMed: 15199963]

Girija UV, Mitchell DA, Roscher S, Wallis R. Carbohydrate recognition and complement activation by rat ficolin-B. Eur J Immunol. 2011; 41:214–223. [PubMed: 21182092]

Hajela K, Kojima M, Ambrus G, Wong KH, Moffatt BE, Ferluga J, Hajela S, Gal P, Sim RB. The biological functions of MBL-associated serine proteases (MASPs). Immunobiology. 2002; 205:467–475. [PubMed: 12396008]

Hansen S, Selman L, Palaniyar N, Ziegler K, Brandt J, Kliem A, Jonasson M, Skjoedt MO, Nielsen O, Hartshorn K, Jorgensen TJ, Skjodt K, Holmskov U. Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. J Immunol. 2010; 185:6096–6104. [PubMed: 20956340]

Harboe M, Mollnes TE. The alternative complement pathway revisited. J Cell Mol Med. 2008; 12:1074–1084. [PubMed: 18419792]

Hiemstra PS, Biewenga J, Gorter A, Stuurman ME, Faber A, van Es LA, Daha MR. Activation of complement by human serum IgA, secretory IgA and IgA1 fragments. Mol Immunol. 1988; 25:527–533. [PubMed: 3173354]

Keshi H, Sakamoto T, Kawai T, Ohtani K, Katoh T, Jang SJ, Motomura W, Yoshizaki T, Fukuda M, Koyama S, Fukuzawa J, Fukuoh A, Yoshida I, Suzuki Y, Wakamiya N. Identification and characterization of a novel human collectin CL-K1. Microbiol Immunol. 2006; 50:1001–1013. [PubMed: 17179669]

Liu Y, Endo Y, Iwaki D, Nakata M, Matsushita M, Wada I, Inoue K, Munakata M, Fujita T. Human M-ficolin is a secretory protein that activates the lectin complement pathway. J Immunol. 2005; 175:3150–3156. [PubMed: 16116205]

Matsushita M, Thiel S, Jensenius JC, Terai I, Fujita T. Proteolytic activities of two types of mannose-binding lectin-associated serine protease. J Immunol. 2000; 165:2637–2642. [PubMed: 10946292]

McMullen ME, Hart ML, Walsh MC, Buras J, Takahashi K, Stahl GL. Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. Immunobiology. 2006; 211:759–766. [PubMed: 17113913]

Megyeri M, Mako V, Beinrohr L, Doleschall Z, Prohaszka Z, Cervenak L, Zavodszky P, Gal P. Complement protease MASP-1 activates human endothelial cells: PAR4 activation is a link between complement and endothelial function. J Immunol. 2009; 183:3409–3416. [PubMed: 19667088]

Moller-Kristensen M, Thiel S, Sjoholm A, Matsushita M, Jensenius JC. Cooperation between MASP-1 and MASP-2 in the generation of C3 convertase through the MBL pathway. Int Immunol. 2007; 19:141–149. [PubMed: 17182967]

Motomura W, Yoshizaki T, Ohtani K, Okumura T, Fukuda M, Fukuzawa J, Mori K, Jang SJ, Nomura N, Yoshida I, Suzuki Y, Kohgo Y, Wakamiya N. Immunolocalization of a novel collectin CL-K1 in murine tissues. J Histochem Cytochem. 2008; 56:243–252. [PubMed: 18040075]

Pangburn MK, Schreiber RD, Muller-Eberhard HJ. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b–like activities by spontaneous hydrolysis of the putative thioester in native C3. J Exp Med. 1981; 154:856–867. [PubMed: 6912277]

Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010; 11:785–797. [PubMed: 20720586]

Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. J Immunol. 2001; 167:2861–2868. [PubMed: 11509633]

Rosen BS, Cook KS, Yaglom J, Groves DL, Volanakis JE, Damm D, White T, Spiegelman BM. Adipsin and complement factor D activity: an immune-related defect in obesity. Science. 1989; 244:1483–1487. [PubMed: 2734615]

Rossi V, Cseh S, Bally I, Thielens NM, Jensenius JC, Arlaud GJ. Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and −2. J Biol Chem. 2001; 276:40880–40887. [PubMed: 11527969]

Runza VL, Schwaeble W, Mannel DN. Ficolins: novel pattern recognition molecules of the innate immune response. Immunobiology. 2008; 213:297–306. [PubMed: 18406375]

Skjoedt M-O, Palarasah Y, Munthe-Fog L, Ma JY, Weiss G, Skjodt K, Koch C, Garred P. MBL-associated serine protease-3 circulates in high serum concentrations predominantly in complex with Ficolin-3 and regulates Ficolin-3 mediated complement activation. Immunobiology Epub ahead of print. 2010

Takahashi M, Endo Y, Fujita T, Matsushita M. A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. Int Immunol. 1999; 11:859–863. [PubMed: 10330290]

Takahashi M, Ishida Y, Iwaki D, Kanno K, Suzuki T, Endo Y, Homma Y, Fujita T. Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D. J Exp Med. 2010; 207:29–37. [PubMed: 20038603]

Takahashi M, Iwaki D, Kanno K, Ishida Y, Xiong J, Matsushita M, Endo Y, Miura S, Ishii N, Sugamura K, Fujita T. Mannose-binding lectin (MBL)-associated serine protease (MASP)-1 contributes to activation of the lectin complement pathway. J Immunol. 2008; 180:6132–6138. [PubMed: 18424734]

Terai I, Kobayashi K, Matsushita M, Fujita T. Human serum mannose-binding lectin (MBL)-associated serine protease-1 (MASP-1): determination of levels in body fluids and identification of two forms in serum. Clin Exp Immunol. 1997; 110:317–323. [PubMed: 9367419]

Thiel S. Complement activating soluble pattern recognition molecules with collagen-like regions, mannan-binding lectin, ficolins and associated proteins. Mol Immunol. 2007; 44:3875–3888. [PubMed: 17768106]

Thiel S, Petersen SV, Vorup-Jensen T, Matsushita M, Fujita T, Stover CM, Schwaeble WJ, Jensenius JC. Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein MAp19. J Immunol. 2000; 165:878–887. [PubMed: 10878362]

Thielens NM, Cseh S, Thiel S, Vorup-Jensen T, Rossi V, Jensenius JC, Arlaud GJ. Interaction properties of human mannan-binding lectin (MBL)-associated serine proteases-1 and −2, MBL-associated protein 19, and MBL. J Immunol. 2001; 166:5068–5077. [PubMed: 11290788]

Thurman JM, Holers VM. The central role of the alternative complement pathway in human disease. J Immunol. 2006; 176:1305–1310. [PubMed: 16424154]

Thurman JM, Kraus DM, Girardi G, Hourcade D, Kang HJ, Royer PA, Mitchell LM, Giclas PC, Salmon J, Gilkeson G, Holers VM. A novel inhibitor of thealternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice. Mol Immunol. 2005; 42:87–97. [PubMed: 15488947]

Wallis R, Dodd RB. Interaction of mannose-binding protein with associated serine proteases: effects of naturally occurring mutations. J Biol Chem. 2000; 275:30962–30969. [PubMed: 10913141]

White RT, Damm D, Hancock N, Rosen BS, Lowell BB, Usher P, Flier JS, Spiegelman BM. Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue. J Biol Chem. 1992; 267:9210–9213. [PubMed: 1374388]

Yamauchi Y, Stevens JW, Macon KJ, Volanakis JE. Recombinant and native zymogen forms of human complement factor D. J Immunol. 1994; 152:3645–3653. [PubMed: 8144940]

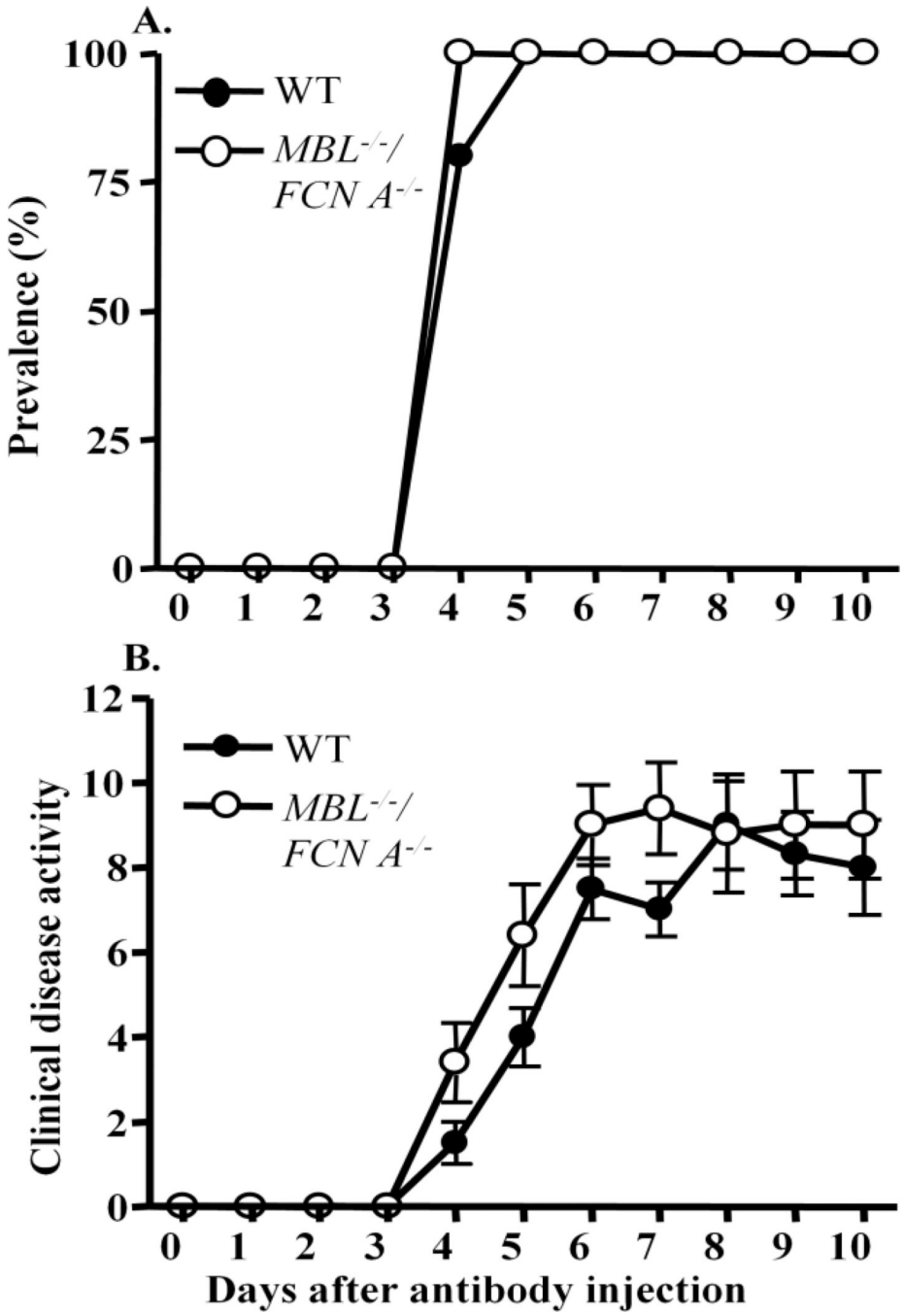

FIGURE 1.

Prevalence and clinical disease activity score in WT and *MBL*$^{-/-}$/*FCN A*$^{-/-}$ mice with CAIA. Four mAb to CII (Arthrogen-CIA) were injected i.p. on day 0 followed by an injection of LPS at day 3 to cycle the development of arthritis. *A.* Prevalence of disease in percentage (%). *B.* Clinical disease activity score with a maximum score of 12. The data shown in panels A and B are expressed as mean ± SEM based on WT mice n = 6 and *MBL*$^{-/-}$/*FCN A*$^{-/-}$ mice n = 5.

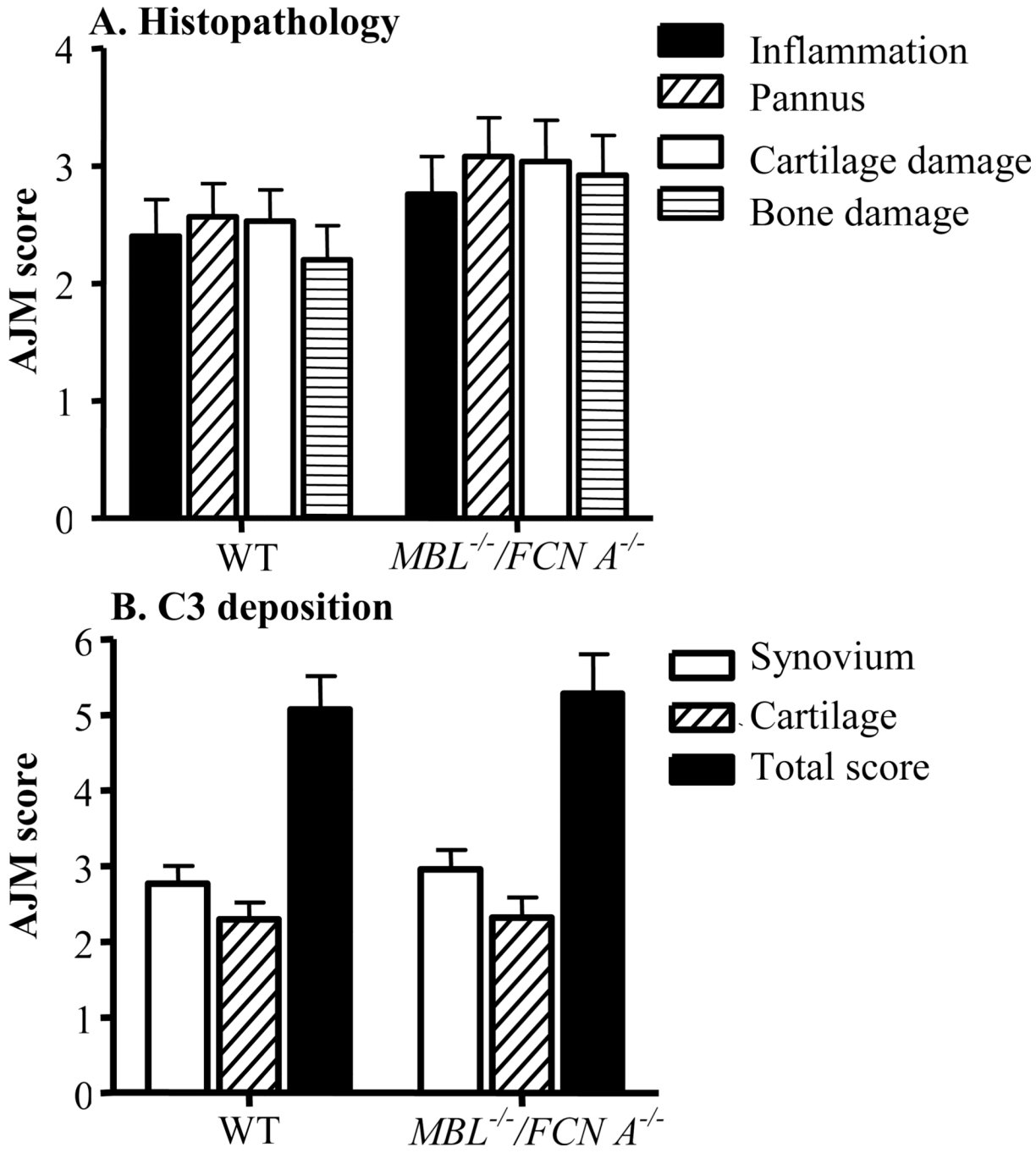

FIGURE 2.

Histopathological and immunohistochemical analysis of C3 deposition in the knee joint of WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice with CAIA. Five formalin fixed and processed joints (two forepaws and right hind ankle, knee and paw) were analyzed for various parameters of histopathology (inflammation, pannus, cartilage damage, and bone damage). *A.* Histopathology scores from WT (n = 6), and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice (n = 5). *B.* C3 deposition in the synovium, on the surface of cartilage, and total score (synovium plus cartilage) from the knee joints of WT n = 6, and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice n = 5. These data are expressed as all joint mean score (AJM) ± SEM.

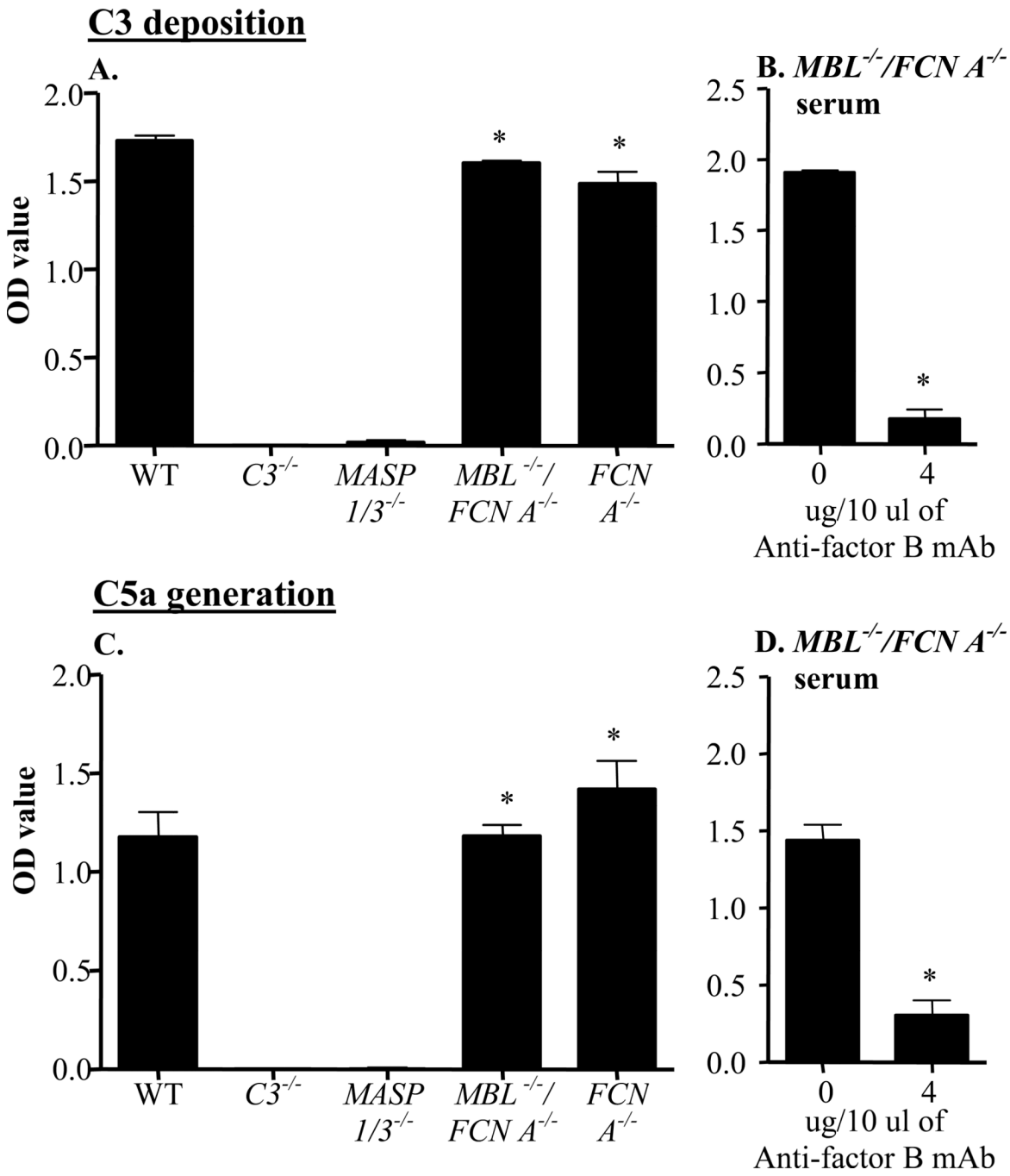

FIGURE 3.

Sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and *FCN A*$^{-/-}$ mice activate the AP of complement and are blocked specifically by an inhibitory mouse mAb to factor B. All sera for this experiment were diluted in a calcium-deficient buffer with 5 mM $MgCl_2$ and 10 mM EGTA. Levels of C3 deposition and C5a generation were measured by ELISA after induction by adherent mAb to CII. Serum from a *MASP-1/3*$^{-/-}$ mouse was used as a negative control for this experiment. *A.* C3 deposition using sera from WT, *C3*$^{-/-}$,*MASP-1/3*$^{-/-}$,*MBL*$^{-/-}$*/FCN A*$^{-/-}$ and *FCN A*$^{-/-}$ mice. *B.* Inhibition of C3 deposition by a mAb to factor B added to the sera after dilution. *C.* C5a generation using sera from WT, *C3*$^{-/-}$,*MASP-1/3*$^{-/-}$,*MBL*$^{-/-}$*/FCN A*$^{-/-}$ and *FCN A*$^{-/-}$ mice. *D.* Inhibition of C5a generation by a mAb to factor B added to the

sera after dilution. The data represent the mean ± SEM based on: WT n = 5, *C3*$^{-/-}$ n = 3, *MASP-1/3*$^{-/-}$ n = 5, *MBL*$^{-/-}$*/FCN A*$^{-/-}$ n = 5 and *FCN A*$^{-/-}$ n = 5. *p < 0.05 in comparison to WT.

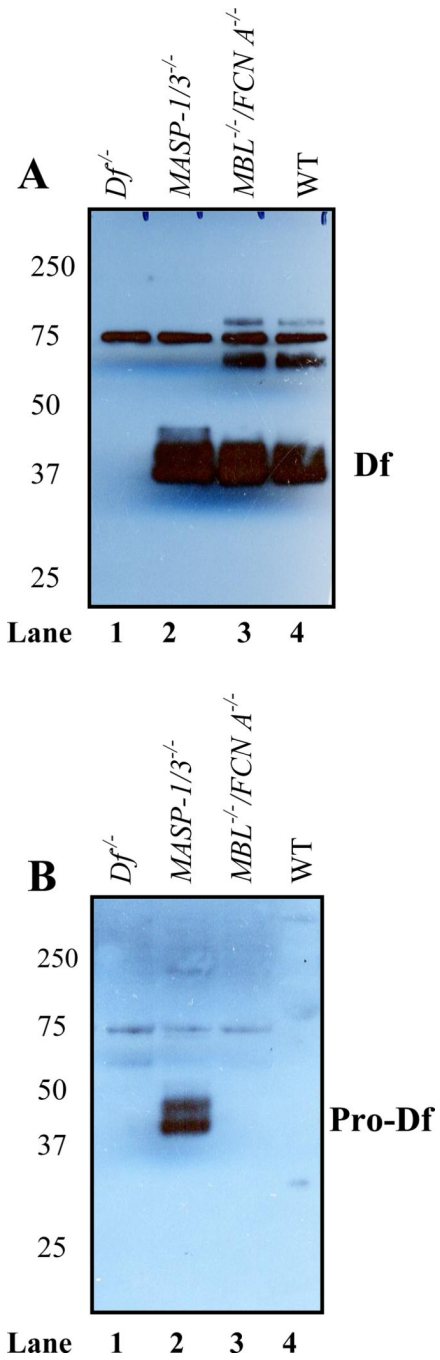

FIGURE 4.

Western blot analysis of pro-Df and mature Df in the sera of *MBL*$^{-/-}$*/FCN A*$^{-/-}$*MASP1/3*$^{-/-}$,*Df*$^{-/-}$, and WT mice without CAIA. One representative serum sample was used from each genotype. Sera from *MASP1/3*$^{-/-}$ and *Df*$^{-/-}$ mice were used as positive and negative controls, respectively. *A.* The blot was probed with a polyclonal goat Ab that recognizes overlapping bands of pro-Df and active factor D (~40 – 44 kDa) in the sera from *MASP1/3*$^{-/-}$,*MBL*$^{-/-}$*/FCN A*$^{-/-}$, and WT mice (lanes 2, 3, 4). The higher MW bands represent, in part, complexes of mature Df with unknown proteins (22). *B.* This blot was probed with a specific polyclonal rabbit Ab that recognizes only five activating residues (QPRGR) present in pro-Df but absent in the mature Df. A band of pro-Df is present in the

sera from *MASP1/3*$^{-/-}$ mice (lane 2); however, pro-Df is completely absent in the sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and WT mice (lanes 3, 4).

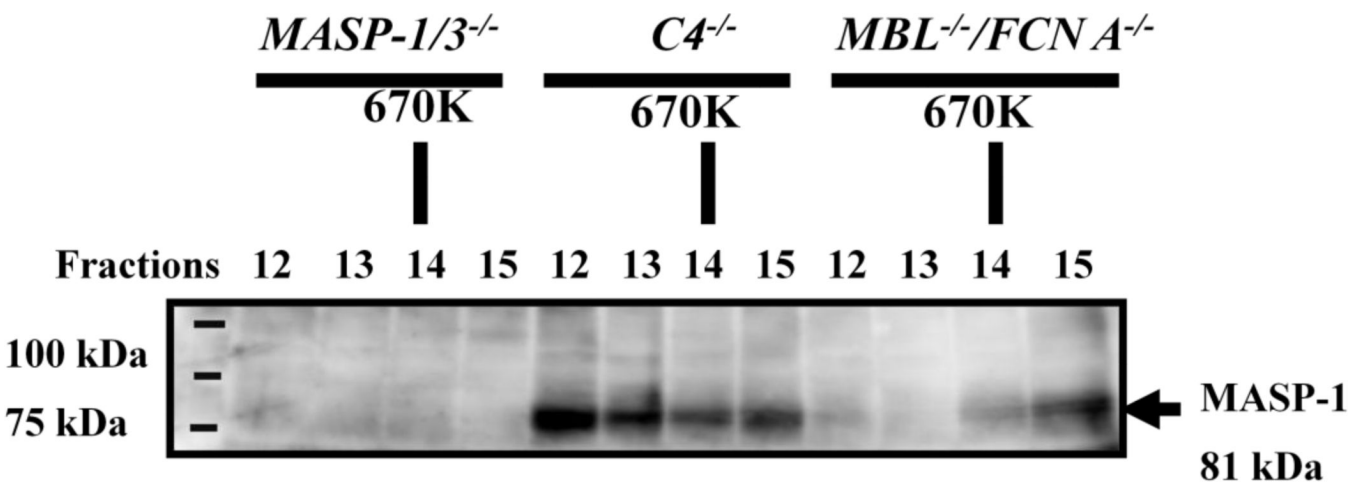

FIGURE 5.

Gel filtration chromatography, followed by SDS-PAGE and Western blot analyses on selected fractions, examining for MASP-1/3 proteins in sera from *MASP1/3*$^{-/-}$*C4*$^{-/-}$ and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice. Each fraction contained 1 ml; thus, fractions 12–15 represent elution volumes of 12 to 15 ml. The horizontal lines at top show the eluted position of a 670 kDa standard. Fractions 12–15 were further separated by SDS-PAGE under reducing conditions with Western blot analysis. Fractions 12 and 13 from a WT serum contained MBL-C and fractions 13 and 14 contained MBL-A (data not shown). In the serum from a *C4*$^{-/-}$ mouse, bands of MASP-1/3 protein (81 kDa) were observed by Western blot analyses in gel filtration fractions 12–15. However, the serum of a *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mouse contained MASP-1/3 proteins primarily in gel filtration fraction 15, apparently in association with an unknown protein of a MW smaller than MBL-A and MBL-C.

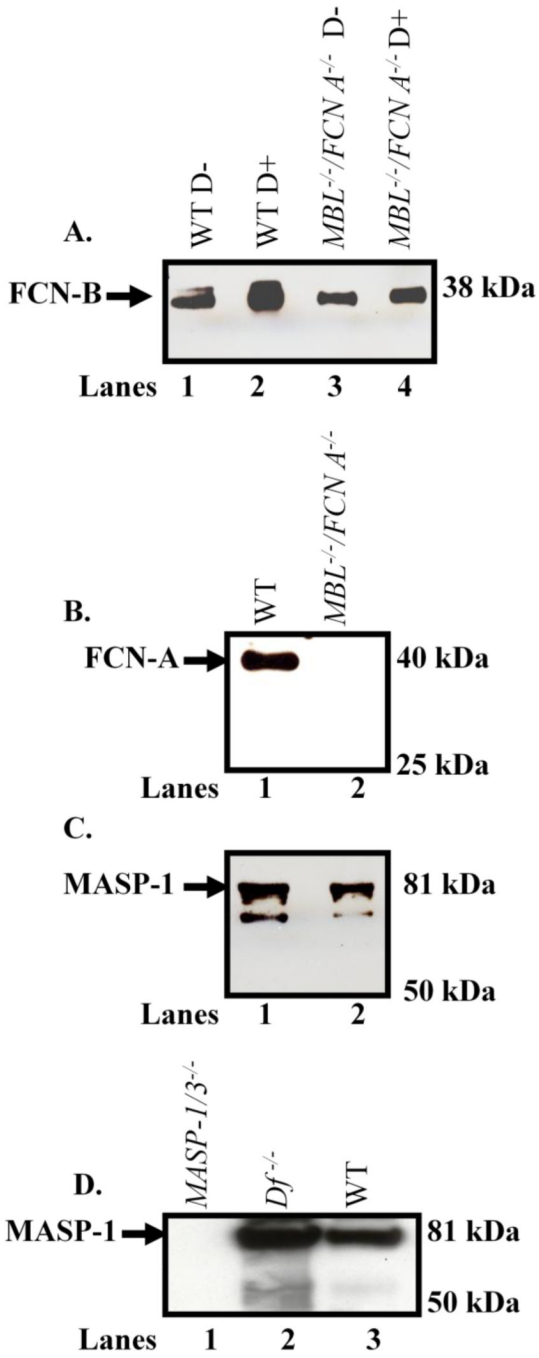

FIGURE 6.

Selective adsorption of FCN-A and FCN-B from sera of WT, *MBL*$^{-/-}$*/FCN A*$^{-/-}$ and *Df*$^{-/-}$ mice was performed with GlcNAc beads. The beads with the adsorbed proteins were examined by SDS-PAGE and Western blot analysis. *A*. The blot was probed with a polyclonal rabbit Ab to murine FCN-B using sera from WT and *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice either without (D−) or with (D+) CAIA. The upper band seen with sera from WT mice represented a slight cross-reactivity with FCN-A as it was not seen with sera from *MBL*$^{-/-}$*/FCN A*$^{-/-}$ mice (lanes 3 and 4). *B*. The blot was probed with a polyclonal rabbit Ab to murine FCN-A. *C*. The blot was probed with a polyclonal rabbit Ab to murine MASP-1/3. The lower band in each lane may represent degraded MASP-1. *D*. The blot was probed with

a polyclonal rabbit Ab to murine MASP-1/3 using sera from *MASP-1/3*$^{-/-}$*Df*$^{-/-}$ and WT mice (lanes 1, 2 and 3 respectively).

Table 1

Levels of complement components in sera from WT and complement deficient mice[a]

Mice	C1q	C3	C4	factor B	factor D[b]	MASP-1
WT (9)	0.474 ± 0.03	1.46 ± 0.15	0.72 ± 0.11	0.948 ± 0.03	1.06 ± 0.08	0.375 ± 0.02
MBL$^{-/-}$/	0.527 ± 0.05	1.18 ± 0.13	0.519 ± 0.15	0.988 ± 0.04	1.264 ± 0.04	ND[c]
FCN A$^{-/-}$(5)						
p	*0.349*	*0.235*	*0.322*	*ND*[c]	*0.091*	
FCN A$^{-/-}$ (5)	0.469 ± 0.043	1.62 ± 0.15	0.702 ± 0.15	0.649 ± 0.06	1. 26 ± 0.04	0.392 ± 0.04
P	*0.936*	*0.518*	*0.936*	*ND*[c]	*0.091*	*0.672*

[a] These data are expressed as optical density units with mean ± SEM based on the indicated number of sera (n).

[b] The anti-Df antibody used to measure the absolute levels of Df recognizes both mature Df and pro-Df.

[c] ND = not done; this assay could not be performed due to technical issues.

Sera from at least three *C1q*$^{-/-}$,*C3*$^{-/-}$,*C4*$^{-/-}$,*fB*$^{-/-}$ and *Df*$^{-/-}$ were used as negative control for respective ELISAs; all of the values using these sera were identical to the background levels.