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Chapter 7

The Study of MASPs Knockout Mice

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1. Introduction

Plasma proteases, e.g. thrombin, factor X, complement factor D and C1s are responsible for the physiological activities, such as coagulation and complement system. These proteases circulate as their zymogen in blood and are activated by various stimulations. In this chapter, we focus on a family of plasma serine proteases, called MASP (MBL/ficolin-associated serine protease) that can activate the complement. Three distinct MASP, MASP-1, MASP-2 and MASP-3 have been identified in many species of vertebrates. Although the contribution of MASP-2 in activation of complement was well defined, the substrates for MASP-1 and MASP-3 were still obscure. We have generated MASP-1- and MASP-3-deficient mice (*Masp1/3*$^{-/-}$) to verify roles of MASP-1 and MASP-3 proteases *in vivo*. One major finding is that MASP-1, considered being a lectin pathway component—also acts as a pro-factor D (Df) convertase, the initiator of the alternative pathway. Our results emphasize a unique feature of MASP-1, participating two complement pathways. We also generated MASP-2 deficient mice. In here, we would like to summarize the results obtained from these knockout mice.

2. Complement system

The complement system is an important part of the innate immune system, mediating several major effector functions, such as directly killing pathogens, promoting phagocytosis, and clearance of immune complexes and apoptotic cells and modulating adaptive immune responses, as describing in some excellent reviews (Ricklin, et al., 2010) (Fujita, et al., 2004) (Carroll, 2004). On the other hand, inappropriate activation of complement affects the pathogenesis of inflammatory diseases (Holers, 2003). Therefore, well-understanding of the mechanisms of its activation is very important. More than 30 proteins in plasma consist of the complement system. The most abundant protein among them is the third component (C3). Once the complement system is activated, a chain of reactions involving restricted proteolysis and assembly occurs, resulting in cleavage of C3 into C3b and C3a. The cascade

up to C3 cleavage is called the activation pathway. There are three distinct activation pathways of the complement cascade; the classical, alternative, and lectin pathways, that all converge on factor C3 and lead to activation of complement effector functions as above (Walport, 2001a)(Fig. 1).

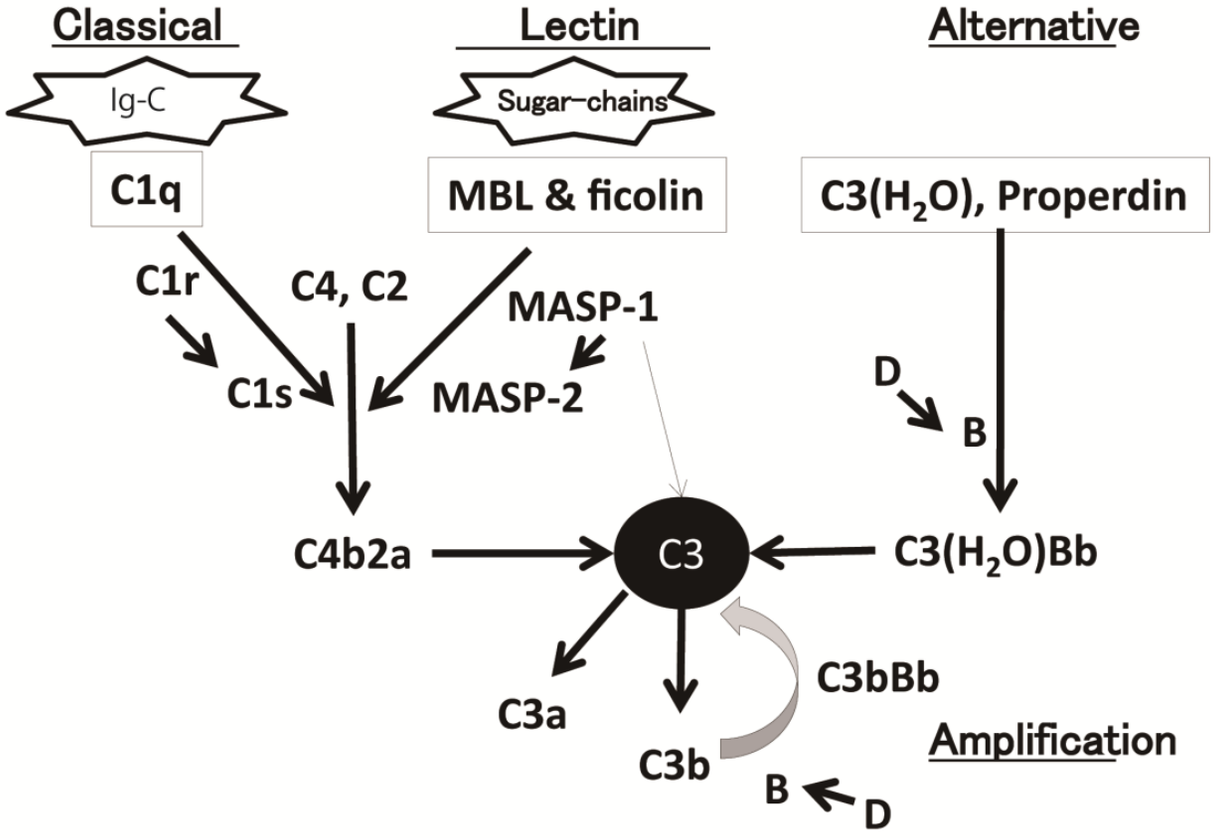

Figure 1. Activation pathways for complement system.

In the mammalian complement system, the pivotal molecule circulating C3 is cleaved into C3a and C3b by two different C3 convertases, C4b2a and C3bBb. C4b2a is generated by the classical and lectin pathway and C3bBb is generated by the alternative pathway.

2.1. The classical pathway

The classic pathway is initiated by recognition of the first C1 binding to a variety of targets, most prominently immune complexes (Walport, 2001a) (Walport, 2001b). C1 consists of a single C1q molecule associated with dimers of C1r and C1s(Lepow, et al., 1963). C1r and C1s are plasma serine proteases, normally existing in an inactive pro-enzyme form. The conformational exchange of C1q by binding to immune complexes results in the activation of C1r. C1r is thought to be cleaved in some autocatalytic manner and once C1r molecule is activated, it activate C1s, which in turn cleaves C4 and then C2(Arlaud, et al., 2002). The C4 cleavage products are C4a and C4b. The latter molecule may be bound to non-self surfaces on pathogens and is bound to C2 to form the classical pathway C3 convertase.

2.2. The alternative pathway

In the alternative pathway, spontaneous hydrolysis of C3, designated C3(H_2O) results in triggering complement activation with complement factor B, making another C3 convertase, C3(H_2O)Bb on foreign cells (Muller-Eberhard and Gotze, 1972, Pangburn, et al., 1981). This leads to the cleavage of factor B by factor D, giving rise to an active enzyme complex with the fragment Bb as the enzyme. The alternative pathway does not involve specific recognition molecules and also functions to amplify C3 activation (amplification loop) (Brouwer, et al., 2006).

2.3. The lectin pathway

Activation of the lectin pathway is similar with that of the classical pathway (Degn, et al., 2010). The lectin pathway is initiated by some serum lectins binding to pathogen-associated molecular patterns, mainly carbohydrate structures present on bacterial, fungal, or viral pathogens. In 1978, a serum lectin, designated mannose-binding lectin (MBL), which recognizes carbohydrates such as mannose and N-acetylglucosamine was first isolated from rabbit liver (Kawasaki, et al., 1978). MBL acts as the pattern recognition molecule, which recognizes sugar chains on some foreign pathogens. MBL is also found to have an avidity of complement activation (Ikeda, et al., 1987) (Holmskov, et al., 2003) (Turner, 1996). It has been thought that MBL activates complement by $C1r_2C1s_2$ protease complex that consists of classical pathway (Ohta, et al., 1990). However, in 1992, Matsushita and Fujita found a new plasma serum protease designated MBL-associated serine protease (MASP) that binds MBL (Matsushita and Fujita, 1992) (Matsushita, et al., 1998). Recent studies identified ficolins that are also plasma proteins with binding activity for carbohydrates to associate with MASP and to activate complement (Matsushita, et al., 2000, Matsushita, et al., 2001) (Cseh, et al., 2002). Ficolins has a collagen-like domain and a fibrinogen-like domain. Furthermore, CL-K1 (Keshi, et al., 2006) was also identified as a collectin that associates with MASP(Hansen, et al., 2010).

3. MBL-associated serine proteases

3.1. Three MASP proteins were associated with MBL and ficolins

MASP is homologue of C1r and C1s of the classical pathway, sharing the well-described domains structure in the order from N-terminus, CUB-I, EGF, CUB-II, CCP-I, CCP-II and SP (Sato, et al., 1994). The CUB (C1r/C1s, embryonic sea Urchin protein [Uefg], and Bone-morphogenetic protein 1 [Bmp1]) domain is approximately 110 aa, predicting a molecular structure of an antiparallel beta-barrel similar to those in immunoglobulins (Bork and Beckmann, 1993). The EGF (epidermal growth factor-like) domain of approximately 50 aa is also found in many proteins and is known to mediate protein-protein interactions via calcium ion. The N-terminal three domains consisting of CUB-I, EGF and CUB-II of the MASP are responsible for dimerization and for the calcium-dependent binding to MBL and ficolins(Feinberg, et al., 2003). The two contiguous CCPs (complement control protein) of

MASP, especially the second CCP domain, have been implicated in the binding of macromolecular substrates. The CCP domains of around 60 aa are found in a number of complement factors and other proteins(Chou and Heinrikson, 1997). The SP (serine protease) domain is the catalytically active unit of the proteases and defines them as part of the S1A family of chymotrypsin-like proteases (Yousef, et al., 2004). MASP is able to cleave C4 and C2 to generate a C3 convertase, C4b2a. Recent studies isolated two additional MASPs in human MBL complex (Thiel, et al., 1997) (Dahl, et al., 2001). These newly identified MASPs are called as MASP-2 and MASP-3 and the former one is MASP-1(Schwaeble, et al., 2002).

3.2. Substrates for MASP

It is apparently defined that MASP-2 cleaves C4 that is similar with C1s in the classical pathway (Vorup-Jensen, et al., 1998) (Ambrus, et al., 2003). However, substrates for MASP-1 and MASP-3 are still obscure. Several candidates were demonstrated by recent studies as shown in Table 1.

MASP	Substrates (reference)
MASP-1	C3 (Matsushita and Fujita, 1995), C2, fibrinogen, Factor XIII(Hajela, et al., 2002), PAR4 (Megyeri, et al., 2009), Df(Takahashi, et al., 2010)
MASP-2	C4, C2(Ambrus, et al., 2003), prothrombin
MASP-3	IGFBP-5 (Cortesio and Jiang, 2006), Df(Iwaki, et al., 2011)
C1r	C1s
C1s	C4, C2

Table 1. Substrates for MASPs

3.3. *MASP* genes

3.3.1. MASP1

MASP1 is located on chromosome 3q27-q28 in human and chromosome 16 (B2-B3) in mouse (Takada, et al., 1995). Three gene products, MASP-1, MASP-3 and MAP44 are encoded from this gene by alternative splicing. MAP44 is a truncated protein of MASP-1/3 and lacks serine protease domain (Degn, et al., 2009) (Skjoedt, et al., 2010). MAP44 is thought to be a regulatory factor, attenuating activation of the lectin pathway. *MASP1* gene has a unique structure. A single exon, encoding whole MASP-3 light-chain and the six sprit exons, encoding MASP-1 are tandem located (Dahl, et al., 2001). Therefore, MASP-1 and MASP-3 consist of a common heavy-chain and the distinct light-chain.

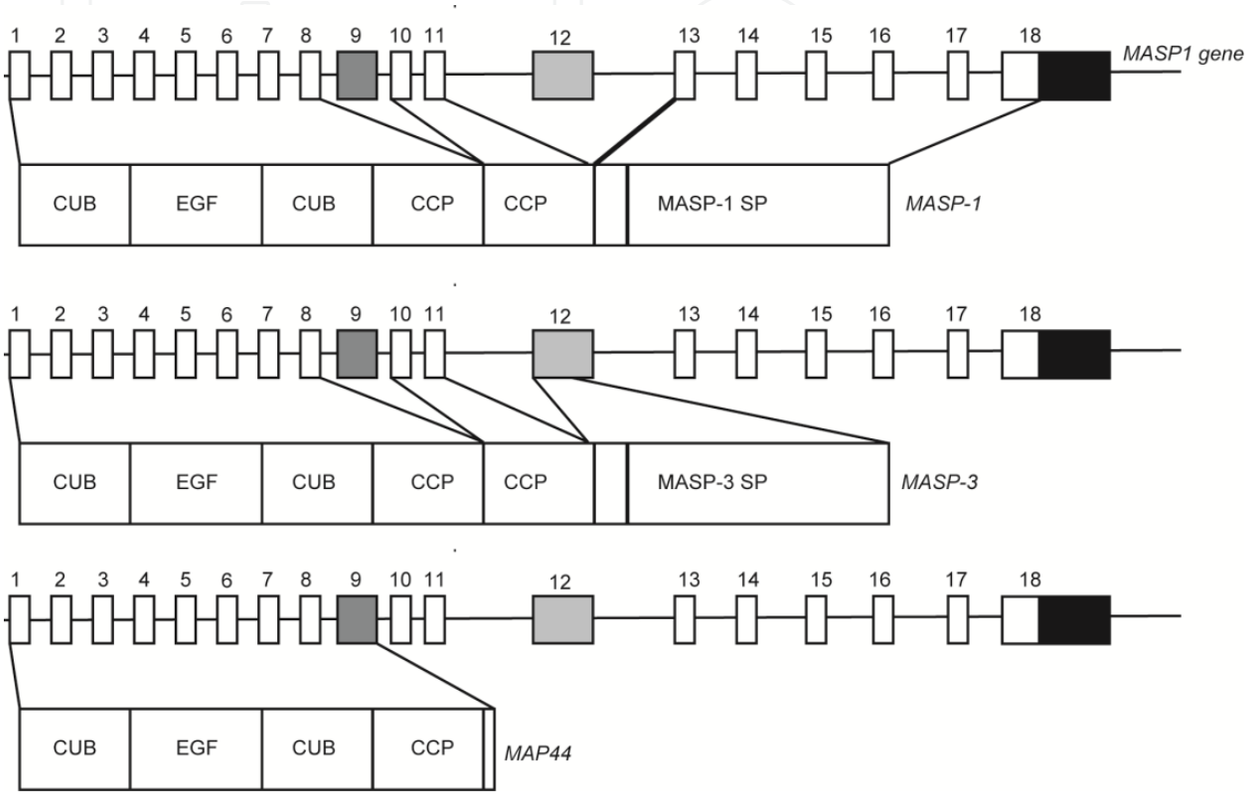

Figure 2. Schematic representation of *MASP1* gene

MASP1 gene consists of 18 exons, encoding three gene products, MASP-1, MASP-3 and MAP44 by alternative splicing.

3.3.2. MASP2

MASP2 gene is located on human chromosome 1p36.3-p36.2(Stover, et al., 1999a). And mouse Masp2 gene is located on chromosome 4(Lawson and Reid, 2000). It was shown that the MASP2 gene encodes two gene products, the 76 kDa MASP-2 serine protease and a plasma protein of 19 kDa, termed sMAP/MAp19 by alternative splicing (Takahashi, et al., 1999) (Stover, et al., 1999b). sMAP/MAp19 consist of only CUB-I and EGF-like domain of MASP-2, lacking catalytic domain.

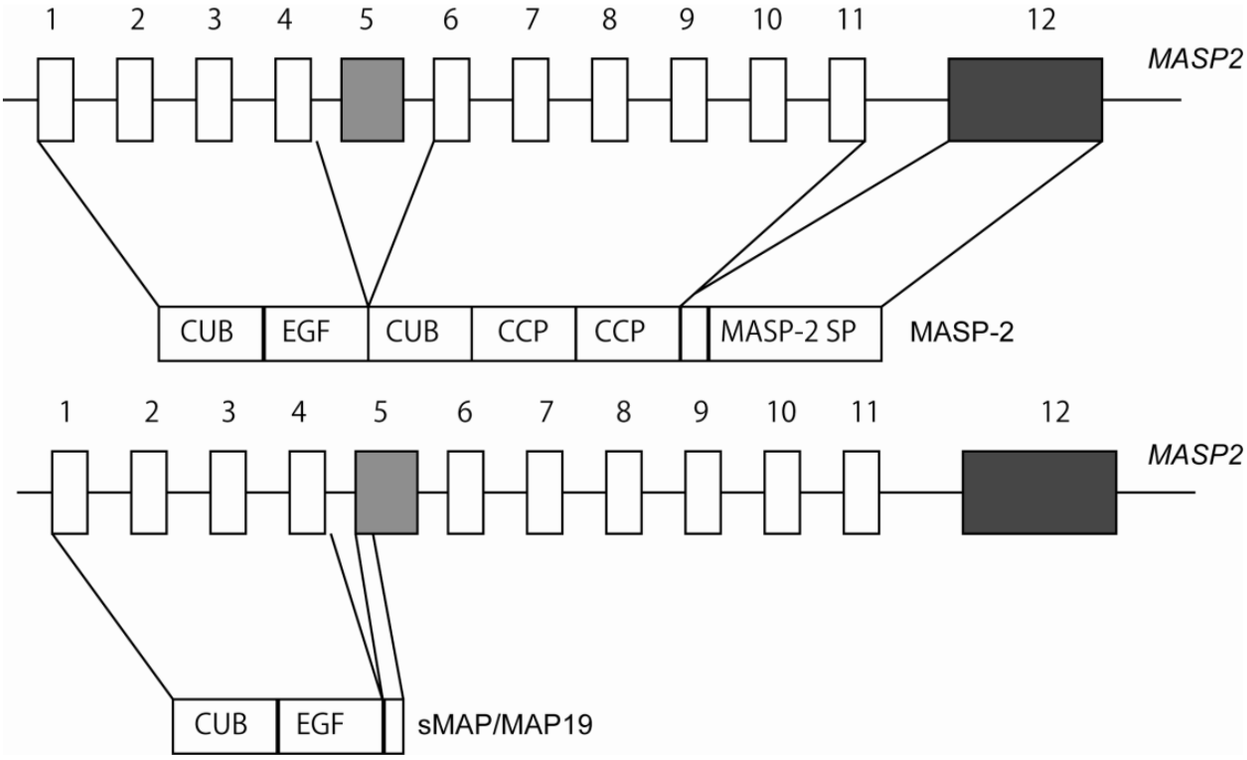

Figure 3. Schematic representation of *MASP2* gene

MASP2 gene consists of 12 exons, encoding two gene products, MASP-2 and sMAP/Map19 by alternative splicing.

4. Studies for the Masp-knockout mice

Knockout mice	Mutant allele	chromosome	Targeted exon
Masp1/3$^{-/-}$	Masp1^{tm1Tefu}	16	2
sMAP/Masp2$^{-/-}$	Masp2^{tm1Tefu}	4	5
Masp2$^{-/-}$	Masp2^{tm1Wjsc}	4	11 &12

Table 2. Masps knockout mice

4.1. MASP-1 and MASP-3-deficient mice (*Masp1/3*$^{-/-}$)

To investigate the role of MASP-1 in complement activation, we planned to disrupt the second exon of Masp1 gene by a conventional gene targeting (Takahashi, et al., 2008). When this project was proceeding, MASP-3 was identified (Dahl, et al., 2001). Surprisingly, both gene products were produced from *MASP1* gene. Since the targeted second exon is at upstream of both transcripts, it was predicted that MASP-3 is also absent in this knockout mice. It was confirmed that not only MASP-1, but also MASP-3, is absent in *MASP1/3*$^{-/-}$ mice (Takahashi, et al., 2008).

4.1.1. Masp1/3$^{-/-}$ shows the abnormality of the lectin pathway activation

Serum from *Masp1/3*$^{-/-}$ shows the abnormality of both C4 and C3 activation on mannan and it is restored by adding recombinant MASP-1. This result supported that MASP-1 contributes the lectin pathway through C4 activation. Furthermore, MASP-2 activation is delayed in *Masp1/3*$^{-/-}$ to be compared with that of wild type. This result reveals that MASP-1 and/or MASP-3 may involve in the lectin pathway activation through the acceleration of MASP-2 activation (Takahashi, et al., 2008).

4.1.2. Masp1/3$^{-/-}$ shows the abnormality of the alternative pathway activation

Further study noticed us that not only lectin pathway but also alternative pathway is abnormal in *Masp1/3*$^{-/-}$. We found that complement factor D (Df) circulates as a zymogen in *Masp1/3*$^{-/-}$ (Takahashi, et al., 2010). Df was known to be active-form, but not a zymogen in circulation (Lesavre and Muller-Eberhard, 1978). However, it has become evident that most proteases in blood are secreted as zymogen. Df was thought to be an exception. We also found that Df is synthesized as zymogen from adipocytes (Takahashi, et al., 2010) (Fig. 4). This result supports the general consensus for Df. Interestingly, increasing evidence suggests that the alternative pathway is involved in human disease, such as inflammatory arthritis and ischemia/reperfusion injury (Thurman and Holers, 2006).

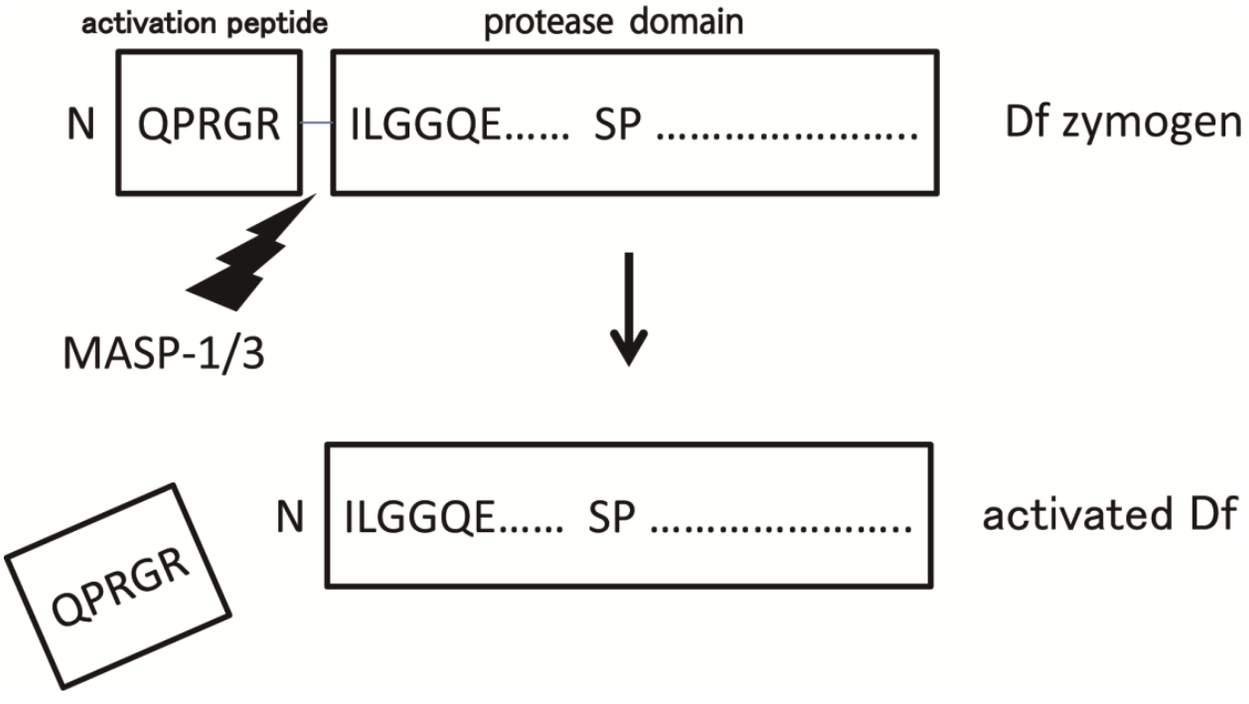

Figure 4. MASP-1 and/or MASP-3 involve in activating a zymogen of complement factor D

Complement factor D (Df) is synthesized as a zymogen (Pro-Df) from adipocytes. In serum of *Masp1/3*$^{-/-}$, Pro-Df that has an activation peptide (QPRGR) at N-terminal of Df was observed.

4.1.3. MASP-1 and/or MASP-3 involve the fat metabolism through Df activation

It was also reported that the alternative pathway is involved in fat metabolism in adipose tissue (Paglialunga, et al., 2008). Recent studies have indicated that acylation-stimulating protein (ASP), which is identical to C3adesArg, stimulates fat storage in adipocytes (Yasruel, et al., 1991) (Maslowska, et al., 1997). ASP is a derivative of complement C3; thus, *C3*$^{-/-}$ mice are lean owing to ASP deficiency. Furthermore, plasma ASP levels are decreased in Bf-deficient and Df-deficient mice, indicating that the alternative pathway stimulates production of ASP. We found that *Masp1/3*$^{-/-}$ mice are also apparently lean (Takahashi, et al., 2008), strongly indicating a contribution of MASP-1 to fat metabolism via alternative pathway. We measured the plasma concentration of leptin and TNF-alpha (Fig. 5). Leptin plays a critical role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure. Leptin appears to be a hormone secreted by adipocyte (Zhang, et al., 1994). It was shown that level of leptin in *Masp1/3*$^{-/-}$ significantly decrease. Furthermore, we determined that one of inflammatory factor, TNF-alpha increases in *Masp1/3*$^{-/-}$. As shown in Fig. 4, adipose tissues in Masp1/3-/- apparently show atrophy. Therefore, fat metabolisms in Masp1/3-/- adipose tissue might be reduced.

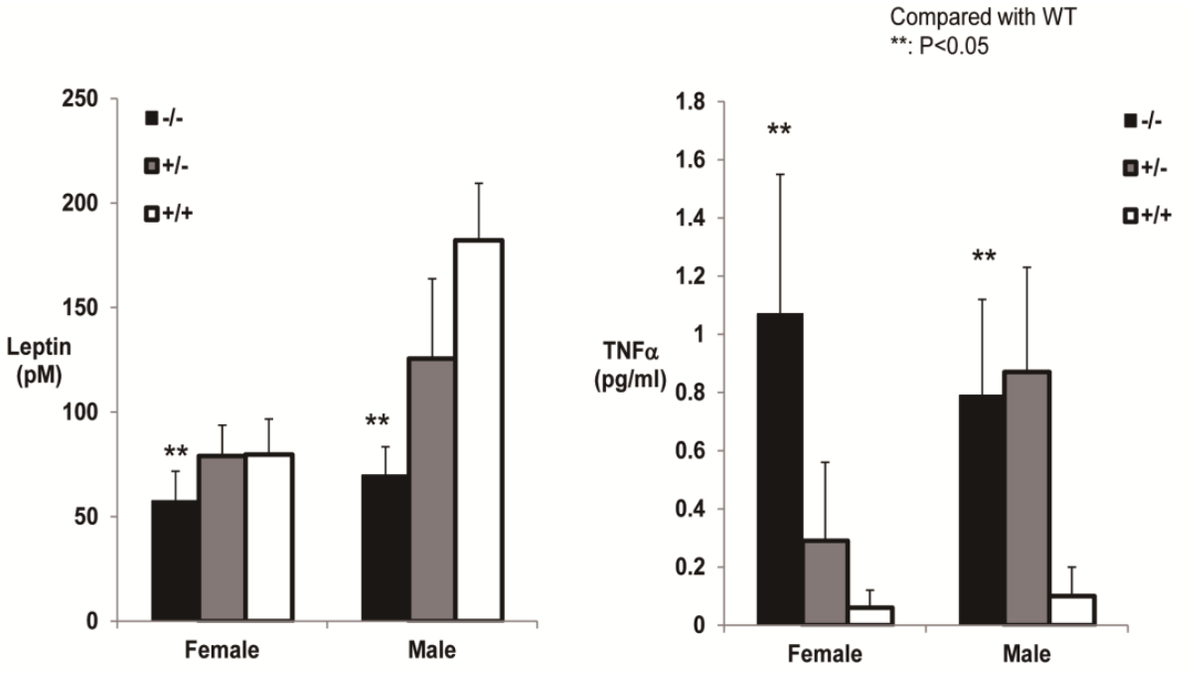

Figure 5. Serum leptin and TNF-alpha level in *Masp1/3*$^{-/-}$

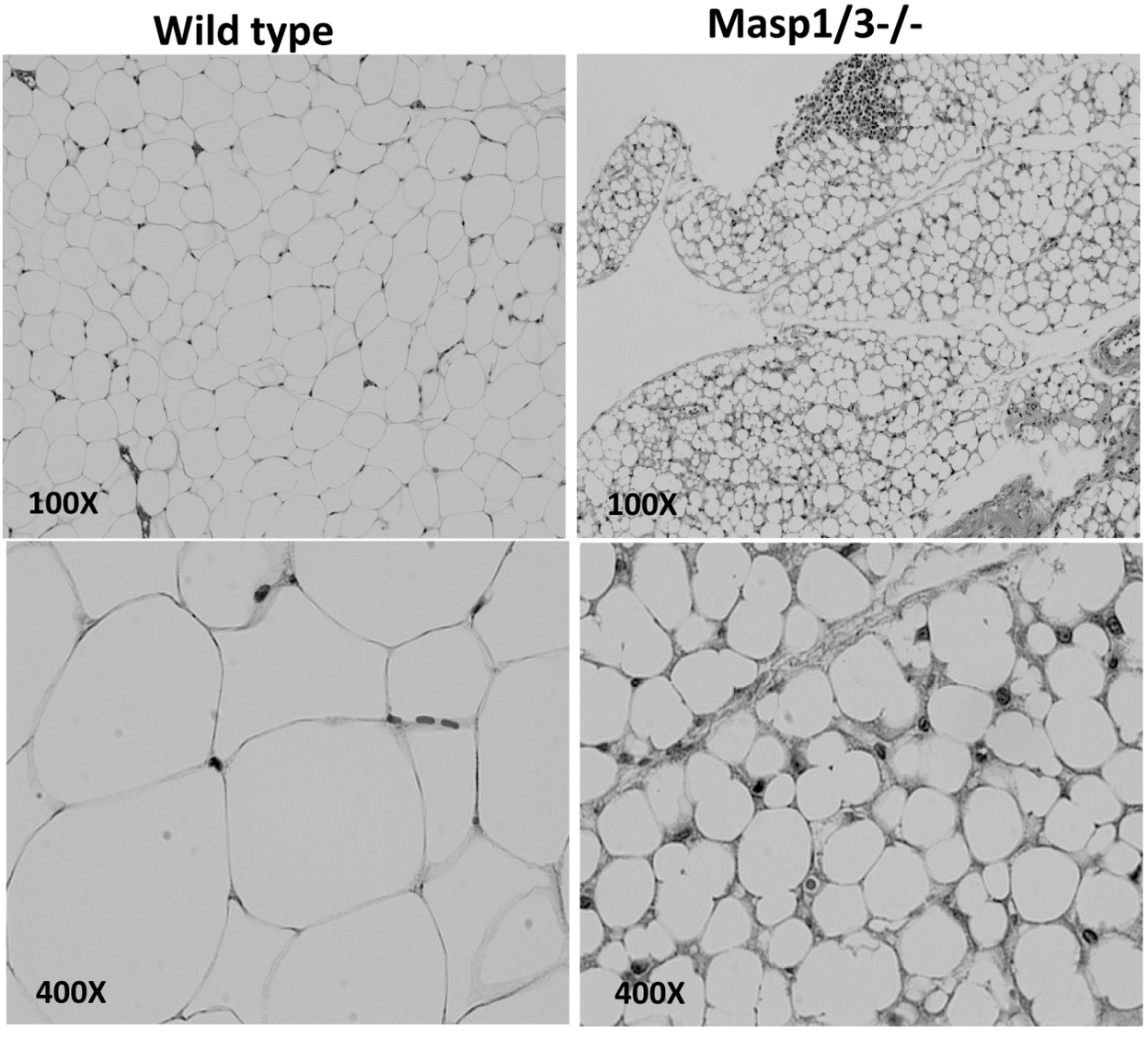

Figure 6. H&E staining of mouse adipose tissue

4.1.4. Masp1/3$^{-/-}$ is resistant to AP-mediated joint damage

Banda et al. demonstrated that *Masp1/3*$^{-/-}$ mice are highly resistant to CAIA as evidenced by a significant decrease in the histological scores as compared with WT mice (Banda, et al., 2010). Recent studies supported that the alternative pathway is both necessary and sufficient to induce disease in murine collagen Ab-induced arthritis (CAIA) (Banda, et al., 2006) (Banda, et al., 2007). This model mouse confirmed that *Masp1/3*$^{-/-}$ shows the abnormality of the alternative pathway.

4.1.5. MASP3 mutation causes 3MC syndrome

3MC syndrome (Malpuech-Michels-Mingarelli-Carnevale syndrome) are four rare autosomal recessive disorders (Carnevale, et al., 1989) (Mingarelli, et al., 1996) (Malpuech, et al., 1983) (Michels, et al., 1978). This syndrome shows facial dysmorphic traits. Recent

observations for families, including patients who suffer from 3MC syndrome found the genetic mutations in *CL-K1* and *MASP1* genes (Rooryck, et al., 2011). This result was very interesting, since a possibility was raised that MASP-3 may be responsible to not only complement system, but also development system with a recognition molecule, CL-K1. In 2010, Sirmaci, et al. also found the mutations of *MASP1* gene in two Turkish familes (Sirmaci, et al., 2010). Preliminary results was obtained that *Masp1/3* knockout mice have some developmental disorders (publication preparing).

4.2. sMAP and MASP-2-deficient mice (*sMAP/Masp2*$^{-/-}$)

To clarify the role of sMAP/Map19, we also generated another mutant mice, disrupting the fifth exon of *MASP2* gene by replacement with *neo*^{r}-gene (Iwaki, et al., 2006). Since this targeted region is the sMAP/Map19-specific exon, it was predicted that MASP-2 might be intact in this knockout mice. However, MASP-2 was not detected in their serum. Therefore, these mutant mice were named as *sMAP/Masp2*$^{-/-}$.

When recombinant sMAP and recombinant MASP-2 (rMASP-2) reconstituted the MBL-MASP-sMAP complex in deficient serum, the binding of these recombinant proteins to MBL was competitive, and the C4 cleavage activity of the MBL-MASP-sMAP complex was restored by the addition of rMASP-2. On the other hand, the addition of recombinant sMAP attenuated the activity. Therefore, MASP-2 is essential for the activation of C4 and sMAP plays a regulatory role in the activation of the lectin pathway(Iwaki, et al., 2006).

4.3. MASP-2-deficient mice (*Masp2*$^{-/-}$)

An England group generated MASP-2-deficient mice(Schwaeble, et al., 2011). This strain lacks exon 11 and 12 of *Masp2* gene, encoding the C-terminal part of the CCPII and the SP domains. In their knockout mice, sMAP/Map19 is predicted to be intact. In vitro analysis of *MASP2*$^{-/-}$ plasma showed a total absence of lectin pathway-dependent C4 cleavage on mannan- and zymosan-coated surfaces. They investigated whether MASP-2 affect the inflammatory process using a model of myocardial ischemia reperfusion injury (MIRI). It was observed that *MASP2*$^{-/-}$ was protected from MIRI.

5. Conclusion

Here, we focus on analyses of three strains for *Masps* knockout mice, *Masp1/3*$^{-/-}$, *sMAP/Masp2*$^{-/-}$ and *Masp2*$^{-/-}$. All strains show that activation of lectin pathway is deficient. We also detected the abnormality of the alternative pathway in *Masp1/3*$^{-/-}$. But *Masp2*-deficient phenotype does not affect the activity. MASPs are associated with MBL, ficolins and CL-K1. MBL-deficient mice were generated and analysed (Takahashi, et al., 2002, Shi, et al., 2004). Surprisingly, MBL-null mice show the comparable level of the alternative pathway with that of wild type. If MASP-1 and/or MASP-3 involve the activation of alternative pathway with MBL, MBL-null mice must be affected. Other recognition molecules, ficolin or CL-K1 might be involved in this phenomenon. This problem should be resolved in future study.

Recently MASP1 mutants were identified in human patients, suffering from 3MC syndrome. However, the mechanisms how MASP-1 and/or MASP-3 contribute the facial development are still unclear. Further study using Masp1/3-/- would provide a powerful tool to resolve this problem.

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