Activities of MASPs, The Complement Proteases Associated with Collectins and Ficolins

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Brief History

Mannose-binding lectin (MBL)-associated serine proteases (MASPs) are the enzymatic components of the supramolecular complexes that are responsible for the activation of the lectin pathway of the complement system. The pattern recognition subunit of these complexes can be MBL, ficolins 1, 2, 3 (aka M-, L-, H-ficolins), collectin kidney 1 (CL-K1, aka CL-11) or collectin liver 1 (CL-L1, aka CL-10). Historically, the first clue that such complexes exist came from the discovery of the Ra-reacive factor (RaRF), a bactericidal factor that binds to Ra chemotype strains of Salmonella (Kawakami et al. 1982). Later, it turned out that RaRF is a complex consisting of MBL and a serine protease (P100, later named MASP-1) capable of cleaving both C4 and C2, consequently activating the complement cascade very similarly to the C1 complex of the classical pathway (Matsushita and Fujita 1992; Takayama et al. 1999). This discovery dismissed the earlier view that MBL is associated with C1r and C1s and activates the complement cascade through the classical pathway (Ikeda et al. 1987). When a second MASP (MASP-2) was discovered (Thiel et al. 1997) and it was shown that this minor component of the complex is actually responsible for C4 and C2 cleavage, it was tempting to believe that MASP-1 and MASP-2 act like C1r and C1s in the C1 complex. It was also shown in *in vitro* experiments, however, that MASP-2 can autoactivate and therefore it can initiate the complement cascade independently from any other proteases (Vorup-Jensen et al. 2000). The view that MASP-2 alone is sufficient for complement activation was the "central dogma" of the lectin pathway for about a decade and degraded the status of MASP-1 as being a supporting enzyme only. Finally, the picture became even more complicated when the third MASP enzyme (MASP-3) was discovered (Dahl et al. 2001). MASP-3 binds to collectins and ficolins but it

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does not cleave any downstream complement components (i.e. C4, C2, C3); it seemed that its proteolytic activity does not contribute to complement activation. Interestingly, no physiological inhibitor of MASP-3 has been identified.

Nevertheless, the domain structures of C1r, C1s, MASP-1, MASP-2 and MASP-3 are identical: the serine protease (SP) domain is preceded by five non-catalytic domains in the order CUB1-EGF-CUB2-CCP1-CCP2-SP (Fig. 1). This fact and other data indicate that these proteases are evolutionary closely related (Gál et al. 2007; Fujita et al. 2004). In which the MASPs differ from C1r and C1s is that truncated forms of MASPs have been identified in the circulation. MAp 19 (aka MAP-2, sMAP) is an alternative splice product of the *MASP2* gene containing the CUB1-EGF domain pair plus four unique C-terminal residues (Stover et al. 1999; Takahashi et al. 1999). Similarly, MAp 44 (aka MAP-1) is an alternative splice product of the *MASP1* gene consisting of the CUB1-EGF-CUB2-CCP1 domains and a 17 amino-acid-long peptide at the C-terminus (Degn et al. 2009; Skjoedt et al. 2010). MASP-3 is also an alternative splice product of the *MASP1* gene, the two proteases have identical non-catalytic domains (CUB1-EGF-CUB2-CCP1-CCP2) but they have individual SP domains. The *MASP1* gene is located on chromosome 3q27–28 in human. The level of the expression of the three distinct gene products is quite different depending on the site of the synthesis; MASP-1 is expressed mainly in the liver, MAp 44 is expressed almost exclusively in the heart, while the mRNA of MASP-3 can be detected in many tissues including liver, colon, heart, skeletal muscles, prostate, lung, and cervix. The human *MASP2* gene was mapped to chromosome 1p36.2–3 and the mRNAs of the *MASP2* gene products were detected almost exclusively in the liver. In the recent years, many functions of the different MASPs have been discovered. Most functions are connected to the activation of the complement system, but some functions reach far beyond the traditional complement cascade, and they have not yet been clarified entirely.

The Role of MASPs in Lectin Pathway Activation

The activation of the lectin pathway results in the generation of the classical/lectin pathway C3 convertase complex: C4b2a. The only enzyme, which is capable of cleaving C4 in the lectin pathway is MASP-2. The catalytic efficiency of C4 cleavage has been determined by independent research groups using enzymes from different sources (Ambrus et al. 2003; Rossi et al. 2001; Chen and Wallis 2004) (Table 1). These data show that MASP-2 is a very efficient enzyme at C4 cleavage (the k_{cat}/K_m values are in the 10^5–10^7 M^{-1} s^{-1} range), it is even more efficient than C1s. Kinetic and structural data prove that the CCP domains contribute to the efficient C4 cleavage through providing exosite for the substrate binding (Ambrus et al. 2003; Duncan et al. 2012; Kidmose et al. 2012). Experiments using chimeric molecules containing domains of C1s and MASP-2 proved that the CCP domains of MASP-2 are responsible for the superior C4 cleavage due to their high substrate recognition efficacy (Rossi et al. 2005). The high catalytic power of MASP-2

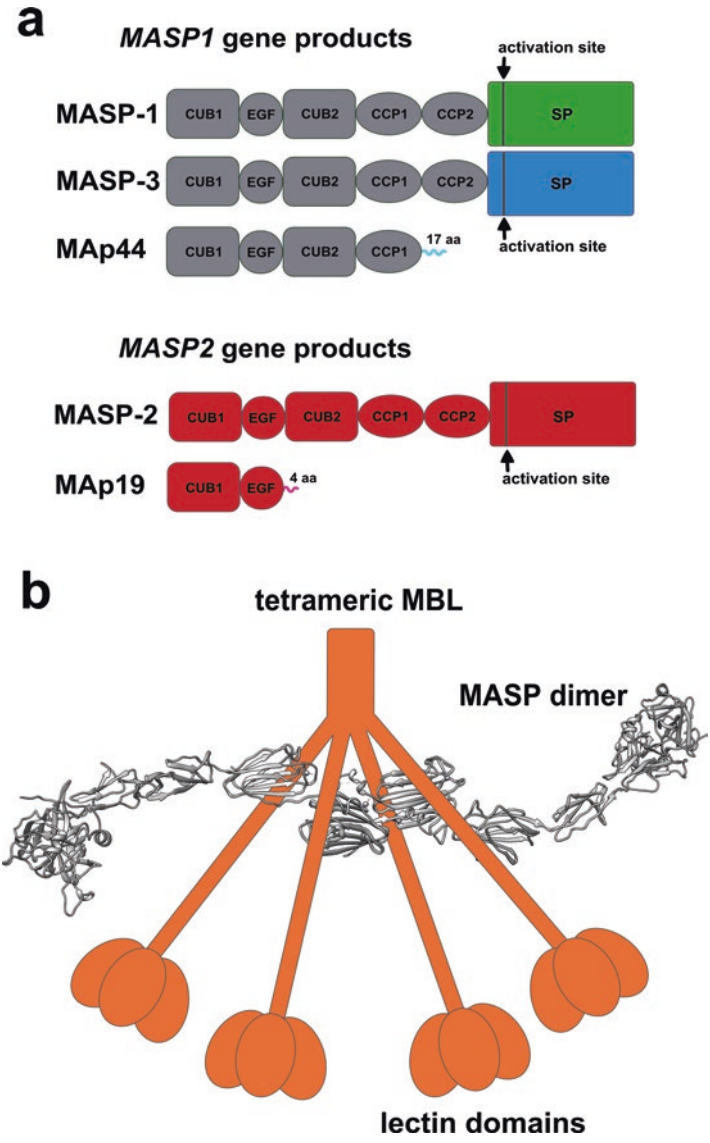

Fig. 1 Domain structure of MASPs and the structure of the MBL-MASP complex. (**a**) MASP-1, MASP-2 and MASP-3 share the same domain organization. The N-terminal CUB (**C**1r/C1s, sea urchin **U**egf and **b**one morphogenetic protein-1) domain is followed by an EGF (epidermal growth factor)-like domain and a second CUB domain. The catalytic region of these proteases consists of two CCP (complement control protein) domains and an SP (serine protease) domain. The one-chain zymogen form is converted to the active form by limited proteolysis at the activation site (arrow). (**b**) Schematic representation of an MBL-MASP complex consisting of a tetrameric MBL and a MASP dimer. Two MASP molecules form a dimer through the CUB1-EGF-CUB2 region and bind to the collagenous stalks of the MBL molecule. The catalytic regions (CCCP1-CCP2-SP) of the MASP protomers are protruding from the complex making possible the cleavage of the protein substrates (C2, C4)

Table 1 Specificity constants for proteolytic cleavage of C2, C4 and C3 by C1s and MASPs

		k_{cat}/K_m (M^{-1} s^{-1})			
Enzyme	Source	C2	C4	C3	References
C1s	Human plasma	4.3×10^5	2.45×10^6	ND[a]	Rossi et al. (2001)
MASP-2cf[b]	Insect cells	1.12×10^6	5.67×10^7	ND[a]	Rossi et al. (2001)
MASP-2	Insect cells	7.5×10^5	2.97×10^7	ND[a]	Rossi et al. (2001)
MASP-2cf[b]	*E. coli*	5.0×10^5	5.5×10^5	3.5×10^2	Ambrus et al. (2003)
MASP-2 CCP2-SP	*E. coli*	1.3×10^6	5.7×10^6	ND[a]	Ambrus et al. (2003)
rat MASP-2K[c]	CHO cells	1.3×10^6	2.8×10^7	ND[a]	Chen and Wallis (2004)
MASP-1cf[b]	*E. coli*	3.0×10^5	NS[d]	3.0×10^2	Ambrus et al. (2003)
rat MASP-1ent[e]	CHO cells	3.4×10^5	–	ND[a]	Chen and Wallis (2004)

[a]*ND* not determined
[b]*MASP-1/2cf* catalytic fragment containing the three C-terminal domains (CCP1-CCP2-SP)
[c]*MASP-2K* catalytically active MASP-2 mutant, in which the Arg^{424} was replaced with a Lys at the activation site
[d]*NS* not significant
[e]*MASP-1ent* catalytically active MASP-1 mutant, in which the ^{425}Lys-His-Ile-Ser-Arg sequence at the activation site was replaced with the recognition sequence of the enterokinase (Asp-Asp-Asp-Asp-Lys)

enables this enzyme to promote efficient lectin pathway activation in spite of its relatively low serum concentration (about 6 nM, 0.4 μg/mL) (Thiel et al. 2012). MASP-2 cleaves C2 with approximately the same rate as C1s. MASP-1 also cleaves C2, although with slightly lower efficiency than C1s and MASP-2. Taking into account the high serum concentration of MASP-1 (about 143 nM, 11 μg/mL) we can presume, that MASP-1 contributes significantly to the formation of the C3 convertase through C2 cleavage. Indeed, it was shown that MASP-1 generates about 60% of C2a present in the lectin pathway C3 convertases (Héja et al. 2012). Since MASP-1 does not cleave C4, it cannot generate C3 convertase complex alone. In *in vitro* experiments it was shown that complexes reconstituted from recombinant MASP-2 and MBL bind to mannan-coated surface and this binding induced the autoactivation of zymogen MASP-2 (Vorup-Jensen et al. 2000). The autoactivation ability of MASP-2 was verified by other laboratories, although the autoactivation capacity of MASP-2 is much less pronounced than that of MASP-1 (Rossi et al. 2001; Chen and Wallis 2004; Gál et al. 2005). Autoactivation of MASP-2 requires much higher concentration than the physiological one and a long incubation at 37 °C. MASP-1 zymogen, however, autoactivates about 3200-times more efficiently than MASP-2 zymogen indicating that MASP-1 is the first enzyme that autoactivates in the course of lectin pathway activation (Megyeri et al. 2013).

The first evidence indicating that MASP-1 plays a pivotal role in the activation of the lectin pathway came from the experiments using serum of MASP1/3$^{-/-}$ mouse (Takahashi et al. 2008). Although the authors claimed that there was a low level lectin pathway activation in this serum, their results showed practically no C4 deposition on mannan-coated surface. Since MASP-2 is the only enzyme in the lectin pathway which can cleave C4, it indicates that there is no active MASP-2 in

the absence of MASP-1. Addition of recombinant MASP-1 restored the lectin pathway activity in the serum of MASP1/3$^{-/-}$ mouse. Finally, using selective inhibitors against MASP-1 and MASP-2 unambiguously proved the central role of MASP-1 in the lectin pathway activation (Héja et al. 2012). SGMI-1, the highly specific MASP-1 inhibitor completely inhibited the lectin pathway in normal human serum. However, when the MASP-1 and MASP-2 proteases were pre-activated before the C4 deposition assay, only the MASP-2 inhibitor (SGMI-2) was efficient at attenuating the lectin pathway. These results clearly show that MASP-1 activates MASP-2 in the course of lectin pathway activation. Although MASP-2 can autoactivate *in vitro* under artificial conditions, this ability does not manifest under physiological conditions in normal human serum. It could be the consequence of its low serum concentration (the autoactivation of MASP-2 requires high enzyme concentration) and the fact that each MASP-2 is surrounded by multiple MASP-1 molecules on the activation surface (the molar ratio of MASP-1 to MASP-2 is approximately 24 to 1 in normal human serum). According to the current model of lectin pathway activation the first enzymatic step is the autoactivation of MASP-1 (Fig. 2). Activated MASP-1 then cleaves MASP-2 which in turn cleaves C4 into C4a and C4b. Deposited C4b binds C2 and the C4b-bound C2 is cleaved by both MASP-1 and MASP-2. This mechanism was corroborated by using serum of a 3MC syndrome patient whose blood lacks both MASP-1 and MASP-3 due to a mutation in the *MASP1* gene (Degn et al. 2012). The serum of this patient does not show lectin pathway activity (there is no C4 deposition), although it contains normal level of MASP-2. The lectin pathway activity could be restored by adding recombinant MASP-1, confirming that MASP-1 is indispensable to activate MASP-2 under physiological conditions. This mechanism was further confirmed by determining

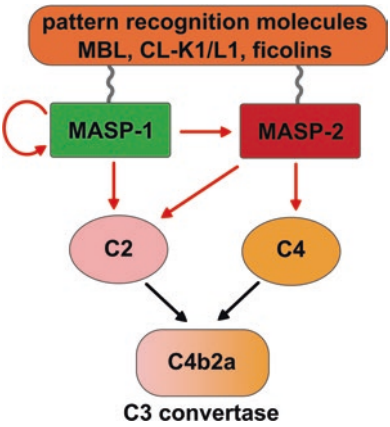

Fig. 2 Mechanism of lectin pathway activation. When the initiation complexes bind to the activator surface, MASP-1 autoactivates and then it activates neighboring MASP-2 molecules. MASP-2 cleaves C4, while C2 is cleaved by both MASP-1 and MASP-2. Red arrows represent proteolytic cleavage pointing from the enzyme to the substrate, while black arrows simply indicate conversion

the rate constants of the autoactivation and cross-activation reactions between MASP-1 and MASP-2 (Megyeri et al. 2013). The autoactivation capacity of zymogen MASP-1 ($k_{cat}/K_m = 4.5 \times 10^2\ M^{-1}\ s^{-1}$) is three-orders of magnitude higher than that of the zymogen MASP-2 ($0.14\ M^{-1}\ s^{-1}$). Activated MASP-1 cleaves zymogen MASP-2 very efficiently ($1.2 \times 10^4\ M^{-1}\ s^{-1}$); actually its rate constant is two orders of magnitude higher than that of the autocatalytic activation of MASP-2 ($6.0 \times 10^2\ M^{-1}\ s^{-1}$) where active MASP-2 cleaves zymogen MASP-2. The rate constants quantitatively highlight the central role of MASP-1 in the initiation of the lectin pathway.

The previously presumed analogy between the proteases of the classical and the lectin pathway has revived, although this analogy is far from perfect. C1r and C1s are present in equimolar concentration in the serum, while MASP-1 is much more abundant than MASP-2. C1r and C1s are present in the same complex with a well-defined stoichiometry as a tetramer ($C1qC1r_2C1s_2$), while MASP-1 and MASP-2 form dimers and mostly occupy separate complexes (e.g. distinct MBL-$(MASP\text{-}1)_2$ and MBL-$(MASP\text{-}2)_2$ complexes) (Dahl et al. 2001; Teillet et al. 2005; Mayilyan et al. 2006). C1r activates C1s inside the C1 complex, while MASP-1 activates MASP-2 probably on the activation surface where the distinct complexes bind next to each other (Degn et al. 2014). Another significant difference is that the function of C1r confines to the activation of C1s, whereas MASP-1 cleaves C2 beside MASP-2 during lectin pathway activation. The presence of the non-enzymatic components (MAp 19 and MAp 44) in the activation complexes of the lectin pathway may provide a regulation that does not exist in the classical pathway.

The above mechanism is valid for the human system. In other species, the enzymatic properties of MASPs can be different. In chicken, for example, MASP-1 is missing but there is a MASP-2-driven lectin pathway (Lynch et al. 2005). It seems very likely that the autoactivation capacity of chicken MASP-2 is much higher than that of human MASP-2, and the activation of chicken MASP-2 does not depend on the activation of other protease. In a mouse model of ischemic brain injury, it was suggested that a MASP-2-driven, MASP-1-independent C4-bypass activation route is responsible for the brain tissue damage (Orsini et al. 2016). The MASP-$2^{-/-}$ mice exhibited significantly reduced infarct volumes compared to the wild type mice, while the MASP-$1/3^{-/-}$ mice were not protected from the ischemia-reperfusion injury (IRI). It cannot be excluded that *in vivo*, during cerebral IRI MASP-2 activates on its own in the MASP-$1/3^{-/-}$ mice, however, *in vitro* experiments with MASP-1 deficient mouse sera do not support this. In the MASP-1 deficient sera no C4 deposition could be detected on mannan- or acetylated BSA-coated surface, although active MASP-2 is an extremely efficient protease at C4 cleavage (Machida et al. 2018). Recently, genome editing by CRISPR/Cas9 system was used to generate mice that mono-specifically lacked MASP-1 in the circulation while the expression of MASP-3 was intact (Hayashi et al. 2019). The sera of these mice failed to elicit C4 deposition on mannan-coated plates, corroborating that MASP-1 is essential for lectin pathway activation. Even if we suppose that the marginal autoactivation ability of MASP-2 may play a role in certain pathophysiological situations, in the sera of wild type mice, where the abundant MASP-1 molecules surround the

MASP-2 molecules on an activation surface, the inhibition of MASP-1 could suppress the possible autoactivation of MASP-2. The physiological role of MASP-1 can only be assessed in wild type mice by selectively inhibiting MASP-1.

Role of the MASPs in the Alternative Pathway Activation

The three activation pathways of the complement system (the classical, the lectin and the alternative pathways) have been considered as independent ones, since they are triggered by different activation surfaces and they use distinct serine protease components. They converge at the cleavage of C3; and from this point, the complement activation proceeds through the unified terminal pathway generating the membrane attack complex (C5b-9). Several interconnections between the different pathways have already been known for a long time. For example, the classical and the lectin pathway generate the same C3 convertase complex by cleaving the same components (C4 and C2), and the alternative pathway acts as an amplifying loop for the classical and the lectin pathways. The alternative pathway ensures an efficient complement activation regardless of the initiation pathway which generates the first C3b molecules (Harboe et al. 2009). It was also shown that the analog components of the classical/lectin and alternative pathway C3 convertases (e.g. C4b, C3b and C2a, Bb) can substitute each-other to a certain degree in *in vitro* experiments (Laich and Sim 2001). In the recent years, however, unexpected novel connections between the alternative and lectin pathways have been discovered, which fundamentally changed our view about the "independent" activation routes.

As we mentioned above, in the serum of the MASP-1/3 knockout mice there is no functional lectin pathway since MASP-2 remains in its zymogen form. It turned out, however, that the alternative pathway activity is also missing (or at least greatly reduced) in these sera: it cannot lyse rabbit erythrocytes and there is no C3 deposition on zymosan-coated plates in Mg^{2+}-EGTA buffer (Takahashi et al. 2010). The reason of this surprising phenomenon is that factor D (FD), the protease which is responsible for cleaving C3b-bound factor B (FB) is also in its zymogen form (pro-FD) in the serum of MASP1/3$^{-/-}$ mice. Previously the general consensus was that pro-FD is activated at the site of its synthesis (in the adipocytes) and it is secreted as a processed enzyme into the blood. Indeed, in the serum only cleaved FD was detected previously. The fact that in the sera of the MASP-1/3 knockout mice pro-FD is not processed suggests that MASP-1 and/or MASP-3 are involved in the processing of pro-FD. The first assumption was that MASP-1 is responsible for the cleavage, since *in vitro*, using purified components, MASP-1 can activate pro-FD. This assumption seemed to be reasonable, because several lines of structural and functional evidence indicated that MASP-1 has a more relaxed substrate specificity than the other complement proteases, which have typically one or two substrates (Dobó et al. 2009). Addition of recombinant MASP-1 to the serum of the knockout animals, however, failed to restore the alternative pathway activity. Another study suggested that MASP-3 is the protease that processes pro-FD and even zymogen

MASP-3 is effective (Iwaki et al. 2011). The picture became more controversial when it was shown that in the serum of a 3MC syndrome patient, where the lectin pathway was non-functional, significant alternative pathway activity could still be detected (Degn et al. 2012).

In order to clarify the role of the MASPs in the pro-FD activation we checked the catalytic efficiency of the three MASPs at the cleavage reaction using purified recombinant enzymes (Oroszlán et al. 2016). The activated form of all three MASPs cleaved pro-FD quite efficiently; the k_{cat}/K_M values were at the 10^3 M^{-1} s^{-1} range: MASP-1, 3.9×10^3; MASP-2, 7.2×10^3; MASP-3, 4.7×10^3. The zymogen form of MASPs, however, did not show significant activity. In the next experiment we added fluorescently labeled pro-FD to human serum and plasma and followed the conversion. Pro-FD was processed in plasma with a half life of ~5 h and in the serum with a half life of ~3 h. The fact that in EDTA plasma pro-FD is cleaved, unequivocally proves that there is a protease in the blood that is able to convert pro-FD into FD prior to the activation of the complement or the coagulation system. Addition of active MASP-1 and MASP-2 did not change the half life of pro-FD. It is very likely that the serpins, especially C1-inhibitor, immediately inactivated these proteases in the plasma. On the other hand, active MASP-3 (200 nM) was very effective: it reduced the half life of pro-FD from ~5 h to ~20 min. Obviously, MASP-3 preserved its activity which is in agreement with the fact, that there is no known inhibitor of this protease in the blood. The experiments using the selective inhibitors also confirmed the key role of MASP-3 in the pro-FD activation; SGMI-1 and SGMI-2 (MASP-1- and MASP-2-selective inhibitors) were inefficient while the MASP-3-selective inhibitor (TFMI-3) blocked the pro-FD conversion completely in plasma and partially in serum (Dobó et al. 2016).

We can conclude that MASP-3 is the exclusive activator of pro-FD in plasma or in "resting" blood, where none of the proteolytic cascade systems are triggered (Fig. 3). This mechanism ensures that nascent pro-FD is permanently activated in the blood even before the appearance of any danger signal. In MASP-3 deficient patients complement and coagulation proteases can serve as back up enzymes providing active FD locally, at the site of infection or injury. It was also shown, that in contrast to the previous assumptions, normal human plasma also contains some pro-FD besides the predominant FD (Wu et al. 2018). It is very unlikely though that MASP-3 can boost the alternative pathway during complement activation via cleaving the residual pro-FD at the activation surface, since only a small percentage of the normal FD level can support significant alternative pathway activity and there is no need to activate the entire pro-FD pool (Wu et al. 2018). The fact that only active MASP-3 can process pro-FD means that MASP-3 should be present in activated form in the circulation. Indeed, according to our measurements more than 70% of the MASP-3 molecules are activated in normal human plasma (Oroszlán et al. 2017). This means that zymogen MASP-3 is activated in the "resting" blood similarly to FD, although we do not know the mechanism yet.

Recently, a novel and unexpected link has been discovered between the lectin and the alternative pathway. We found that the proteolytic activity of MASP-1 is necessary for the efficient alternative pathway activation on LPS-covered surface

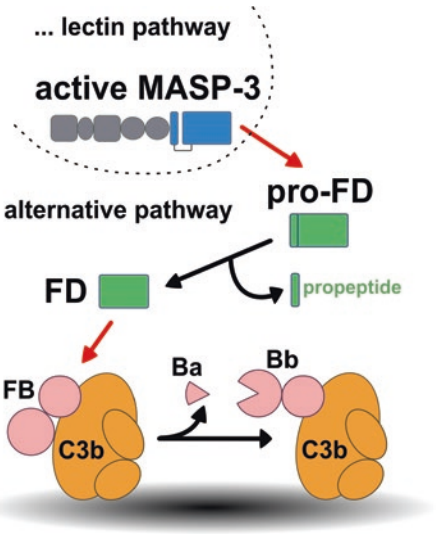

Fig. 3 The role of MASP-3 in the alternative pathway activation. MASP-3 is the exclusive activator of pro-FD in resting human blood. MASP-3 is present predominantly in its active form in the blood, and active MASP-3 cleaves and activates pro-FD. MASP-3 continually activates nascent pro-FD without the prior activation of the complement or the coagulation cascades. Red arrows represent proteolytic cleavage pointing from the enzyme to the substrate, while black arrows indicate conversion

while it has only little effect on zymosan-covered activation surface (Paréj et al. 2018). The MASP-1 specific inhibitor (SGMI-1) significantly reduced the C3 deposition on surfaces covered by LPS of different Gram-negative bacterial strains (e.g. E. coli, S. typhimurium, P. aeruginosa), while the effect was much less pronounced on yeast zymosan and it was negligible at lysing rabbit erythrocytes. A MASP-1 specific polyclonal antibody that was a strong inhibitor of the lectin pathway, also inhibited the LPS-driven alternative pathway, but barely affected the zymosan-driven alternative pathway activation. Depletion of MASP-1 from the normal human serum also confirmed the essential role of MASP-1 in the alternative pathway activation on activators representing the surfaces of Gram-negative bacteria. It seems obvious that MASP-1 directly (cleaving a complement component) or indirectly (activating a protease that cleaves a complement component, or inactivating an inhibitor of the alternative pathway) contributes to alternative pathway activation. We showed that MASP-1 does not cleave C3b-bound FB (does not activate the pro-convertase) and it is not the physiological activator of pro-FD. The MASP-1 specific inhibitor does not inhibit FD or the C3 convertase (C3bBb). On the other hand C3 is cleaved by both MASP-1 and MASP-2; although with low efficiency (Table 1). Interestingly, MASP-1 and MASP-2 cleaved C3i (C3 with a cleaved thioester bond) with 10–20 fold higher efficiency compared to the proteolysis of intact C3, but the physiological relevance of this reaction is unclear (Ambrus et al. 2003). It cannot be ruled out that the low C3 cleaving activity of

MASP-1 contributes to the initiation of the alternative pathway. We have shown, however, that the MASP-1 specific inhibitor attenuates the alternative pathway activation also on surfaces which are partially covered by C3b, suggesting that MASP-1 contributes to the amplification phase of the alternative pathway activation, as well. Taken together it is likely that the protein, which is cleaved by MASP-1 during the alternative pathway activation, is not among the core components of the alternative pathway.

The surface dependence of the MASP-1 action is also an intriguing question. Similar phenomenon was observed earlier using serum from properdin KO mice (Kimura et al. 2008). Properdin, which is a positive regulator of the alternative pathway, was essential for LPS-induced alternative pathway activation, while it was not necessary for zymosan-induced alternative pathway activation. It seems that zymosan, as a strong activator of the alternative pathway, does not need the contribution of properdin and MASP-1, while LPS as a weak activator does. It is very likely that the efficiency of the alternative pathway activation on a given surface depends on the ratio of the various activators and inhibitors present. In this respect it is worth mentioning that factor H (FH), the master regulator of the alternative pathway, binds to LPS more strongly than to zymosan. We can speculate that MASP-1 and properdin may counteract the action of FH on the LPS-covered surfaces.

Another study also supports the role of MASP-1 in the alternative pathway activation on bacterial surfaces. Serum fractions containing MBL-MASP-1 complexes were able to elicit C3b deposition on LPS-covered surface in the absence of C2, whereas complexes containing MASP-2 or MASP-3 failed to do so (Selander et al. 2006). Our experiments also demonstrated that inhibition of MASP-1 in C2-depleted serum attenuated C3b deposition on the surface of Gram-negative bacteria. Taken together, a large body of evidence shows that MASP-1 contributes to alternative pathway activation through a yet unknown mechanism. These findings further emphasize the role of MASP-1 in the antibody-independent defense against Gram-negative bacterial infection.

Cross-Talk Between the Lectin Pathway and Coagulation

The complement system is a proteolytic cascade system that is closely related to the other blood-borne cascade systems (i.e. contact, coagulation and fibrinolytic systems) (Krem and Di Cera 2002). In fact the serine proteases of the blood, all belonging to the chymotrypsin family (Family S1, MEROPS), form a network of proteases that can be divided into distinct cascade systems primarily for practical and didactical reasons only. We should keep in mind, however, that there are several interactions between these cascade systems, and the activation of one cascade system usually influences the activation of the others. (Markiewski et al. 2007; Conway 2015). For example, the complement and the coagulation systems are activated simultaneously at the site of the injury, where pathogen microorganisms

enter into the bloodstream and many interactions have been described between the two proteolytic cascade systems. Recently, a large body of evidences has accumulated indicating that the proteases of the lectin pathway, especially MASP-1, form important links between coagulation and complement (Dobó et al. 2014). It was shown that MBL-null mouse and MASP-1/-3 KO mouse both have prolonged bleeding times *in vivo* (Takahashi et al. 2011). Moreover, in disease models, it was demonstrated that these mice have significantly decreased $FeCl_3$-induced occlusive thrombogenesis (La Bonte et al. 2012; Pavlov et al. 2015).

MASP-1 is an atypical complement serine protease, since it has more substrates than other complement proteases (e.g. C1r, C1s, MASP-2). MASP-1 has been shown to influence the coagulation in several ways (Fig. 4). MASP-1 cleaves fibrinogen and factor XIII (plasma transglutaminase) promoting cross-linked fibrin formation (Hajela et al. 2002; Hess et al. 2012). MASP-1 cleaves the fibrinogen β-chain similarly to thrombin releasing the proinflammatory peptide fibrinopeptide B (Krarup et al. 2008). The fibrinogen α-chain, however, is cleaved differently by the two proteases. Factor XIII is also cleaved and activated by MASP-1, although the catalytic efficiency is lower compared to thrombin cleavage; the thrombin turnover rate of this substrate is about 650 times faster than that of MASP-1 under

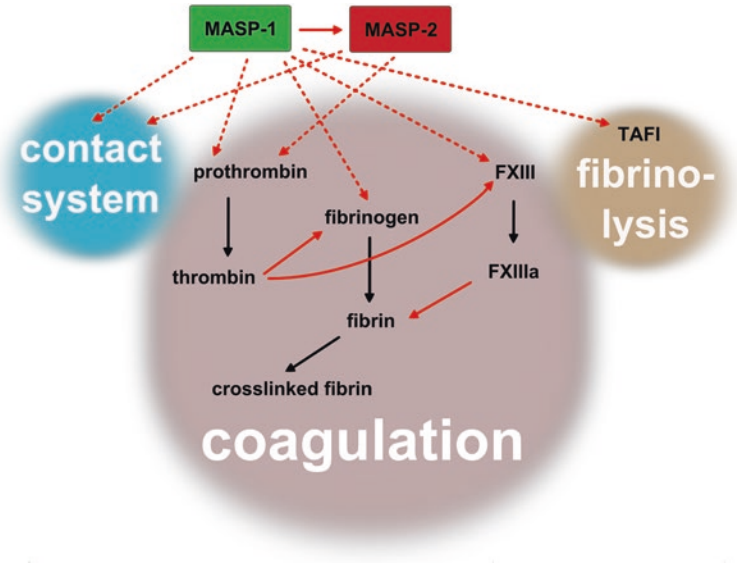

Fig. 4 Cross-talk between the lectin pathway and other blood-borne cascade systems. *In vitro* MASP-1 and MASP-2 can cleave certain components of the coagulation, contact and the fibrinolytic systems. The contribution of MASP-1 to the coagulation and thrombus formation was also corroborated *in vivo* by using mouse models. MASP-1 has thrombin-like activity and it was also shown to activate prothrombin. Red arrows indicate proteolytic cleavage pointing from the enzyme to the substrate, while black arrows indicate conversion. Dashed red arrows indicate cleavage reactions that were demonstrated *in vitro* but their *in vivo* relevance needs further confirmation

physiological circumstances. An interesting feature of the MASP-1 cleavage is that it prefers the Val34 (P4 residue) polymorphic variant of factor XIII to the Leu34 variant, while thrombin shows the opposite preference (Hess et al. 2012). It is speculated that this preference might be disadvantages since the activation of the Val34 variant may increase the probability of the development of unwanted thrombotic events. MASP-1 also cleaves thrombin-activatable fibrinolysis inhibitor (TAFI). Besides being an inhibitor of fibrinolysis, TAFI has direct impact on complement activation, since it is a carboxypeptidase (carboxypeptidase B), and it inactivates the anaphylatoxins (C3a, C5a) very efficiently (Nishimura et al. 2007). MASP-1 can also facilitate fibrin formation in an indirect way, via activating prothrombin. It was shown that addition of MASP-1 to whole blood and platelet-poor plasma samples accelerated the clotting process in a prothrombin-dependent manner (Jenny et al. 2015a). Using purified proteins, it was also demonstrated that MASP-1 cleaves and activates prothrombin in a unique fashion (different from the cleavage pattern of factor Xa and thrombin) generating several active alternative thrombin species (Jenny et al. 2015b). The coagulation promoting effect of MASP-1 was also demonstrated using a microvascular, whole blood flow model, which simulates blood flow through microchannels cultured with endothelial cells representing a situation close to human physiology (Jenny et al. 2018). Besides MASP-1, MASP-2 was also shown to activate prothrombin and to elicit fibrin deposition on the surface of the bacterium *S. aureus* (Krarup et al. 2007). Since MASP-1 is much more abundant than MASP-2, and MASP-2 is activated by MASP-1, it is very likely that MASP-1 plays the major role in the procoagulant activity of the lectin pathway. This procoagulant activity may represent an ancient form of innate immunity. The fibrin clot formation can prevent the spread of the microorganism in the body and facilitate its removal by phagocytosis.

A recently discovered intriguing aspect of the interaction between the complement and the coagulation cascades is that the platelet activation and fibrin formation resulted in the activation of MASP-1 and MASP-2 (Kozarcanin et al. 2016). Both activated platelets and fibrin clots bind lectin pathway components and activate the serine proteases, MASP-1 and-2, which can act as a positive feedback loop to boost the coagulation. This observation further supports the view that the lectin pathway is involved in thrombotic events. Although the exact mechanism has yet to be determined, it seems likely that platelets and fibrin clots activate the lectin pathway differently. The activated platelets generate mainly MASP/C1-inhibitor complexes while on the cross-linked fibrin predominantly MASP/antithrombin complexes can be detected. Taken together, we can conclude that the evolutionary related cascade systems act in a concerted way to eliminate dangerous particles and to restore homeostasis, however the concurrent overactivation of the complement and coagulation systems can lead to the development of disease conditions such as ischemia reperfusion injury, thromboinflammation and atherosclerosis (Ekdahl et al. 2016).

Endothelial Cell Activation by MASP-1

One of the important proinflammatory functions of the complement system is the activation of leukocytes and endothelial cells. The anaphylatoxins (C3a and C5a), soluble proteolytic products of the complement cascade activation, bind to their cognate receptors (C3aR, C5aR1, C5aR2). These receptors are G-protein coupled receptors (GPCRs) and elicit cell activation through increasing the intracellular calcium concentration and triggering signal transduction events mediated by protein kinases. Since MASP-1 and MASP-2 drive the activation of the lectin pathway, these proteases are indirectly responsible for cell activation. During coagulation, the split products of certain coagulation components (e.g. fibrinopeptides) trigger similar cell activation events. Thrombin, the executive protease of the coagulation, however, is able to activate endothelial cells and platelets directly, via the cleavage of protease activated receptors (PARs) on the cell surface (Coughlin 2000). The PARs are also GPCRs, which carry their own ligands (tethered ligand) on the N-terminal end of the polypeptide chain. These ligands become exposed after a protease cleaves in the N-terminal region and unmask a new N-terminus. Cleavage of PAR1 and PAR4 results in morphological changes in endothelial cells and trigger the release of cytokines and other vasoactive substances. Recently, it has been shown that MASP-1 is also able to cleave PARs and stimulate endothelial cells (Megyeri et al. 2009, 2014) (Fig. 5). Using peptide substrates representing the N-terminal regions of the PARs, the kinetic constants of the cleavage reactions have

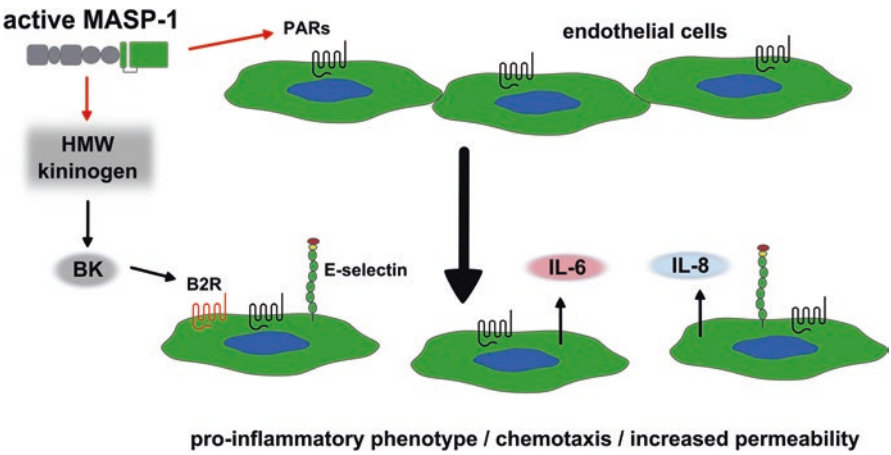

Fig. 5 The role of MASP-1 in endothelial cell activation. MASP-1 can activate endothelial cells by direct and indirect manner. MASP-1 cleaves PARs on the surface of the endothelial cells resulting in the release of chemotactic and pro-inflammatory cytokines, up-regulating E-selectin expression, and also changing endothelial permeability. MASP-1 also cleaves HK and liberates bradykinin. Bradykinin binds to the B2R receptor and induces pro-inflammatory changes in the endothelial cells. Red arrows indicate proteolytic cleavage pointing from the enzyme to the substrate, while black arrows indicate conversion or cellular processes

been determined. MASP-1 cleaved PAR1, PAR2 and PAR4 peptides quite efficiently (k_{cat}/K_M values are 1.1×10^4, 1.5×10^4 and 1.8×10^5 M^{-1} s^{-1}, respectively). Although thrombin is more efficient protease at PAR1 cleavage (1.6×10^6 M^{-1} s^{-1}) than MASP-1, the PAR4 substrate is cleaved by the two proteases with similar efficiency (k_{cat}/K_M value for thrombin: 5.3×10^5 M^{-1} s^{-1}). In cultured human umbilical vein endothelial cells (HUVECs), MASP-1 treatment elicited typical intracellular calcium response, in a similar manner and extent as thrombin. MASP-1 also induced NF-κB nuclear translocation and initiated p38 MAPK signaling. It was also demonstrated that MASP-1 cleaves PAR4 on the surface of HUVECs. These findings unequivocally proved that MASP-1 is able to cleave PARs and trigger pro-inflammatory reactions in the endothelial cells. Other studies corroborated the PAR cleavage-mediated pro-inflammatory role of MASP-1 in endothelial cells. Activation of various signal transduction pathways (e.g. p38 MAPK, pCREB, NFκB, JNK) resulted in cytokine production and altered adhesion molecule pattern in HUVECs (Jani et al. 2014, 2016). MASP-1 induced IL-6 and IL-8 production and it increased the expression of E-selectin and decreased that of ICAM-2. The supernatant of MASP-1-stimulated cells served as chemoattractant for neutrophil granulocytes and the altered adhesion molecule pattern of HUVECs resulted in increased adherence of differentiated PLB-985 cells (neutrophil granulocyte model cells). The neutrophil granulocytes are the predominant contributors to the early cellular response against invading pathogenic microorganisms. MASP-1 forms a link between the humoral and the cellular innate immune response in two ways: in a direct way through activating endothelial cells by PAR cleavage, and in an indirect way through recruiting neutrophil granulocytes by the secreted cytokines and increasing their adhesion to the endothelial cells. A very recent report has demonstrated that MASP-1 dose-dependently increased endothelial permeability (Debreczeni et al. 2019). This effect is mediated by PAR1 and Rho-kinase, and may foster the elimination of invading pathogens by facilitating the extravasation of soluble and cellular components of the immune system to the site of the infection.

A microarray-based transcriptome analysis of inflammation-related gene expression also confirmed the direct proinflammatory effect of MASP-1 on endothelial cells (Schwaner et al. 2017). Analysis of a set consisting of 884 inflammation-related genes showed that the proteolytic activity of MASP-1 changed the expression of 30 genes. The effect of MASP-1 on the gene expression was found to be a rapid process it occurred in the first 2 h of activation. The decisive role of the p38 MAPK and NFκB pathways in the MASP-1-induced pro-inflammatory process was also verified.

Another study demonstrated that MASP-1 can activate hepatic stellate cells (HSCs) and accelerate fibrosis progression in hepatitis C virus liver disease (Saeed et al. 2013). Although the mechanism of this activation has not been deciphered, it is likely that the cleavage of PARs plays a role, since the PARs are expressed at the protein level on quiescent stellate cells, and thrombin also exerts multiple actions on HSCs.

In order to identify other MASP-1 substrates among the serum proteins a proteomic approach was applied. 2D differential gel electrophoresis of MASP-1 treated

serum samples revealed that high-molecular-weight kininogen (HK) is a potential substrate of MASP-1 (Dobó et al. 2011). Using purified HK and MASP-1 we proved that MASP-1 cleaves HK and liberates the vasoactive peptide bradykinin (Fig. 5). Moreover, another study showed that MASP-1 treatment significantly up-regulated the expression of bradykinin receptor B2R in endothelial cells (Debreczeni et al. 2019). Bradykinin is a pro-inflammatory peptide, which is released from kininogens primarily by the proteolytic action of kallikreins. In the blood, plasma kallikrein generates the majority of bradykinin by cleaving HK. Bradykinin is a strong activator of endothelial cells inducing vasodilatation, increasing vascular permeability and triggering the production of second-generation inflammatory mediators. In patients with hereditary angioedema (HAE), typicly caused by C1-inhibitor deficiency, the uncontrolled kallikrein-mediated release of bradykinin results in recurrent tissue swellings. However, we cannot exclude that other proteases can also contribute to this process. Since C1-inhibitor also plays a role in the regulation of MASP-1 activity, the bradykinin generating ability of MASP-1 might contribute to the initiation of HAE attacks and worsening the disease symptoms. MASP-2 also cleaves HK but it does not liberate bradykinin. Plasma kallikrein cleaves HK much more efficiently than MASP-1; the specificity constant of the kallikrein-mediated cleavage (1.5×10^5 M^{-1} s^{-1}) is 375-fold higher than that of MASP-1-mediated cleavage (4.0×10^2 M^{-1} s^{-1}). We should keep in mind, however, that during complement activation, the local concentration of active MASP-1 on a surface can be much higher than in the serum, consequently MASP-1 can cause local endothelial activation by cleaving PARs and HK during infection or oxidative stress. In case of C1-inhibitor deficiency, however, the effect of MASP-1 might be systemic, and it can contribute to the elevated bradykinin level in HAE patients.

Regulation of MASPs

The natural inhibitors of the mammalian blood proteolytic cascade enzymes are almost exclusively serpins. Serpins are suicide inhibitors that change their conformation during the inhibitory reaction and form irreversible covalent enzyme-inhibitor complexes with the target proteases. C1-inhibitor inhibits the proteases of the classical and the lectin pathway: C1r, C1s, MASP-1 and MASP-2 (Davis et al. 2010). It is also a physiological inhibitor of the contact system inhibiting factor XIIa, factor XIa and plasma kallikrein. Antithrombin, an important inhibitor of the coagulation pathway, also inhibits MASP-1 and MASP-2 (Fig. 6). Heparin (and other glycosaminoglycans) can facilitate the formation of the serpin-protease covalent complexes. We have shown that C1-inhibitor and antithrombin are equally efficient inhibitors of the lectin pathway in the presence of heparin (Paréj et al. 2013). The IC_{50} values, determining through C4 deposition in 100-fold diluted normal human serum, are 35.6 and 41.3 nM for the C1-inhibitor and the antithrombin, respectively. Since both MASP-1 and MASP-2 are essential for the lectin pathway activation, inhibition of either protease is enough for the attenuation of lectin

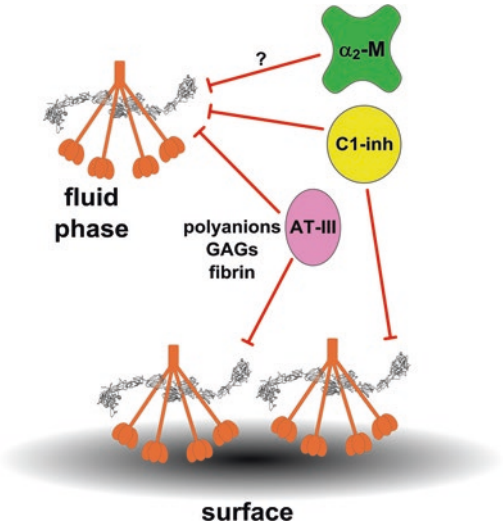

Fig. 6 Regulation of the lectin pathway. The major inhibitors of the lectin pathway are serpins. C1-inhibitor (C1-inh) is an efficient inhibitor of MASP-1 in the presence or absence of heparin. Antithrombin (AT-III) is even a better inhibitor, but only in the presence of heparin (or other glycosamino glycans (GAGs), polyanions, or fibrin). MASP-2 is very efficiently inhibited by C1-inhibitor, which is further enhanced in the presence of heparin. Antithrombin inhibits MASP-2 efficiently only in the presence of heparin. α_2-Macroglobulin (α_2M) is also a potential inhibitors of MASP-1 and MASP-2, but its inhibitory action has only been unequivocally proven in the fluid phase

pathway activation. Inhibition of both proteases at the same time confers an efficient prevention of lectin pathway activation. The second-order association rate constants (k_a) for the serpin-protease reactions were determined in the presence and absence of heparin. The MASP-1-C1-inhibitor reaction is not dependent on the heparin; the k_a values are approximately 6×10^3 M^{-1} s^{-1} in both cases (Dobó et al. 2009). The MASP-1-antithrombin reaction, however, is facilitated by heparin significantly; the k_a value increases from 1.4×10^3 M^{-1} s^{-1} to 4.0×10^4 M^{-1} s^{-1} in the presence of 50 μg/mL heparin. It seems that antithrombin in the presence of heparin is the best natural inhibitor of MASP-1. In the case of MASP-2, the effect of both serpins is heparin dependent. In the presence of heparin, the k_a for the MASP-2-C1-inhibitor reaction increases by an order of magnitude (from 1.6×10^5 M^{-1} s^{-1} to 1.3×10^6 M^{-1} s^{-1}), whereas the k_a for the MASP-2-antithormbin reaction increases even more, by two orders of magnitude (from 4.3×10^2 M^{-1} s^{-1} to 4.7×10^4 M^{-1} s^{-1}). The heparin concentration dependence of the rate of the MASP-2-antithombin reaction shows a bell-shaped curve, suggesting that heparin binds to both proteins and speeds up the formation of the Michaelis complex. The typical example of this "bridging" mechanism is the heparin potentiation of the thrombin-antithrombin reaction. In the case of the MASP-1-antithrombin reaction, however, the "bridging" mechanism does not apply; instead the concentration dependence (saturation curve) suggests an allosteric mechanism. The central role of the serpins in the regulation of the lectin

pathway is also indicated by the fact that significant levels of MASP-1-C1-inhibitor and MASP-1-antithrombin complexes were observed in resting normal human serum (Hansen et al. 2015). It may suggest, that a slow "tick-over" of the lectin pathway takes place in the blood, and the serpins prevent the overall activation of the system before danger signals emerge. On the other hand, it was shown that platelet activation and coagulation lead to the formation of MASP1-/2-serpin complexes, which could explain the presence of such complexes in the serum (Ekdahl et al. 2016).

Besides serpins, inhibitors with different mechanism of action may also contribute to the regulation of the MASPs. The canonical inhibitors are usually small, substrate-like proteins and make a tight but non-covalent complex with the target protease. Although the canonical inhibitors play a significant role in many physiological processes they have almost no influence on the blood cascade systems. The only exemption is tissue factor pathway inhibitor (TFPI), an important inhibitor of the extrinsic coagulation pathway, which was also shown to inhibit MASP-2 (Keizer et al. 2015). The IC_{50} value of TFPI for lectin pathway inhibition was 10 μM in 100-fold diluted serum. Taking into account that the plasma concentration of TFPI is 2.25 nM, it is unlikely that this weak inhibitory effect would play a considerable role in the regulation of lectin pathway activation.

Macroglobulins represent another type of protease inhibitors which are abundantly present in the blood. Like serpins, macroglobulins also undergo a major conformation change after the cleavage of the bait region by the target protease, and sequester the protease by forming a cage-like structure around it. A fraction of the protease molecules may form a covalent bond with the thioester domain of the macroglobulin, but this is not required for the inhibition: the cage will prevent the contact between the protease and its protein substrates. α_2-Macroglobulin (α_2-M) is an abundant inhibitor in the blood and may play a role in the regulation of the plasma cascade systems (Goulas et al. 2017). The published results concerning the lectin pathway regulation, however, are controversial (Fig. 6). Several reports showed that α_2-M forms complexes with MASP-1 and MASP-2. In fluid phase, α_2-M blocked the rMASP-1 mediated C2 cleavage, while it could not prevent C4 deposition on mannan-coated surface (Ambrus et al. 2003; Petersen et al. 2000). Another report claimed that α_2-M protected *Neisseria gonorrhoeae* from lectin pathway mediated killing, and α_2-M and C1-inhibitor acted synergistically to inhibit the lectin pathway on the surface of the bacteria (Gulati et al. 2002). We also demonstrated that α_2-M is able to sequester MASP-1 in the fluid phase, however, we were unable to detect any inhibitory effect on mannan-coated plates (Paréj et al. 2013). α_2-M is a huge tetrameric glycoprotein composed of 1451-residue-subunits. It is very likely that steric hindrance prevents α_2-M to make a contact with the protease in the MBL-MASP complexes immobilized on the activation surface. It is also possible that the rate of the α_2-M-MASP reaction is much slower than the rate of the C4 and C2 cleavage by the MASPs. It is plausible to presume that the role of α_2-M is not the immediate prevention of MASP-1 and MASP-2 proteolytic action during lectin pathway activation, but the slow removal of the activated proteases from the bloodstream. We should also remember that none of the above mentioned

inhibitors react with MASP-3. MASP-3 has no known physiological inhibitor. Like factor D, MASP-3 is regulated differently, maybe through its very narrow substrate specificity and through the self-inhibited structure of the processed protease. Further experiments need to prove these assumptions.

Conclusion and Perspectives

There is no doubt that MASPs are crucially important in developing an efficient innate immune response. MASP-1 and MASP-2 are indispensable for the activation of the lectin pathway, while MASP-3 is necessary for alternative pathway activation. A growing number of evidences suggest that MASP-1 also contributes to the alternative pathway in an activator-dependent manner. In addition to the complement activation, MASP-1 is also capable of activating endothelial cells directly, by cleaving PARs, and indirectly by liberating bradykinin from HK. The activated endothelial cells secret cytokines that attract neutrophil granulocytes, and their altered adhesion molecule pattern facilitates adhesion between neutrophils and endothelial cells. In this way MASP-1 forms a link between the humoral and cellular immune response. The cross-talk between the lectin pathway and the coagulation system has also been studied intensively in the recent years and the results point to the importance of the proteolytic activity of MASPs. Since MASPs are members of a larger proteolytic network, we can predict that further functions, beyond complement activation, will be discovered in the future. MASP-3 is an interesting candidate in this respect. It was shown that deficiency of MASP-3 results in the development of the 3MC syndrome, which manifests in serious developmental abnormalities (Rooryck et al. 2011; Sirmaci et al. 2010). It seems that MASP-3 plays a crucial role in embryogenesis, by cleaving a presently unknown substrate. Although it was reported earlier that MASP-3 can process insulin-like growth factor-binding protein 5 (IGFBP-5) (Cortesio and Jiang 2006), there is no evidence that it would be the key substrate in the developmental processes. Another interesting problem is the activation of MASP-3 in the blood. We know that MASP-3, like factor D, is present predominantly in the active form in the circulation, however, we do not know which protease is responsible for zymogen MASP-3 activation (Oroszlán et al. 2017). Several lines of evidence suggest that the activator protease is not a known member of the complement cascade, but it could rather be a more distant member of the blood's proteolytic network.

While the complement system is a key component of innate immunity, its uncontrolled, excess activation can cause self-tissue damage and contribute to the development of serious disease conditions, including ischemia reperfusion injury (myocardial infarction, stroke), age-related macular degeneration, and several neurodegenerative disorders (Dobó et al. 2018). Inhibiting the activity of the complement proteases is a therapeutic option in these diseases. Targeting MASP-1 or MASP-2 we can block the lectin pathway activation. MASP-2 seems to be a favorable target since its serum concentration is much lower, than that of MASP-1.

Both antibody and small-protein inhibitors have been developed and tested against MASP-2 (Schwaeble et al. 2011; Clark et al. 2018; Szakács et al. 2019). Long-term inhibition of MASP-3 can result in the attenuation of alternative pathway activity. MASP-1 is also a promising target, since it has multiple substrates connected to immune response and inflammation. It is very likely that new anti-complement drugs will be developed and applied in the clinical practice in the near future.

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