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MBL-Associated Serine Protease -1 (MASP-1) is a Significant Contributor to Coagulation in a Murine Model of Occlusive Thrombosis

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Abstract

Bleeding disorders and thrombotic complications constitute a major cause of death and disability worldwide. While it's known that the complement and coagulation systems interact, no studies have investigated the specific role or mechanisms of lectin-mediated coagulation *in vivo*. Ferric chloride ($FeCl_3$) treatment resulted in intra-arterial occlusive thrombogenesis within 10min in wild-type (WT) and C2/fB null mice. In contrast, MBL null and MASP-1/-3 KO mice had significantly decreased $FeCl_3$-induced thrombogenesis. Reconstitution with rhMBL restored $FeCl_3$-induced thrombogenesis in MBL null mice to levels comparable to WT mice, suggesting a significant role of the MBL-MASP complex for *in vivo* coagulation. Additionally, whole blood aggregation demonstrated increased MBL-MASP complex-dependent platelet aggregation. *In vitro*, MBL-MASP complexes were captured on mannan-coated plates and cleavage of a chromogenic thrombin substrate (S2238) was measured. We observed no significant differences in S2238 cleavage between WT, C2/fB null, MBL-$A^{-/-}$ or MBL-$C^{-/-}$ sera, however MBL null or MASP-1/-3 KO mouse sera demonstrated significantly decreased S2238 cleavage. Recombinant human (rh)MBL alone failed to cleave S2238, however cleavage was restored when rMASP-1 was added to either MASP-1/-3 KO sera or rhMBL. Taken together, these findings indicate that MBL-MASP complexes, and specifically MASP-1, play a key role in thrombus formation *in vitro* and *in vivo*.

Keywords

complement; coagulation; thrombosis; MBL; MASP-1; rodent

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INTRODUCTION

Occlusive thrombosis, resulting from atherosclerotic plaque rupture or restenosis plays an important role in the onset of two major causes of death in developed nations: myocardial infarction and ischemic stroke. Typically, acute thrombolytic therapy is the first step taken to reperfuse ischemic tissues downstream of the thrombus. In addition, most patients suffering from cardiovascular disease are prescribed chronic prophylactic thrombolytic drugs. Coagulation is a complex process and there is a delicate balance between bleeding disorders and clot formation. Considering the widespread and critical role of thrombosis in many diseases, significant research efforts have been aimed at the discovery and development of safe and effective antithrombotic (i.e., anti-platelet, anti-thrombin, etc.) drugs for acute and chronic clinical use.

The complement cascade is part of the innate immune system responsible for the initiation of inflammation and elimination of invading foreign cells. There are three independent pathways which can initiate the complement cascade: the classical, alternative, and lectin pathway. All three pathways converge at formation of the C3 convertase and follow a common pathway resulting in the formation of C5b-9 [the membrane attack complex (MAC)]. Lectin pathway activation is initiated via the binding of a multi-molecule complex to carbohydrate structures present on pathogens, or glycosylation patterns on apoptotic, necrotic, or ischemic cells(1–3). The MBL complex consists of MBL and three serine proteases, MASP-1, -2, and -3 (MBL-associated serine proteases 1, 2, and 3), and MAp19 and Map44/MAP-1, two nonenzymatic, truncated products of the *MASP2* gene, which may regulate lectin pathway activation by competing for the binding of MASPs to the carbohydrate recognition complexes(2–10). The system is slightly altered in mice, where two forms of MBL exist, MBL-A and MBL-C(2,7).

Coagulation, like complement, is a highly conserved cascade-style system composed of multiple factors (F) that are activated in a sequential and amplified process, ultimately resulting in the formation of an insoluble fibrin clot. The coagulation cascade is primarily responsible for maintaining hemostasis following vascular injury. Activation of either the intrinsic or extrinsic pathways which make up the coagulation cascade leads to the formation of a prothrombinase complex composed of FVa and FXa, cleaving prothrombin to thrombin. Thrombin formation is a critical central step in coagulation, cleaving fibrinogen, FXIII and activating platelets. Thrombin also plays important roles in the activation of protein C, an anti-coagulative protein with cellular protective actions (11,12).

For many years, it has been recognized that the complement and coagulation systems interact(13). Complement activation is known to contribute to thrombotic tissue injury in systemic lupus erythematosus(14), biomaterial-associated thrombosis(15), and paroxysmal nocturnal hemoglobinuria(16), to name just a few. Additionally, reversal of heparinization with protamine, TPA and streptokinase activate complement(17–19). An important study by Huber-Lang et al demonstrated that thrombin can directly activate C5 to produce C5a and C5b-9 in C3 deficient mice(20). Further, mannose-binding lectin associated protease (MASP)-2 can activate prothrombin to thrombin and may explain the mechanism by which thrombin is produced from prothrombin in C3 deficient mice(21). More recently, work from our group indicated that MBL-MASP complexes are associated with thrombin-like activity *in vitro* and found that MBL null mice have prolonged bleeding times *in vivo*(22).

Endothelial injury and platelet aggregation are key events in the pathogenesis of thrombus formation. Our experimental approach was based on the hypothesis that occlusive thrombosis can be initiated by $FeCl_3$-induced endothelial injury, likely mediated by oxidative radical formation and/or transported intraluminal ferric ion interaction with

platelets, fibrin and other formed blood elements(23). For *in vivo* analysis, the $FeCl_3$ method is the optimal technique as it exhibits many features useful for studying the molecular determinants of arterial thrombosis in transgenic mice, including uniform injury from animal to animal, and the duration of injury can be precisely controlled. In addition, whole blood aggregation has been extensively utilized to elucidate and characterize various signaling pathways and pharmaceuticals involved in platelet stimulation and activation(24,25). Thus, our repertoire of complement-deficient mice (all on a CH57BL/6 background) along with clinically relevant coagulation models allows us to effectively address the role of complement in coagulation.

Here, we report that mannose-binding lectin (MBL) complexes, and specifically MASP-1, have thrombin-like activity and are a significant regulator of thrombus formation both *in vitro* and *in vivo*. The results demonstrate that the innate immune system (complement in particular) has important interactions with the coagulation system hereinto not appreciated. It is proposed that this mechanism may represent a novel non-immunological role for the MBL-MASP complex in hemostasis and coagulopathy.

METHODS

Animals

All procedures were reviewed and approved by the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals. Wild-type (WT), mannose-binding lectin (MBL-A/-C) null, C2/factor B null (C2/fB null)(26), and MBL-associated serine protease-1/-3 knock out (MASP1/3 KO)(27) mice raised on a C57BL/6 background were used for the present study. Previous reports found clotting parameters (PT, aPTT, fibrinogen, and platelets) in MBL null mice are similar to WT mice, indicating that coagulation itself is not compromised in MBL deficiency(22).

Ferric chloride ($FeCl_3$) coagulation model

We used a mouse model of localized thrombus formation as previously described(22,23,28). After isoflurane anesthesia, mice were shaved and prepared for ferric chloride ($FeCl_3$)-induced arterial thrombosis(23). Briefly, an incision was made superficial to the right common carotid artery and an arterial segment was exposed by blunt dissection. Carotid blood flow was measured with a miniature Doppler flow probe (Transonic) as described (29). Localized thrombosis was initiated by applying two pieces of Whatman filter paper saturated with 3.5% $FeCl_3$ to either side of the carotid artery, proximal to the Doppler flow probe. The filter paper was removed three min after application and carotid blood flow measured continuously for 30 min. The carotid arteries (right and left) were collected at euthanasia for further analysis by immunohistochemistry.

Lectin complement pathway restoration in vivo

Recombinant human MBL (rhMBL – gift from NatImmune, Copenhagen, Denmark) was administered to MBL null mice via i.p. injection 10 min prior to $FeCl_3$ exposure at 75 μg(30) as previously described(31).

Serum Collection

Mice were exsanguinated via cardiac puncture and the whole blood samples were allowed to clot for 2 hrs at room temperature then placed at 4° C overnight. The following day, the clotted samples were centrifuged at 3000×g for 10 min and the serum was collected, aliquoted, and stored at −80° C for future use.

MBL-dependent Whole Blood Aggregation

Aggregation was analyzed using a Chrono-Log Whole Blood Aggregometer as described previously with slight modifications(24). Mannan (5 mg/ml in $NaHCO_3/Na_2CO_3$, pH 9.6) was bound to cuvettes (Chrono-Log Corp., Havertown, PA) overnight at 4°C as described(32). The following day, cuvettes were blocked with 3% BSA for two hrs at room temperature then washed with PBS + 0.05% Tween, PBS, and veronal buffered saline (VBS). Negative control cuvettes were made by omitting the mannan from the initial plating step. Blood from individual animals was tested in both control and mannan-coated cuvettes and run simultaneously to ensure equal platelet counts. Aggregometry was performed at 37°C with constant stirring. Blood samples were collected in sodium citrate from anesthetized mice via the inferior vena cava. Whole blood was then diluted (1:4) with 0.9% saline and placed in either control or mannan-coated cuvettes. The blood was incubated for 5 min at 37°C, and the aggregation probe was then immersed in the diluted blood sample and incubated at 37°C for 2 min. Aggregation was induced via 1 μg/mL collagen (Chrono-Log Corp., Havertown, PA). Aggregation (Ohms of electrical impedance) was recorded for 25 min and all experiments were performed within 3 hrs of blood acquisition. Data are expressed as the difference between the impedance measured in the mannan-coated cuvette compared to the control cuvette.

Thrombin substrate cleavage assay

The MBL-MASP complex capture assay has been described previously(1) and recently enhanced to a multiplexed system(32). A variation of this assay was developed for the mouse system. Briefly, we captured mouse MBL-MASP complexes on mannan-coated plates for *in vitro* analysis of complement and coagulation interactions. To examine the individual components of the MBL-MASP complex in the absence of other serum components, we captured recombinant human MBL and recombinant MASP-1 (rMASP-1 – a gift from Dr. Minoru Takahashi(27)). Plates were washed to remove sera components and retain MBL complexes, and a synthetic chromogenic thrombin substrate (S2238, H-D-Phe-Pip-Arg-pNA·2HCl – DiaPharma; 25 mM) was added to each well. As S2238 is enzymatically cleaved, p-nitroaniline (pNA) is released. Thus, the rate of pNA formation is proportional to the enzymatic activity. A SpectraMax Plus spectrophotometer (Molecular Diagnostics) was used to measure optical density (OD) every five min for 1.5 hrs at 405nm. Data are expressed as the normalized optical density.

Immunostaining

Following experimental thrombosis, one set carotid artery sections were removed and placed in formalin. Samples were paraffin embedded and sectioned by AML Laboratories (Baltimore, MD). Thrombus formation was examined via hematoxylin-eosin staining as described(33). Additional carotid artery sections from a second group of mice were removed and quickly embedded in OCT and frozen in 2-methylbutane chilled in liquid nitrogen. Frozen sections (5um) were stained for MBL using monoclonal rat anti-mouse MBL-A and MBL-C antibodies (Hycult Biotech, The Netherlands) (1:100 in PBS 0.05% Triton X-100 supplemented with 1mM $CaCl_2$) or mouse monoclonal anti-human 2A9 (anti-hMBL; 1:2000). Briefly, slides were air dried after sectioning. Tissues were fixed with 4% paraformaldehyde for 10 min, rinsed with PBS, followed by incubation with monoclonal rat anti-mouse MBL-A and MBL-C for one hr. Slides were rinsed with PBS 0.05% Triton X-100 and MBL antibody was detected by biotinylated polyclonal rabbit anti-rat IgG or goat anti-mouse IgG (Dako, CA) (1:600 in PBS for 45 min). All slides were incubated with Vectastain ABC-AP Kit Standard (Vector Laboratories, CA) and MBL deposition was visualized using Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, CA). Images were taken on a Nikon Eclipse E400 microscope, and analyzed using SPOT Imaging

software (Diagnostic Instruments, Inc). Negative control staining was performed as described above with omission of the primary antibody.

Statistical Analysis

All values are expressed as means ± standard error (SEM). Comparisons between groups were made using RM-ANOVA (in vivo studies) or t-test (in vitro studies) followed by post-hoc analysis. Differences were considered significant at $p \leq 0.05$. SigmaStat 3.1 software (Jandel Scientific) was used for statistical analysis.

RESULTS

Ferric Chloride ($FeCl_3$)-Induced Thrombosis

Ferric chloride exposure results in reproducible thrombus formation within the carotid artery(23,28). As shown in Figure 1, 3.5% $FeCl_3$ application resulted in complete loss of carotid artery blood flow after approximately 10 min in WT mice. Similarly, C2/fB null mice responded with complete loss of blood flow by 20 min, suggesting that downstream complement activation (C3; C5b-9) plays no role in thrombus formation. In contrast, we observed no decrease in blood flow in MBL null or MASP-1/-3 mice following ferric chloride exposure (Fig. 1A; *$p<0.01$). Furthermore, reconstitution with rhMBL (75 μg) in MBL null mice restored thrombogenesis, supporting a significant role for the MBL-MASP complex in coagulation *in vivo*.

Carotid arteries were fixed and sectioned transluminally to visualize the thrombus composition. H&E staining shows a distinct thrombus in sections from WT mice following $FeCl_3$ exposure, whereas very little thrombus formation is observed in arteries from MBL null mice (Fig. 1B). When the lectin pathway was restored with rhMBL in MBL null mice, thrombus formation was again observed. Similar to MBL null arteries, MASP-1/-3 KO arteries demonstrated no thrombi, suggesting a complete MBL-MASP complex is necessary for thrombogenesis following $FeCl_3$ exposure. These results strongly coincide with the *in vivo* carotid blood flow measurements.

MBL Deposition on the Vascular Endothelium

To determine if MBL deposition occurred in the regions of $FeCl_3$-initiated thrombus formation, we stained arterial sections for MBL-A and MBL-C. Figure 2 illustrates MBL deposition along the vascular endothelium in WT and C2/fB null arteries. As anticipated, no MBL staining is present on the endothelium of MBL null arteries, yet reconstitution with rhMBL again results in MBL deposition and thrombus formation. This strongly supports the $FeCl_3$ findings in C2/fB null mice, in that the C2/fB null mice cannot activate downstream complement components (C3; C5b-9) yet we still observed occlusive thrombogenesis. No MBL staining was observed in contralateral arteries or arteries treated with vehicle (data not shown). Thus, our data indicate that the complement components necessary for thrombogenesis are within the MBL-MASP complex alone, and downstream complement activation has little to no role in the thrombogenesis.

MBL-dependent Whole Blood (WB) Aggregation

To determine whether lectin complement pathway activation results in increased platelet aggregation, we tested blood samples from WT and MBL null mice using a Chrono-Log whole blood aggregometer in the presence or absence of immobilized mannan. The increase in impedance correlates with the amount of platelet aggregates deposited on the electrodes after the addition of a platelet agonist, in our case, collagen. Figure 3 demonstrates significantly increased platelet aggregation in whole blood samples from WT mice placed in mannan-coated cuvettes compared to control cuvettes, suggesting that MBL activation on

the cuvette's internal surface enhances the thrombotic potential of the blood sample, therefore leading to increased platelet aggregation. In contrast, there is no significant difference in the maximum aggregation in MBL null mouse blood incubated in control or mannan-coated cuvettes, indicating that the enhanced aggregation observed in WT blood in mannan-coated cuvettes is mediated by the MBL-MASP complex.

In Vitro Thrombin-Substrate Cleavage Assays

Following our *in vivo* observations, we used sera from mice deficient in specific complement components to elucidate the molecular mechanisms responsible for MBL-MASP complex-mediated coagulopathy. Figure 4 demonstrates that capture of MBL-MASP complexes from MBL null mouse serum cleaves significantly less thrombin substrate (S2238) compared to WT serum, likely resulting from no MBL available for capture. Capture of MBL-A or MBL-C complexes from respective MBL-$C^{-/-}$ and MBL-$A^{-/-}$ mouse sera results in no significant difference in S2238 cleavage compared to WT (Fig. 4) as these genotypes are capable of forming a complete MBL-MASP complex. Furthermore, serum (and thus MBL-MASP complexes) from C2/fB null mice resulted in S2238 cleavage similar to WT, indicating that downstream complement components (i.e. C3, C5b-9) do not play a role in thrombin substrate cleavage. N-acetyl glucosamine (GlcNAc) is competitive inhibitor of MBL binding to mannan, thereby serving as a negative control. When serum from WT mice is treated with GlcNAc (100mM), MBL complexes do not bind to the microtitre plate and S2238 cleavage is significantly inhibited. These data suggest that an intact MBL-MASP complex is needed for thrombin-like activity in the mouse *in vitro* system.

To tease out whether the thrombin-like activity was mediated by either MBL or MASPs, we tested S2238 cleavage using MASP-1/-3 KO serum and recombinant MASP-1 (rMASP1). No thrombin-like activity was observed in MBL-MASP complexes captured from MASP-1/-3 KO serum (Fig. 5A; *$p<0.05$) compared to WT sera. However, when rMASP1 was added to MBL complexes captured from MASP-1/-3 KO serum, thrombin-like activity was restored in a dose-related manner. Further, we observed no thrombin substrate cleavage from the capture of rhMBL (panel B) alone, yet when rMASP1 was co-incubated with rhMBL, thrombin-like activity was again observed (Fig. 5B). These data indicate that within the MBL-MASP complex, MASP-1 is the necessary complement component for thrombin-like activity *in vitro*.

DISCUSSION

MBL is an interesting complex whereby deficiency in humans is associated with severe atherosclerosis and recurrent infections(34,35). Our earlier studies demonstrated that ischemic injury results in complement activation and MBL deposition within the vasculature(36,37). Ischemic injury in a variety of vascular beds initiates lectin-complement pathway activation, and inhibition of MBL or its removal reduces post-ischemic myocardial reperfusion injury(31,38). Thus, MBL appears to be a double-edge sword in human disease, where the disease dictates whether MBL's presence or absence influences the clinical outcome. The present study advances our previous findings to suggest that MBL-MASP complexes play a significant role in thrombus formation *in vivo*.

MASP-2 can cleave prothrombin to thrombin *in vitro*(21). Other studies report that MASP-1 may also have thrombin-like activity as it was found to cleave fibrinogen and factor XIII and lead to a fibrin clot in vitro(39–42). More recently, Takahashi and colleagues demonstrated that both MASP-1 and thrombin cleaved a synthetic substrate identical to the activation peptide of factor D, further linking MASP-1 to thrombin-like activity(43). Furthermore, Megyeri et al (2009) demonstrated that MASP-1 activates PAR4, an interesting finding since PAR4 activation by thrombin is responsible for platelet activation in humans(44,45).

However, all of these previous studies were conducted in vitro and the present studies were undertaken to observe whether these pro-coagulant activities occur *in vivo*. These findings may represent a mechanism by which $FeCl_3$-induced thrombogenesis is attenuated in the MBL null and MASP-1/-3 KO mice, as shown in Figure 1. Further, these findings demonstrate that a MBL complex comprised of only MASP-2, which is present in the MBL complex of MASP-1/3 KO mice, does not play a sufficient role to induced occlusive thrombosis *in vivo* in this model.

Westrick et al (2007) published a thorough review of various murine models for examining thrombosis and hemostasis, a significant number of which use the $FeCl_3$ method(28). $FeCl_3$ was first discovered to be a thrombogenic agent by Reimann-Hunziker in 1944(46) and has since been widely used to investigate specific mechanisms involved in thrombosis. Many studies suggest that the strongly oxidizing $FeCl_3$ compound injures the endothelial cell wall via oxidative stress(47). Tseng et al (2005) demonstrated that ferric ions permeate the endothelial basal lamina before entering the arterial lumen via an endocytic-exocytic pathway, ultimately resulting in complete endothelial denudation and occlusive thrombosis(48). Further, Saldeen and collegues found that antioxidant therapy significantly decreased $FeCl_3$-induced thrombogenesis(49). Along these lines, we have previously shown that intracellular oxidative stress leads to MBL ligand expression, MBL complex binding and complement activation *in vitro*(1). Thus, the oxidative stress produced by $FeCl_3$ administration likely results in MBL ligand expression and MBL deposition along the vascular endothelium as we have shown (Fig. 2).

We demonstrate that downstream complement activation is not necessary for thrombin-like activity *in vitro* or *in vivo*, as demonstrated by the similar results observed between the WT and C2/fB null mice since the C2/fB null mice cannot activate complement components C3 through the C5b-9 membrane attack complex. Further, we demonstrate occlusive thrombogenesis in rhMBL reconstituted MBL null mice *in vivo* (Fig. 1). These data suggest that MBL inhibition or deficiency decreases coagulation parameters, whereas other complement inhibitors that act on components below MBL in the complement cascade would not attenuate or eliminate coagulation induced by MBL. This hypothesis might explain why a randomized clinical trial involving pexelizumab, a recombinant anti-C5 monoclonal antibody, in coronary artery bypass graft (CABG) surgery did not have a statistically significant effect on the primary endpoint (composite endpoint of death or myocardial infarction)(50). While MBL levels were not measured during these clinical trials, low MBL levels were correlated to a significant reduction in mortality in the APEX-MI trial(51), suggesting an important role of MBL following MI/R in this clinical trial. Further, these data suggest that inhibition of the MBL complex and/or MASP-1 will prevent MBL pathway mediated coagulation, whereas inhibition of MASP-2 would be ineffective(52) as Takahashi et al (2008) reported that MASP-1 is required for enhanced MASP-2-dependent complement activation through direct cleavage and activation of MASP-2 (27).

MBL binding activates the lectin complement pathway, resulting in a pathophysiologic state that may be worse in high-MBL expressing patients/mice compared to MBL-deficient patients/mice. Further, coagulopathy may result from direct MBL complex-mediated activation of thrombin substrates and coagulation factors. The *in vitro* and *in vivo* data presented in this report support this hypothesis. In particular, by capturing and activating MBL complexes on the internal surface of mannan-coated cuvettes used for whole blood aggregation, we demonstrated a significant increase in platelet aggregation compared to control cuvettes in WT mouse blood. No difference in platelet aggregation was observed between the negative control cuvette and the mannan-coated cuvette using MBL null mouse blood, as would be expected in the absence of MBL complexes. These data further support

the *in vitro* thrombin substrate data suggesting that MASP-1 may be the active component of the MBL complex responsible for thrombin-like activity.

Based on published *in vitro* observations and the findings obtained in the present study, we propose the following model for $FeCl_3$-mediated occlusive thrombogenesis (Figure 6). External $FeCl_3$ application results in the translocation of ferric ions and reactive oxygen species (ROS) via Fenton chemistry, whereby the ferrous iron is oxidized by hydrogen peroxide to form ferric iron, a hydroxyl radical and a hydroxyl anion(47,48). This hypothesis is further supported by limited thrombogenesis in this model following antioxidant therapy(49). We hypothesize that, during the immediate acute phases of endothelial injury, such as $FeCl_3$ application or ischemia/reperfusion, MBL binds to stressed endothelial cells. Indeed, our laboratory demonstrated that stressed endothelial cells activate the lectin complement pathway via ROS generation and induction of an MBL ligand(1,53,54). Upon MBL binding to injured endothelial cells, we hypothesize the serine proteases, MASP-1 and MASP-2, play a significant role in the amplification of coagulation via the cleavage of prothrombin to thrombin(21), the direct cleavage of thrombin substrates including fibrinogen and factor XIII(41–43), and activation of platelets through PAR4(44,45). This sequence of events ultimately results in thrombus formation *in vivo*. Furthermore, the data presented herein represent the first evidence that MBL and/or MASP-1 deficiency leads to coagulation abnormalities *in vivo*.

Results from this study may have important implications for the early identification, treatment and prevention of common vascular complications such as myocardial infarction and stroke. Our data extend previously published 0 findings and show further interactions of lectins (MBL complexes) to activate components of the coagulation system both *in vitro* and *in vivo*. The data yields new information on the importance of the MBL complex alone in mediating pathologies without the need for complement activation per se.

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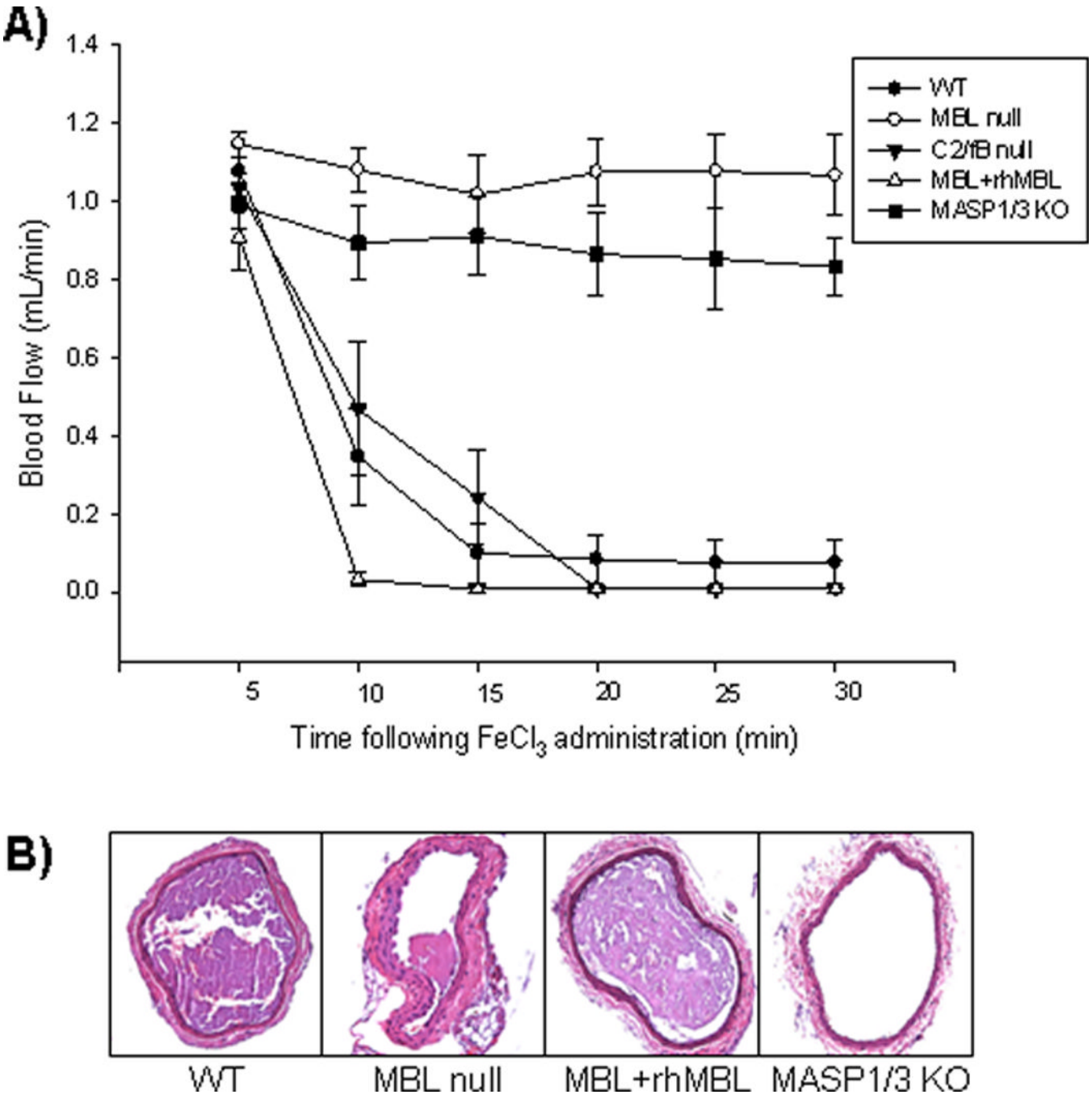

Figure 1. The MBL complex with MASP-1/-3 is necessary for $FeCl_3$-induced thrombogenesis *in vivo*

A) MBL null (open circle) and MASP1/3 KO (closed square) mice are protected from $FeCl_3$-induced thrombogenesis. WT (closed circles) and C2/fB null (closed triangle) mice experience significant thrombogenesis and cessation of carotid blood flow within15–20min. Reconstitution with rhMBL in MBL null mice (open triangle) resulted in a significant decrease in blood flow that was not different from WT and C2/fB null. B) Representative panels of the H&E staining for thrombus composition in carotid arteries following $FeCl_3$, 20X magnification.

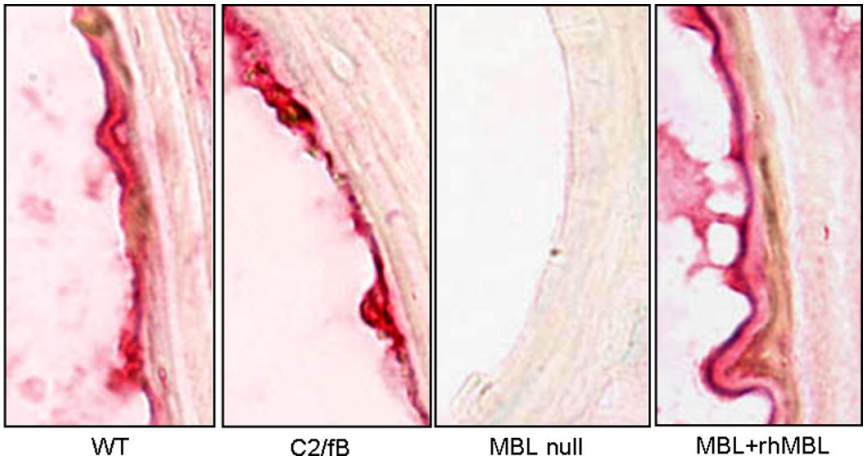

Figure 2. Complement deposition on the vascular endothelium is MBL dependent

Representative panels of the immunohistochemistry used to identify MBL deposition on the vascular endothelium of carotid arteries following $FeCl_3$-induced thrombogenesis. MBL deposition (red) on the vascular endothelium, 100X magnification. Images were obtained using a Nikon Eclipse E400 microscope (Nikon) and representative images were captured and analyzed using SPOT Imaging software (Diagnostic Instruments, Inc).

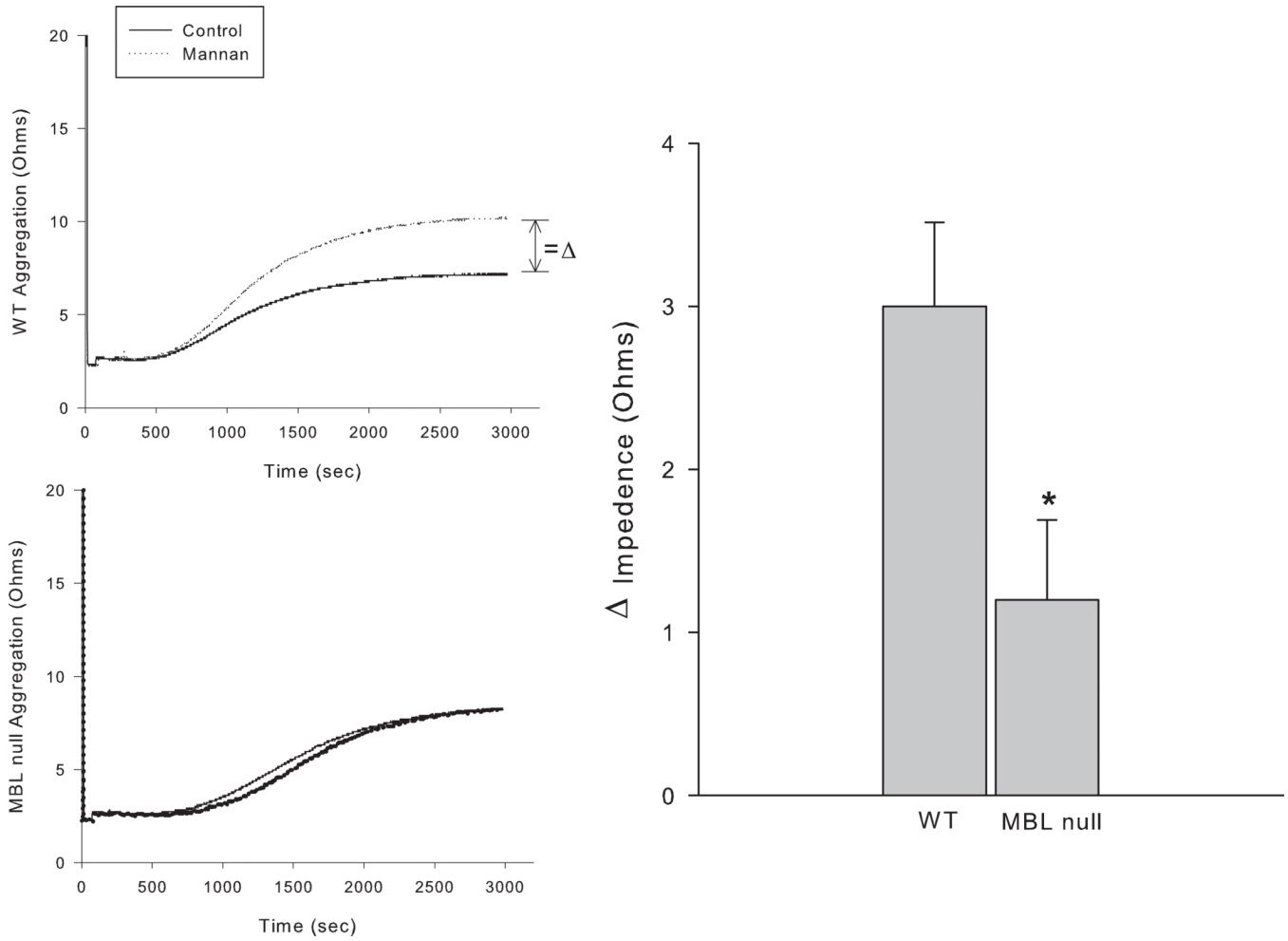

Figure 3. WB aggregation is enhanced following MBL complex activation

Whole blood samples were taken from WT and MBL null mice, aliquot into control and mannan-coated cuvettes and aggregation was stimulated using 1ug/mL collagen. Data are expressed as the difference (Δ) between the impedance measured in the mannan-coated cuvette compared to the control cuvette. Representative aggregation tracings are shown on the left (WT top; MBL null bottom). *$p<0.05$

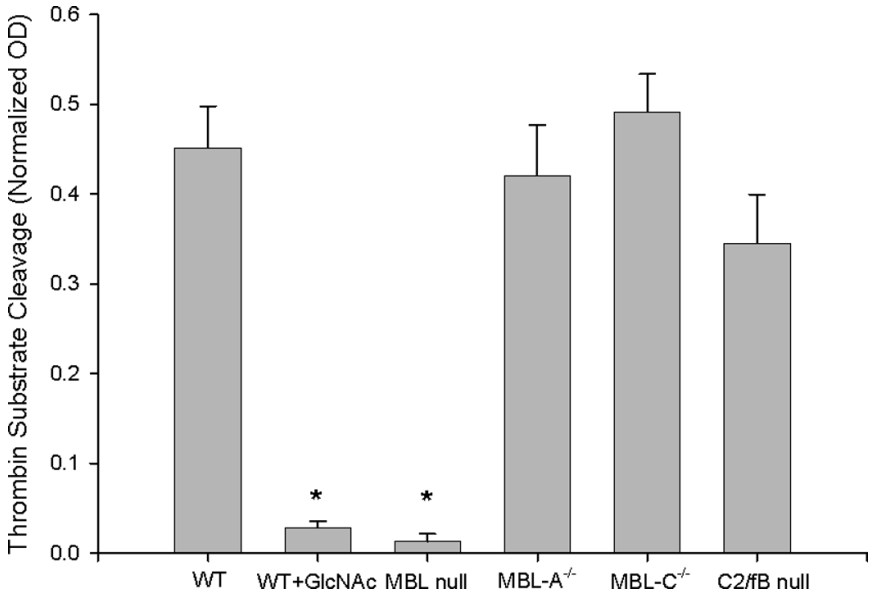

Figure 4. MBL deficiency results in significantly decreased thrombin substrate cleavage

Lack of thrombin-like activity was observed in both MBL null mouse serum and in serum incubated with N-acetyl Glucosamine (GlcNAc), a competitive MBL inhibitor. No significant difference in thrombin substrate cleavage was observed between WT and mice containing intact MBL complex components. *$p<0.05$*

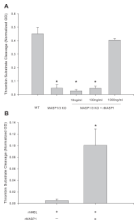

Figure 5. MASP-1 is necessary for thrombin substrate cleavage

A) Reconstituting the MBL-MASP complex with rMASP1 results in a dose-dependent increase in thrombin substrate cleavage. B) No thrombin-like activity is observed with rhMBL alone. Thrombin substrate cleavage is partially restored when rMASP-1 is added to rhMBL. Together, these results suggest that MASP-1 is primarily responsible for the thrombin-like activity. *$p<0.05$*

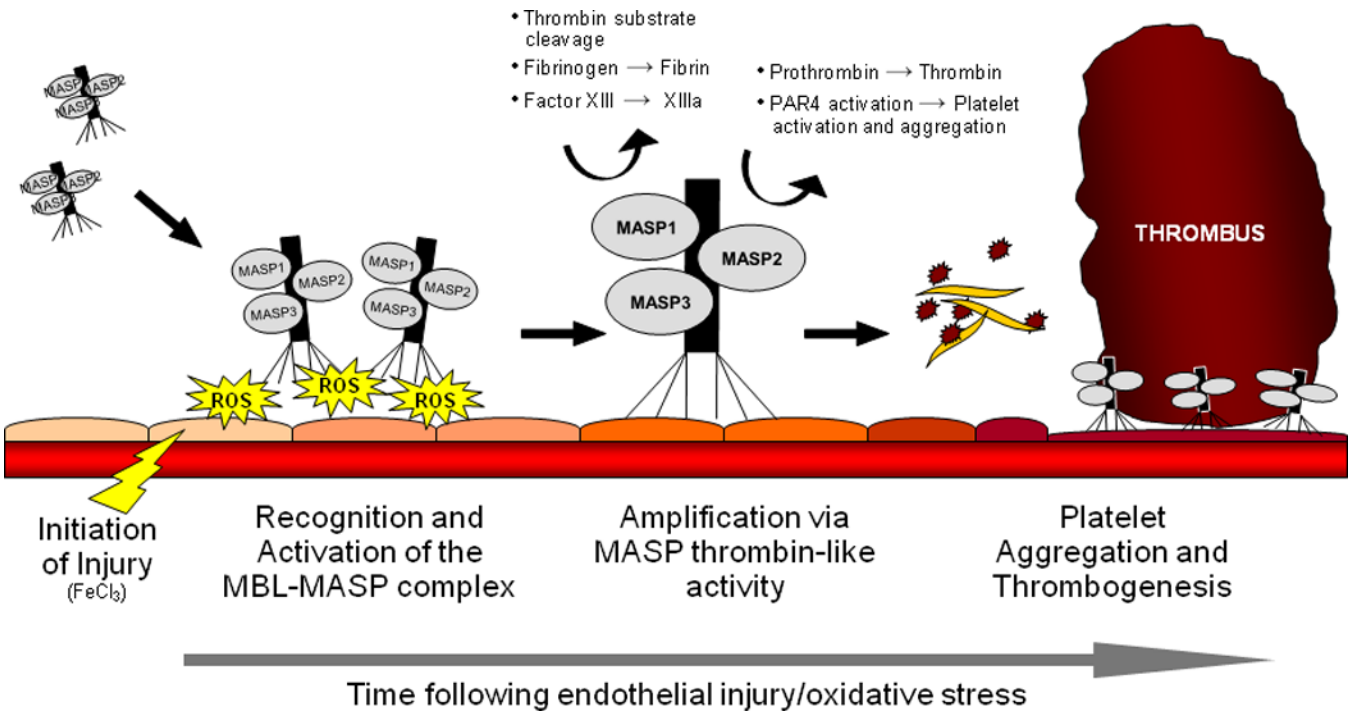

Figure 6. Proposed Mechanisms

Injury to the vascular endothelium, in this case by external application of ferric chloride, results in the endo- and exocytosis of oxygen free radicals (reactive oxygen species; ROS) (47,48). Endothelial generation of reactive oxygen species (ROS) results in the expression of an MBL ligand and subsequent MBL-MASP binding and activation(1). Previous in vitro studies demonstrated that MASP-2 cleaves prothrombin to thrombin(21), and MASP-1 cleaves fibrinogen and factor XIII leading to a fibrin clot(39–42). Here, we've extended those findings to include evidence of MBL deposition along the vascular endothelium along with a role for MASP-1 in cleavage of thrombin substrates, enhancement of platelet aggregation, and in vivo thrombogenesis.