Published in final edited form as:

Immunobiology. 2011 ; 216(1-2): 96–102. doi:10.1016/j.imbio.2010.02.005.

Mannose-binding lectin and its associated proteases (MASPs) mediate coagulation and its deficiency is a risk factor in developing complications from infection, including disseminated intravascular coagulation

Kazue Takahashi^{1,*}, Wei-Chuan Chang¹, Minoru Takahashi², Vasile Pavlov³, Yumi Ishida², Laura La Bonte³, Lei Shi^{1,#}, Teizo Fujita², Gregory L. Stahl^{3,‡}, and Elizabeth M. Van Cott^{4,‡}

¹ Developmental Immunology Program, Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, MA02114, USA

² Department of Immunology, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan

³ Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA02115, USA

⁴ Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

Abstract

The first line of host defense is the innate immune system that includes coagulation factors and pattern recognition molecules, one of which is mannose-binding lectin (MBL). Previous studies have demonstrated that MBL deficiency increases susceptibility to infection. Several mechanisms are associated with increased susceptibility to infection, including reduced opsonophagocytic killing and reduced lectin complement pathway activation. In this study, we demonstrate that MBL and MBL-associated serine protease (MASP)-1/3 together mediate coagulation factor-like

Address correspondence to Dr Kazue Takahashi, 55 Fruit Street, GRJ1402, Boston, MA02114., Phone: 1-617-726-1394. Fax: 1-617-724-3248. ktakahashi1@partners.org.

[‡]Co-senior authors.

[#]Current address: Centocor Inc. 145 King of Prussia Rd, Radnor, PA, USA.

Author Contributions

Kazue Takahashi – Responsible for the entire project and performed studies of infection, bleeding time, in vitro assays, data analysis and wrote manuscript

Wei-Chuan Chang - Performed thrombin-like activity assay

Minoru Takahashi - Performed bleeding time assay and prepared serum

Vasile Pavlov - Performed blood flow assay

Yumi Ishida - Performed bleeding time assay

Laura La Bonte - Performed thrombin-like activity assay

Lei Shi - Worked on infection studies and bacterial titer assays

Teizo Fujita - Generated and provided MASP null mice

Gregory L. Stahl - Generated and provided mAb 3F8, oversaw blood flow assay and wrote manuscript

Elizabeth M. Van Cott - Guided the coagulation experimental plan and performed coagulation assays and wrote manuscript

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

activities, including thrombin-like activity. MBL and/or MASP-1/3 deficient hosts demonstrate *in vivo* evidence that MBL and MASP-1/3 are involved with hemostasis following injury. *Staphylococcus aureus* infected MBL null mice developed disseminated intravascular coagulation (DIC), which was associated with elevated blood IL-6 levels (but not TNF- α and multi-organ inflammatory responses). Infected MBL null mice also develop liver injury. These findings suggest that MBL deficiency may manifest into DIC and organ failure during infectious diseases.

Keywords

mannose-binding lectin; MASP; deficiency; infection; coagulation; inflammation

Introduction

The innate immune system protects against invading pathogens by orchestrating innate immune cells, innate immune soluble factors, coagulation factors and pattern recognition molecules, such as MBL (Takahashi and Ezekowitz, 2005, Takahashi, et al., 2006). MBL, through its carbohydrate recognition domains (CRD), recognizes molecular patterns, which are present on many pathogens or exposed neoepitopes on apoptotic cells and/or injured/inflamed tissues (Takahashi and Ezekowitz, 2005, Takahashi, et al., 2006). These molecular patterns contain sugars, such as D-mannose and N-acetyl-D-glucosamine (GlcNAc). MBL functions as an opsonin and initiates lectin complement pathway activation via MBL-associated serine proteases (MASPs) (Fujita, et al., 2004, Takahashi, et al., 2006). MASPs are homologues to C1r and C1s of the classical complement pathway, although they are evolutionarily older (Fujita, et al., 2004). Three MASPs and a related protein, which associate with MBL, include MASP-1, MASP-2, MASP-3 and sMAP (Map19). The latter two are alternative splicing products of the former two genes, respectively (Fujita, et al., 2004). MASP-2 is necessary for MBL-mediated complement activation via the lectin pathway, while the functional role of MASP-1 in the MBL complex is unknown.

The human MBL gene has single nucleotide polymorphisms (SNPs) in the promoter region and exon 1 of the coding region (Madsen, et al., 1998). Individuals may be heterozygous or homozygous for these MBL SNPs, which results in reduced blood concentration and/or dysfunctional MBL forms (Garred, et al., 2006, Steffensen, et al., 2000). As a result, human MBL blood levels vary significantly with MBL deficiency, a common immunodeficiency arising in 5 – 30% of the population (Garred, et al., 2003).

Compelling clinical studies have associated MBL deficiency with increased infection susceptibility (Sumiya, et al., 1991, Takahashi and Ezekowitz, 2005). Animal disease model studies have confirmed that MBL null mice are susceptible to certain infections, including *S. aureus* (Gadjeva, et al., 2004, Moller-Kristensen, et al., 2006, Shi, et al., 2004). The infected phenotype is a result of MBL deficiency because reconstitution of MBL null mice with recombinant human MBL (rhMBL) reverses the disease phenotype (Moller-Kristensen, et al., 2006, Shi, et al., 2004). Infection arises as a result of bacteremia from reduced opsonophagocytic killing and reduced lectin complement pathway activation (Moller-Kristensen, et al., 2006, Shi, et al., 2004). Recent clinical studies have reported an association of MBL deficiency with increased morbidity and mortality (Garred, et al., 1999, Mullighan and Bardy, 2004, Satomura, et al., 2006, Tran, et al., 2007).

Disseminated intravascular coagulation (DIC) is a coagulation disorder, in which thrombosis (clotting) and bleeding simultaneously progress, leading to increased mortality (Bakhtiari, et al., 2004, Smith, et al., 2004). DIC is also known as an independent predictor of mortality and adverse events in many clinical diseases (Dhainaut, et al., 2005, Smith, et al., 2004). Not

all patients with similar clinical conditions and symptoms develop DIC, suggesting genetic components are involved. However, no gene or biomarker has been identified. Previous *in vitro* studies have suggested that MASP-1 and MASP-2 are involved with coagulation (Ambrus, et al., 2003, Hajela, et al., 2002, Krarup, et al., 2008, Krarup, et al., 2007, Presanis, et al., 2004), however with the advent of novel genetically modified mice, the roles of these MASPs *in vivo* have not been investigated. In the present study, we investigated the potentially significant role that MBL and these MASPs may play in host defense and coagulation using mouse models of MBL deficiency.

Materials and Methods

Assays for thrombin-like activity and fibrin clotting

The assay was designed to detect MBL-MASP complex-mediated activities by using plates that were coated with one of mannan, BSA or GlcNAc-BSA as indicated in each experiment at 20 µg/ml in carbonate binding buffer, pH 9.5. Mannan was used as a representative ligand of MBL (Holmskov, et al., 1993). After rinsing with TBS, pH 7.4, supplemented with 10 mM CaCl₂ (TBS-CaCl₂), the wells were incubated with diluted mouse serum or rhMBL with or without 1% MBL null serum as a MASP source. Wells were incubated at room temp for 1 hr and then rinsed 4 times in order to wash off endogenous prothrombin and thrombin. Thrombin-like activity of MBL/MASP complex was measured by incubating the wells with a rhodamine 110 based thrombin substrate (tosyl Gly-Phe-Arg-amide, R22124, Invitrogen) at 10 µM in TBS-CaCl₂, except for the experiment in the Figure 1D, in which another structurally different thrombin substrate, Phe-pipecolyl-Arg p-Nitroanilide Chromogenix S-2238 (Diapharma) at 10 µg/ml was used, and read at 500 excitation/520 emission and OD at 405 nm, respectively.

For the fibrin clot assay, wells were coated with mannan, GlcNAc or GalNAc as above. After rinsing, wells were incubated with fibrinogen at 1 mg/ml in TBS-CaCl₂ and OD at 340 nm, adsorption of which indicates fibrin clot, was recorded. Thrombin (King Pharmaceuticals, TN) was used as a standard and relative clotting activity was evaluated.

Mice

WT, MBL null, C3 null, MASP-1/3 null, MASP-2/sMAP null and all MASP null mice were on C57BL/6J genetic background as described previously (Iwaki, et al., 2006, Prodeus, et al., 1997, Shi, et al., 2004, Takahashi, et al., 2005, Takahashi, et al., 2008). All animal experiments were performed under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital and Fukushima Medical University.

Bleeding time

Bleeding time was measured as previously described (Bhole and Stahl, 2004). Briefly, mice were anesthetized with i.p. injection of avertin (Sigma, MO) and the terminal 2 mm of the tail tip was excised with a scalpel blade. The tail was then immersed in PBS maintained at 37°C and the time required for stoppage of blood flow (stoppage for 60 s or more) was recorded. Assays were terminated after 15 min, if bleeding did not cease.

Reconstitution of MBL in MBL null mice was performed by injecting 75 µg of rhMBL in 0.2ml saline via the tail vein 1 hr prior to commencing the bleeding time test. This amount of MBL was previously confirmed to fully restore MBL-mediated lectin complement pathway (Shi, et al., 2004). The rhMBL was a gift from Enzon Pharmaceuticals.

Coagulation assays

Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, platelet counts, and platelet aggregation were performed at the Coagulation Laboratory (Director, Elizabeth Van Cott), Massachusetts General Hospital.

Anesthesia in mice was induced with isoflurane (3–4 MAC) and then maintained during surgery at 1–2 MAC. After induction, mice were shaved and prepared for FeCl₃-induced arterial thrombosis as previously described (Wang, X 2004). Briefly, an incision was made superficial to the right common carotid artery, and then a segment of the artery was bluntly dissected and exposed. Carotid artery blood flow was measured with a miniature Doppler flow probe (Transonic) as described (Zhu 1999, Konstantinides 2001). Localized thrombosis was initiated by applying 2 pieces of filter paper saturated with 3.5% ferric chloride to either side of the artery, proximal to the Doppler flow probe. The filter paper was removed 3 min after application to the vessel and carotid artery blood flow was measured continuously for 30 min. Results were expressed as percentage of base line blood flow.

Disseminated intravascular coagulation (DIC) was detected by an abnormal biphasic waveform of the aPTT prior to the development of prolonged PT and aPTT (Downey, et al., 1998). The waveform represents the change in light transmittance (T) through a plasma specimen as the aPTT reaction takes place. A biphasic waveform is characterized by an initial steep negative slope, which is called slope₁ (%T/sec) and the normal slope range is from -0.1 to +0.05, according to the reference range determined in the Coagulation Laboratory.

Infection experiments

S. aureus (Reynolds CP5+) was prepared as previously described (Shi, et al., 2004) and 1×10^7 cfu/0.2 ml saline was inoculated via the tail vein. Blood with appropriate anticoagulants and organs were harvested from euthanized mice following 15 hrs of bacterial inoculation.

Bacterial loads in blood and organs were measured as described previously (37). Briefly, serial dilutions of blood and organ homogenates were prepared and cultured on tryptic soy agar II plates (Sigma-Aldrich) overnight in order to determine bacterial titers. Plasma and supernatants of homogenized tissues were also used for cytokine and chemokine assays. TNF- α , IL-6, MIP-2, and MCP-1 were measured by ELISA kits (R&D System), according to the manufacturer's instructions. ALT and AST, creatinine, and blood urea nitrogen (BUN) were measured at the Chemistry Laboratory (Director, James G. Flood), Massachusetts General Hospital.

Statistical analysis

Statistical analysis methods used were ANOVA, Student's t-test or Wilcoxon/Kruskal-Wallis tests for in vitro studies and Log-Rank for survival study provided by JMP software (SAS Institute Inc., NC).

Results

Thrombin substrates (fibrinogen, Factor XIII and synthetic substrates) have been cleaved by recombinant MASPs (Krarup, et al., 2007, Presanis, et al., 2004 Ambrus et al, 2003, Hajela et al, 2002). MASPs are thought to be activated upon conformational change induced by MBL-binding to its ligand in its native environment (Wallis, 2007). We captured MBL complexes on mannan-coated wells in order to mimic natural activation in an *in vitro* biochemical experiment using a thrombin specific fluorogenic substrate. We first investigated whether the recombinant human MBL (rhMBL) can associate with thrombin-

like activity, which is a crucial step of blood clotting (Figure 1A). When rhMBL, in the presence of aMASP source, bound to mannan-coated wells, thrombin-like activity was detectable in the wells. This did not occur with BSA-coated wells (Figure 1B). The thrombin-like activity was observed only when MBL null mouse serum (1%) was used as a MASP source (data not shown). Thrombin-like activity was dependent on MBL binding, as it was inhibited by an anti-MBL monoclonal antibody (3F8 mAb), which inhibits MBL-ligand binding, in a dose-dependent manner (Figure 1C) (Zhao, et al., 2002)(Walsh, et al., 2005). Further, thrombin-like activity demonstrated time dependency, confirming that the activity is not only MBL-dependent but also likely to depend on enzyme activation (Figure 1D). Induction of thrombin-like activity was also inhibited by GlcNAc (Figure 1D), another inhibitor of MBL binding to mannan (Hansen, et al., 2000, Wallis, 2007).

We next addressed whether MBL in complex with MASPs can activate fibrinogen to form a fibrin clot as another thrombin-like activity. Indeed, rhMBL supplemented with 1% MBL null serum (a MASPs source) exhibited fibrin clot formation on wells coated with mannan or GlcNAc-BSA but not with N-acetyl-D-galactosamine (GalNAc-BSA), which is not recognized by MBL (Figure 1E) (Wallis, 2007) (Hansen, et al., 2000). Taken together these results demonstrate that the human MBL-MASPs complex is able to mimic thrombin and initiate coagulation.

MBL-MASPs complexes captured from WT mouse serum demonstrated thrombin-like activity while MBL deficient mouse serum lacked this activity (Figure 2A), confirming that the MBL-MASPs complex is associated with thrombin-like activity upon binding to its target. Generation of thrombin-like activity was inhibited by excess mannan (to prevent MBL from binding to the plate), confirming that thrombin-like activity was initiated by target binding of the CRD of MBL (Figure 2B). MBL-binding to its targets results in a conformational change in MASPs (Wallis, 2007) and recent reports have suggested that MASP-1 and MASP-2 possess thrombin-like activity in vitro and can generate fibrin clots (Krarup, et al., 2007, Presanis, et al., 2004, Gulla et al, 2010). Thrombin-like activity was undetectable in MASP-1/3 (M-1) deficient serum, whereas MASP-2/sMAP (M-2) deficient serum demonstrated comparable activity to WT serum (Figure 2C), suggesting that thrombin-like activity is mediated by MASP-1/3. In contrast, the MBL initiated activation of the lectin complement pathway was MASP-2 dependent as C4 deposition was abolished in MASP-2/sMAP deficient serum unlike MASP-1/3 deficient serum (data not shown) in agreement with a previous report (Matsushita, et al., 2000). In order to determine whether MASP-1/3 is involved with coagulation in vivo, we assessed bleeding time using the tail tip excision model. MASP-1/3 (M-1) null mice demonstrated a significantly prolonged bleeding time compared with MASP-2/sMAP (M-2) null mice, further confirming the thrombin-like activity of MASP-1/3 in vitro (Figure 2D). Taken together, these findings provide in vivo evidence that MBL in complex with MASP-1/3 is involved with hemostasis.

We hypothesized that MBL null mice would also demonstrate coagulopathy. As expected, following tail tip excision MBL null mice demonstrated a prolonged bleeding time that was reversed by reconstitution with rhMBL (Figure 3A). We also tested the involvement of complement activation because it is thought to play a role in coagulation (Laudes, et al., 2002) and MBL activates the lectin complement pathway. All complement pathways require C3 for activation, therefore, we performed a bleeding test on C3 null mice; however, the results were comparable to WT mice (Figure 3A). Thus, the coagulopathy observed is before the level of C3 activation and does not appear to involve complete complement activation.

We assayed routine coagulation parameters including PT, aPTT, fibrinogen, and platelet counts. Platelet aggregation function was also comparable in MBL null and WT mice as the maximum aggregation was similar in the two groups (20% vs. 25%). (Figure 3B). All

clotting parameters in MBL null mice were similar to WT mice (Table 1). These data indicate that coagulation itself is not compromised in MBL deficient hosts.

In a previous study, 100% of MBL null mice succumbed by 48 hrs from *S. aureus* bacteremic infection (Shi, et al., 2004). The phenotype was reversed to wild type mice by pretreating MBL null mice with rhMBL, confirming that the high mortality was a result of MBL deficiency. In the present study, 1×10^7 cfu inoculums induced 90% mortality in MBL null mice by day 8, which was significantly greater than that observed in WT mice (Figure 4A). *S. aureus* infection reportedly influences coagulation (Bokarewa and Tarkowski, 2001, Rivera, et al., 2007). We then investigated whether MBL deficiency would have an effect on coagulation following 15 hr of *S. aureus* inoculation. We undertook two independent methods to evaluate the role of MBL on *S. aureus* induced alterations in coagulation (i.e., FeCl₃ induced thrombogenesis and the waveform of aPTT). FeCl₃ decreased carotid artery blood flow to less than 10% of initial flow by 15 min and remained low for 30 min in both naïve and infected WT mice (Figure 4B), suggesting that coagulation was not affected by *S. aureus* infection in WT mice. In contrast, FeCl₃ increased blood flow in naïve MBL null mice over this same time frame, thus demonstrating that MBL deficiency decreases FeCl₃-induced thrombogenesis. The blood flow in MBL null mice following *S. aureus* infection was slightly reduced between 10 and 25 min but went back to baseline flow by 30 min (Figure 4B). These data suggest that *S. aureus* infection had little effect on coagulation.

The second method, the aPTT waveform, is used clinically to diagnose DIC. The waveform was normal in infected WT plasma (-0.011 slope₁) whereas infected MBL null plasma displayed the abnormal biphasic waveform (-0.420 slope₁; the normal slope₁ range is from -0.10 to +0.05) (Figure 4C). Both PT and aPTT were comparable between MBL null and WT mice at this time point (data not shown). Taken together, these data demonstrate that reduced coagulation persists in infected MBL deficient hosts and MBL deficiency is associated with developing DIC from *S. aureus* infection.

It has become clear that inflammation and coagulation influence each other (Levi and van der Poll, 2005). Plasma IL-6 levels were significantly elevated in infected MBL null mice while TNF- α levels were comparable in infected WT and MBL null mice (Figure 4D). Similarly, blood bacterial load was comparable between WT and MBL null mice (Figure 4D). These data suggest that elevated IL-6 blood level is associated with DIC but not blood bacterial load at this early infection stage.

Organ damage has been clinically associated with DIC (Bakhtiari, et al., 2004, Smith, et al., 2004). Even at the 15 hr time point, bacterial load in the liver and the kidney was high in MBL null mice compared to WT mice, although statistical significance was obtained only in the kidney (Figure 5A and 5B, respectively). Interestingly, when comparing infected MBL null to WT mice, indices of renal injury (i.e., BUN or creatinine) were similar or low in MBL null mice (Figure 5B). Further, indices of liver injury (i.e., ALT and AST) were significantly higher in infected MBL null mice compared to WT mice (Figure 5A). Organ injury is thought to be associated with inflammation (Andersson, 2005) and MBL can modify inflammatory responses as previously reported (Ip, et al., 2008, Moller-Kristensen, et al., 2006, Shi, et al., 2004). However, proinflammatory cytokines, such as IL-6, TNF- α , MCP-1, and MIP-2 in the organs of infected MBL null mice were comparable to WT mice (data not shown). Nevertheless, these results demonstrate that MBL deficiency affects not only bacterial clearance but also organ function and that the MBL's effect may be organ dependent during infectious insults.

Discussion

MBL binding to its targets results in a conformational change and autoactivation of MASP-1 and MASP-2 (Wallis, 2007). Recent reports have shown that MASP-1 possess thrombin-like activity *in vitro* (Krarup, et al., 2008, Presanis, et al., 2004) and that MASP-2 activates prothrombin (Krarup et al 2007). Our findings have demonstrated that the MBL-MASP complexes mediate thrombin-like activity when MBL binds to a surface coated with mannan or GlcNAc, suggesting an additional 'player' in the coagulation pathway (Figure 6). We have also demonstrated that thrombin-like activity is MASP-1/3 dependent (Figure 2), confirming previous reports (Krarup, et al., 2008, Presanis, et al., 2004, Hajela et al, 2002). These findings open many questions as to how endothelial cells might be involved mechanistically with the clotting activity of the MBL and MASP-1/3 complex. One proposed mechanism involves a protease-activated receptor PAR4, on endothelial cells, which is cleaved by MASP-1 (Megyeri, et al., 2009). Intriguingly, MASP-1/3 protein deficiency has not been observed in a cohort of healthy Japanese (Terai, et al., 1997) although MASP-1 null mice demonstrated increased susceptibility to viral infection (Takahashi, et al., 2007), suggesting that the deficiency may have an adverse effect in infectious diseases. Genotyping of MASP-1/3 in Caucasians identified amino acid substitution polymorphisms at low frequency although phenotypic effects have yet to be determined (Weiss, et al., 2007).

Our results demonstrate, for the first time that MBL and MASP-1/3 are involved with hemostasis *in vivo* as mice lacking MBL or MASP-1/3 demonstrated a significantly prolonged bleeding time following a tail tip excision (Figure 3). We speculated that the mechanism might be through complement activation, which has been thought to play a role in coagulation (Krarup, et al., 2007, Laudes, et al., 2002), since MBL initiates lectin complement pathway activation. This hypothesis was not correct as C3 null mice displayed similar bleeding times as WT mice, suggesting complement activation has little effect in hemostasis. We have previously found that C6 is associated with coagulation, and platelet aggregation is altered in C6 deficient rodents, resulting in a prolonged bleeding time (Bhole and Stahl, 2004). However, platelet aggregation was similar in WT and MBL null mice, suggesting that platelet function is not a part of the mechanism and is not compromised in MBL deficient hosts. Similarly, other coagulation parameters investigated (e.g., PT, aPTT) were not altered in MBL deficient hosts.

Infected MBL null mice developed DIC, which was detected by a clinically used test (abnormal aPTT waveform) and the persistent coagulation disorder was confirmed by an independent method, examining FeCl₃-induced thrombogenesis during bacterial infection. Although DIC is often observed in sepsis patients (Smith, et al., 2004) blood bacterial counts were not associated with DIC, suggesting that it is not a useful predictor of DIC, at least in the early infection stage. However, DIC was associated with elevated blood IL-6 levels in MBL null mice. This observation is supported by a report that anti-IL-6 antibody treatment reduced coagulation activation during sepsis (van der Poll, et al., 1994). We diagnosed DIC using the abnormal biphasic aPTT waveform, which is sensitive and can be detected prior to development of a prolonged PT and aPTT (Toh and Giles, 2002). The cause of the negative slope (slope₋₁) in the aPTT waveform during DIC (Figure 4) has been identified as calcium-mediated precipitation of C-reactive protein (CRP) complexed with very-low-density lipoprotein (Toh, et al., 2002). MBL binding also requires calcium, as it is a C-type lectin. Moreover, it has been suggested that MBL and ficolins, which are structurally similar to MBL and also activate the lectin complement pathway, may associate with CRP to activate the lectin complement pathway (Ng, et al., 2007, Suresh, et al., 2006).

In this investigation using mouse infection models, we demonstrate, for the first time, that MBL deficiency is involved with developing organ injury and DIC, both of which have been associated with high mortality (Dhainaut, et al., 2005, Smith, et al., 2004, Toh, et al., 2003). We have previously concluded that MBL null mice die from bacteremia, resulting from reduced opsonophagocytic killing, which was also associated with reduced lectin complement pathway activity (Moller-Kristensen, et al., 2006, Shi, et al., 2004). Our current investigation identified liver injury as an additional mechanism of increased mortality in MBL deficient hosts. MBL's effect during infection was organ dependent as renal injury was comparable or less severe in MBL null compared to WT mice. Organ injury could not be attributed to inflammatory responses because cytokine responses were not significantly different (data not shown). One potential explanation might be that since the liver is the primary organ that synthesizes MBL (Uemura, et al., 2002), the lack of MBL synthesis may also affect production of other liver proteins and influence function. Further investigation is required to elucidate the role of MBL in protecting the liver in infected hosts.

In conclusion, our findings demonstrate that MBL deficiency manifests as DIC from infection and that the DIC progresses along with organ injury at an early infection stage. DIC has been associated with adverse events, even among less severely ill patients (Smith, et al., 2004). Thus development of DIC in MBL deficient hosts may explain several important clinical studies associated with high mortality and complications in MBL deficient hosts (Garred, et al., 1999, Mullighan and Bardy, 2004, Satomura, et al., 2006, Tran, et al., 2007). We propose that MBL deficiency is a risk factor in developing complications in certain diseases and that screening for MBL deficiency may improve clinical care because MBL deficiency is one of the most common immunodeficiencies, occurring in 5 – 30% of the population. Further investigations on coagulation revealed that MBL and MASP-1/3 were involved with hemostasis, in which a thrombin-like activity appeared to be a property of MASP-1/3. Moreover, lectin-mediated coagulation is not surprising because ficolins, which are closely related to horseshoe crab hemolymph (Kairies, et al., 2001, Krarup, et al., 2004) that clots lipopolysaccharide and β -glucan (Muta and Iwanaga, 1996), thus providing innate immune protection.

Acknowledgments

The work was supported by grants from NIH NIH: KT, WC, and SM by R21AI077081 and U01 AI074503; GS and LL by R01HL52886, RC1HL099130, R01HL56086, R56HL056086, R21HL092469; and TF, MT and YI by MECSSTJ and CREST and STA. We thank Dr. Michael C. Carroll, Jean C. Lee and Enzon Pharmaceuticals for providing C3 null mice breeding pairs, *S. aureus* and rhMBL, respectively. We also thank Dr. Andrew Kolodziej and Ms. Sheree McClear for providing the fibrin clot assay protocol and excellent technical assistance, respectively.

Abbreviations

MBL	mannose-binding lectin
MASP	MBL-associated serine protease
DIC	disseminated intravascular coagulation
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BUN	blood urea nitrogen
MAC	Monitored Anesthesia Care
CRD	carbohydrate recognition domain
GlcNAc	N-acetyl-D-glucosamine

SNPs	single nuclear polymorphisms
rhMBL	recombinant human MBL
C3	complement component C3
PT	Prothrombin time
aPTT	activated prothomboplastin time
TNF-α	tumor necrosis factor- α
IL-6	interleukin-6
MIP-2	macrophage migration inhibition factor-2
MCP-1	macrophage chemotactic factor-1

References

- Ambrus G, Gal P, Kojima M, Szilagyi K, Balczer J, Antal J, Graf L, Laich A, Moffatt BE, Schwaebler W, Sim RB, Zavodszky P. Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. *J Immunol* 2003;170:1374–1382. [PubMed: 12538697]
- Andersson J. The inflammatory reflex--introduction. *J Int Med* 2005;257:122–125.
- Bakhtiari K, Meijers JC, de Jonge E, Levi M. Prospective validation of the International Society of Thrombosis and Haemostasis scoring system for disseminated intravascular coagulation. [see comment]. *Crit Care Med* 2004;32:2416–2421. [PubMed: 15599145]
- Bhole D, Stahl GL. Molecular basis for complement component 6 (C6) deficiency in rats and mice. *Immunobiol* 2004;209:559–568.
- Bokarewa MI, Tarkowski A. Thrombin generation and mortality during *Staphylococcus aureus* sepsis. *Microb Pathog* 2001;30:247–252. [PubMed: 11312618]
- Dhainaut JF, Shorr AF, Macias WL, Kollef MJ, Levi M, Reinhart K, Nelson DR. Dynamic evolution of coagulopathy in the first day of severe sepsis: relationship with mortality and organ failure. [see comment][comment]. *Crit Care Med* 2005;33:341–348. [PubMed: 15699837]
- Downey C, Kazmi R, Toh CH. Early identification and prognostic implications in disseminated intravascular coagulation through transmittance waveform analysis. *Thromb Haemost* 1998;80:65–69.
- Fujita T, Endo Y, Nonaka M. Primitive complement system--recognition and activation. *Mol Immunol* 2004;41:103–111. [PubMed: 15159055]
- Gadjeva M, Paludan SR, Thiel S, Slavov V, Ruseva M, Eriksson K, Lowhagen GB, Shi L, Takahashi K, Ezekowitz A, Jensenius JC. Mannanbinding lectin modulates the response to HSV-2 infection. *Clin Exp Immunol* 2004;138:304–311. [PubMed: 15498041]
- Garred P, Larsen F, Madsen HO, Koch C. Mannose-binding lectin deficiency--revisited. *Mol Immunol* 2003;40:73–84. [PubMed: 12914814]
- Garred P, Larsen F, Seyfarth J, Fujita R, Madsen HO. Mannosebinding lectin and its genetic variants. *Genes Immun* 2006;7:85–94. [PubMed: 16395391]
- Garred P, Pressler T, Madsen HO, Frederiksen B, Svejgaard A, Hoiby N, Schwartz M, Koch C. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis [see comments]. *J Clin Invest* 1999;104:431–437. [PubMed: 10449435]
- Hajela K, Kojima M, Ambrus G, Wong KH, Moffatt BE, Ferluga J, Hajela S, Gal P, Sim RB. The biological functions of MBL-associated serine proteases (MASPs). *Immunobiol* 2002;205:467–475.
- Hansen S, Thiel S, Willis A, Holmskov U, Jensenius JC. Purification and characterization of two mannan-binding lectins from mouse serum. *J Immunol* 2000;164:2610–2618. [PubMed: 10679100]

- Holmskov U, Teisner B, Willis AC, Reid KB, Jensenius JC. Purification and characterization of a bovine serum lectin (CL-43) with structural homology to conglutinin and SP-D and carbohydrate specificity similar to mannan-binding protein. *J Biol Chem* 1993;268:10120–10125. [PubMed: 8486682]
- Holthofer H, Virtanen I, Kariniemi AL, Hormia M, Linder E, Miettinen A. *Ulex europaeus* I lectin as a marker for vascular endothelium in human tissues. *Lab Invest* 1982;47:60–66. [PubMed: 6177923]
- Ip WK, Takahashi K, Moore KJ, Stuart LM, Ezekowitz RA. Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome. *J Exp Med* 2008;205:169–181. [PubMed: 18180310]
- Iwaki D, Kanno K, Takahashi M, Endo Y, Lynch NJ, Schwaible WJ, Matsushita M, Okabe M, Fujita T. Small mannose-binding lectin-associated protein plays a regulatory role in the lectin complement pathway. *J Immunol* 2006;177:8626–8632. [PubMed: 17142762]
- Kairies N, Beisel HG, Fuentes-Prior P, Tsuda R, Muta T, Iwanaga S, Bode W, Huber R, Kawabata S. The 2.0-Å crystal structure of tachylectin 5A provides evidence for the common origin of the innate immunity and the blood coagulation systems. *Proc Natl Acad Sci U S A* 2001;98:13519–13524. [PubMed: 11707569]
- Krarup A, Gulla KC, Gal P, Hajela K, Sim RB. The action of MBL-associated serine protease 1 (MASP1) on factor XIII and fibrinogen. *Biochim Biophys Acta* 2008;1784:1294–1300.
- Krarup A, Thiel S, Hansen A, Fujita T, Jensenius JC. L-ficolin is a pattern recognition molecule specific for acetyl groups. *J Biol Chem* 2004;279:47513–47519. [PubMed: 15331601]
- Krarup A, Wallis R, Presanis JS, Gal P, Sim RB. Simultaneous activation of complement and coagulation by MBL-associated serine protease 2. *PLoS ONE* 2007;2:e623. [PubMed: 17637839]
- Laudes JJ, Chu JC, Sikrath S, Huber-Lang M, Guo RF, Riedemann N, Sarma JV, Schmaier AH, Ward PA. Anti-c5a ameliorates coagulation/fibrinolytic protein changes in a rat model of sepsis. *Am J Pathol* 2002;160:1867–1875. [PubMed: 12000738]
- Levi M, van der Poll T. Two-way interactions between inflammation and coagulation. *Trends Cardiovasc Med* 2005;15:254–259. [PubMed: 16226680]
- Madsen HO, Satz ML, Høgh B, Svejgaard A, Garred P. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *J Immunol* 1998;161:3169–3175. [PubMed: 9743385]
- Matsushita M, Thiel S, Jensenius JC, Terai I, Fujita T. Proteolytic activities of two types of mannose-binding lectin-associated serine protease. *J Immunol* 2000;165:2637–2642. [PubMed: 10946292]
- Megyeri M, Mako V, Beinrohr L, Doleschall Z, Prohaszka Z, Cervenak L, Zavodszky P, Gal P. Complement protease MASP-1 activates human endothelial cells: PAR4 activation is a link between complement and endothelial function. *J Immunol* 2009;183:3409–3416. [PubMed: 19667088]
- Møller-Kristensen M, Ip WK, Shi L, Gowda LD, Hamblin MR, Thiel S, Jensenius JC, Ezekowitz RA, Takahashi K. Deficiency of Mannose-Binding Lectin Greatly Increases Susceptibility to Postburn Infection with *Pseudomonas aeruginosa*. *J Immunol* 2006;176:1769–1775. [PubMed: 16424207]
- Mullighan CG, Bardy PG. Mannose-binding lectin and infection following allogeneic hemopoietic stem cell transplantation. *Leuk Lymph* 2004;45:247–256.
- Muta T, Iwanaga S. Clotting and immune defense in Limulidae. *Prog Mol Subcell Biol* 1996;15:154–189. [PubMed: 8963461]
- Ng PM, Le Saux A, Lee CM, Tan NS, Lu J, Thiel S, Ho B, Ding JL. C-reactive protein collaborates with plasma lectins to boost immune response against bacteria. *EMBO* 2007;26:3431–3440.
- Presanis JS, Hajela K, Ambrus G, Gal P, Sim RB. Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. *Mol Immunol* 2004;40:921–929. [PubMed: 14725788]
- Prodeus AP, Zhou X, Maurer M, Galli SJ, Carroll MC. Impaired mast cell-dependent natural immunity in complement C3-deficient mice. *Nature* 1997;390:172–175. [PubMed: 9367154]
- Rivera J, Vannakambadi G, Hook M, Speziale P. Fibrinogen-binding proteins of Gram-positive bacteria. *Thrombosis & Haemostasis* 2007;98:503–511. [PubMed: 17849038]
- Satomura A, Endo M, Fujita T, Ohi H, Ohsawa I, Fuke Y, Matsumoto K, Sudo S, Matsushita M. Serum mannose-binding lectin levels in maintenance hemodialysis patients: impact on all-cause mortality. *Nephron* 2006;102:c93–99. [PubMed: 16282701]

- Shi L, Takahashi K, Dundee J, Shahroor-Karni S, Thiel S, Jensenius JC, Gad F, Hamblin MR, Sastry KN, Ezekowitz RA. Mannose-binding lectin-deficient mice are susceptible to infection with *Staphylococcus aureus*. *J Exp Med* 2004;199:1379–1390. [PubMed: 15148336]
- Smith EY, Charles LA, Van Cott EM. Biphasic activated partial thromboplastin time waveform and adverse events in non-intensive care unit patients. *American J Clin Pathol* 2004;121:138–141.
- Stefani MR, Dunlap JA, Yorek MA. Effect of L-fucose on proliferation and myo-inositol metabolism in cultured cerebral microvessel and aortic endothelial cells. *J Cell Physiol* 1992;153:321–331. [PubMed: 1429852]
- Steffensen R, Thiel S, Varming K, Jersild C, Jensenius JC. Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. *J Immunol Methods* 2000;241:33–42. [PubMed: 10915847]
- Sumiya M, Super M, Tabona P, Levinsky RJ, Arai T, Turner MW, Summerfield JA. Molecular basis of opsonic defect in immunodeficient children. *Lancet* 1991;337:1569–1570. [PubMed: 1675710]
- Suresh MV, Singh SK, Ferguson DA Jr, Agrawal A. Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. *J Immunol* 2006;176:4369–4374. [PubMed: 16547275]
- Takahashi K, Ezekowitz RA. The role of the mannose-binding lectin in innate immunity. *Clin Infect Dis* 2005;41(Suppl 7):S440–444. [PubMed: 16237644]
- Takahashi K, Ip WE, Michelow IC, Ezekowitz RA. The mannosebinding lectin: a prototypic pattern recognition molecule. *Curr Opin Immunol* 2006;18:16–23. [PubMed: 16368230]
- Takahashi K, Shi L, Gowda LD, Ezekowitz RA. Relative roles of complement factor 3 and mannose-binding lectin in host defense against infection. *Infect Immun* 2005;73:8188–8193. [PubMed: 16299314]
- Takahashi M, Iwaki D, Kanno K, Ishida Y, Xiong J, Matsushita M, Endo Y, Miura S, Ishii N, Sugamura K, Fujita T. Mannose-binding lectin (MBL)-associated serine protease (MASP)-1 contributes to activation of the lectin complement pathway. *J Immunol* 2008;180:6132–6138. [PubMed: 18424734]
- Takahashi, M.; Mori, S.; Shigeta, S.; Fujita, T. Role of MBL-associated serine protease (MASP) on activation of the lectin complement pathway. Springer New York; New York: 2007. p. 93-104.
- Terai I, Kobayashi K, Matsushita M, Fujita T. Human serum mannosebinding lectin (MBL)-associated serine protease-1 (MASP-1): determination of levels in body fluids and identification of two forms in serum. *Clin Exp Immunol* 1997;110:317–323. [PubMed: 9367419]
- Toh CH, Giles AR. Waveform analysis of clotting test optical profiles in the diagnosis and management of disseminated intravascular coagulation (DIC). *Clin Lab Haematol* 2002;24:321–327. [PubMed: 12452811]
- Toh CH, Samis J, Downey C, Walker J, Becker L, Brufatto N, Tejidor L, Jones G, Houdijk W, Giles A, Koschinsky M, Ticknor LO, Paton R, Wenstone R, Nesheim M. Biphasic transmittance waveform in the APTT coagulation assay is due to the formation of a Ca(++)-dependent complex of Creactive protein with very-low-density lipoprotein and is a novel marker of impending disseminated intravascular coagulation. *Blood* 2002;100:2522–2529. [PubMed: 12239165]
- Toh CH, Ticknor LO, Downey C, Giles AR, Paton RC, Wenstone R. Early identification of sepsis and mortality risks through simple, rapid clotwaveform analysis. Implications of lipoprotein-complexed C reactive protein formation. *Inten Care Med* 2003;29:55–61.
- Tran CT, Kjeldsen K, Haunso S, Hoiby N, Johansen HK, Christiansen M. Mannan-binding lectin is a determinant of survival in infective endocarditis. *Clin Exp Immunol* 2007;148:101–105. [PubMed: 17286761]
- Uemura K, Saka M, Nakagawa T, Kawasaki N, Thiel S, Jensenius JC, Kawasaki T. L-MBP is expressed in epithelial cells of mouse small intestine. *J Immunol* 2002;169:6945–6950. [PubMed: 12471128]
- van der Poll T, Levi M, Hack CE, ten Cate H, van Deventer SJ, Eerenberg AJ, de Groot ER, Jansen J, Gallati H, Buller HR. Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. *J Exp Med* 1994;179:1253–1259. [PubMed: 8145042]
- Wallis R. Interactions between mannose-binding lectin and MASPs during complement activation by the lectin pathway. *Immunobiol* 2007;212:289–299.

- Weiss G, Madsen HO, Garred P. A novel mannose-binding lectin-associated serine protease 1/3 gene variant. *Scandinavian J Immunol* 2007;65:430–434.
- Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP, Solomon SD, Ezekowitz RA, Stahl GL. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* 2005;75:541–6. [PubMed: 15972690]
- Zhao H, Wakamiya N, Suzuki Y, Hamonko MT, Stahl GL. Identification of human mannose binding lectin (MBL) recognition sites for novel inhibitory antibodies. *Hybrid Hybridomics* 2002;21:25–36. [PubMed: 11991814]

Figure 1.

Capture of thrombin-like activity on mannan-coated plates (A) Diagram of fibrinogen cleavage (B) Capture of thrombin-like activity is dependent on rhMBL dose. The assay was performed on plates coated with mannan or BSA as a negative control. 1% MBL null serum was used to supply MASPs. (C) Inhibition of capture of thrombin-like activity by anti-human MBL mAb 3F8. (D) MBL-MASPs complexes from human serum (30%) possess thrombin-like activity, which is inhibited by GlcNAc (20mg/ml) or mAb 3F8 (10 $\mu\text{g/ml}$). This assay was performed using a different thrombin substrate in order to confirm results. (E) Fibrin clot formation activity captured on wells coated with mannan, GlcNAc-BSA or GalNAc-BSA. Closed bars represent rhMBL (10 $\mu\text{g/ml}$) mixed with 1% MBL null serum as MASPs source and open bars represent rhMBL alone. The assay was performed in duplicate.

Figure 2.

Native MBL and MASP-1/3 together are required for thrombin-like activity. (A) Lack of thrombin-like activity in MBL null mice serum. The assay was performed on a mannan-coated assay plate in duplicate. Pooled serum of three mice was used. (B) Inhibition of capture of thrombin-like activity by soluble mannan. Pooled 10% WT mouse serum was mixed with indicated concentration of mannan. (C) Lack of thrombin-like activity in MASP-1/3 (M-1) null mouse serum. M-1, MASP-1/3 null; M-2, MASP-2/sMAP null and M-1/2, all MASP null. MBL, MBL null. Sera from three mice of each MASP null mouse strain were assayed in duplicate. Bars indicate mean $\pm$ SE. *, $p = 0.0024$ (ANOVA). (D) Prolonged bleeding time in MASP-1/3 (M-1) null mice. Y axis shows minutes The experiment was performed as in Figure 3A using 5 MASP-1/3 (M-1) null and 3 MASP-2/sMAP (M-2) null mice. Bars indicate mean $\pm$ SE. *, $p = 0.0245$ (Wilcoxon/Kruskal-Wallis tests).

Figure 3.

MBL is involved with hemostasis. (A) Prolonged bleeding time in MBL null mice. The duration of bleeding after tail tip excision was recorded and expressed in min. 13 WT, 12 MBL null, 6 MBL null + rhMBL, and 7 C3 null mice were used. Bars indicate mean $\pm$ SE. *, $p < 0.05$ and **, $p < 0.005$ (ANOVA). (B) Platelet aggregation test. Platelets of WT and MBL null mice were tested for adenosine diphosphate (ADP)-stimulated aggregation.

Figure 4.

MBL null mice develop a coagulation anomaly, including disseminated intravascularcoagulation (DIC) following infection. (A) Survival curve following *S. aureus* infection. Mice were intravenously inoculated with *S. aureus* 1×10^7 cfu/0.2 ml. 32 wild type (WT) mice and 30 MBL null mice were used. (B) FeCl_3 induced thrombogenesis. Carotid blood flow expressed as changes from baseline. $\circ$, wild type (WT); Δ , MBL null; $\bullet$, WT + *S. aureus*; $\blacktriangle$ MBL null + *S. aureus*. Representative data from three experiments are shown. (C) Waveform from activated partial thromboplastin time (aPTT) of infected WT and MBL null mice. Citrated plasma from 5 mice, in order to reduce sampling errors, were pooled and aPTT was performed. Numbers and arrows indicate slope_1 (% T/sec). The slope_1 normal range is -0.10 to $+0.05$. (D) Cytokines and bacterial loads in blood. $\text{TNF-}\alpha$, IL-6 and bacterial load in blood following *S. aureus* infection. 10 WT and 12 MBL null mice were used. Bars indicate mean $\pm$ SE. *, $p < 0.01$ (Student t-test).

Figure 5.

MBL null mice have high mortality and develop liver damage following *S. aureus* infection. (A) Bacterial load in the liver, Alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Samples were collected at 15 hrs following infection. 10 WT and 12 MBL null mice were used. Bars indicate mean $\pm$ SE. *, $p < 0.01$ (Student's t-test). (B) Bacterial load in the kidney, BUN and creatinine. Samples were collected at 15 hrs following infection. 10 WT and 12 MBL null mice were used. Bars indicate mean $\pm$ SE. *, $p < 0.05$ (Student's t-test).

Figure 6. Updated diagram of fibrinogen turnover demonstrating a role for the MBL-MASPs complex in coagulation.

Table 1

Baseline clotting factors and parameters.

Parameters	Genotype	
	WT	MBL null
PT (sec)	10.3 ± 0.3	10.3 ± 0.1
aPTT (sec)	22.4 ± 1.6	22.4 ± 3.5
Fibrinogen (mg/dL)	434	491
Platelets (th/cmm)	755 ± 64	761 ± 131

Note.. Three mice in each group were used except for fibrinogen, for which plasma of three mice were pooled for the analysis. Data are expressed as mean ± SE.