Mannose-Binding Lectin Is a Regulator of Inflammation That Accompanies Myocardial Ischemia and Reperfusion Injury[1]

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The mannose-binding lectin (MBL), a circulating pattern recognition molecule, recognizes a wide range of infectious agents with resultant initiation of the complement cascade in an Ab-independent manner. MBL recognizes infectious non-self and altered self in the guise of apoptotic and necrotic cells. In this study, we demonstrate that mice lacking MBL, and hence are devoid of MBL-dependent lectin pathway activation but have fully active alternative and classical complement pathways, are protected from cardiac reperfusion injury with resultant preservation of cardiac function. Significantly, mice that lack a major component of the classical complement pathway initiation complex (C1q) but have an intact MBL complement pathway, are not protected from injury. These results suggest that the MBL-dependent pathway of complement activation is a key regulator of myocardial reperfusion ischemic injury. MBL is an example of a pattern recognition molecule that plays a dual role in modifying inflammatory responses to sterile and infectious injury. ***The Journal of Immunology,*** **2005, 175: 541–546.**

Injury following restoration of blood flow after transient ischemia in the heart is known as postmyocardial ischemia reperfusion (MI/R)[3] injury. Whereas reestablishment of oxygenated blood flow is critical for myocardial salvage, the accompanying reperfusion results in significant morbidity and mortality. The sterile injury evokes intrinsic changes in myocytes that become targets for the innate immune system (1). In human and animal models, the complement system has been evoked as a key effector cascade in mediating MI/R injury.

The complement system is an important component of the humoral innate immune system (2, 3). Complement activation is catalyzed by recognition of Ags by Abs (classical pathway), foreign surfaces (alternative pathway), or exposed carbohydrates (lectin pathway). More than a decade ago, Weissman et al. (4) demonstrated that complement components are deposited in reperfused myocardium. Subsequently, animal models of ischemia/reperfusion of the gut, kidney, and muscle indicate that the complement system is a key mediator of postischemic damage in other organ systems, further establishing that the complement system is required for manifestation of reperfusion injury (5–9). For injury to occur, there must be direct recognition of neo-Ags that are exposed as a result of relative hypoxia.

Classical pathway activation usually requires Ab recognition by the subcomponent, C1q; however, C1q may also recognize apoptotic cells in the absence of Ab (10–12). Mannose-binding lectin (MBL) and ficolins are carbohydrate recognition subcomponents of the lectin pathway, each of which may initiate complement activation. The lectin or classical pathway initiation then may form a structurally common C3 convertase (e.g., C4b2a). Alternative complement pathway initiation appears not to require MBL, Ab, or C1q but instead results from direct binding of C3b to target surfaces. Alternative pathway activation may form another structurally different C3 convertase (e.g., C3bBb). Thus, all complement pathways converge at the cleavage of the third complement component, C3 (2, 3, 13).

What has been more controversial and not fully resolved is the relative importance of classical Ab-dependent complement pathway, the alternative complement pathway and the more recently discovered lectin pathway of complement activation as triggers for reperfusion injury. It may well be that as yet undetermined tissue-specific ligands are induced by hypoxia and hence form distinct targets for MBL and natural Ab. In this regard, recent animal models of intestinal reperfusion injury indicate a key role for a specific self-reactive IgM Ab as the trigger for complement-mediated injury (14). In contrast, work from our laboratory (G.L.S.) suggests that MBL recognizes myocardial neo-Ags following MI/R in rats (15, 16), which leads to lectin pathway activation as the key pathway in MI/R injury.

Although these rat studies (15, 16) suggest a role for MBL-dependent injury in MI/R, we were unable to dissect out the role of the classical pathway in this model. Another shortcoming of our previous studies is that rodents have two homologous MBL proteins, MBL-A and MBL-C, both of which may initiate activation of the MBL-dependent portion of the lectin complement pathway (17). The conclusions of the previous rat study were based on inhibition studies using an Ab that inhibited rat MBL-A only (15). In the present study, we used genetically altered mice to investigate the role of the classical pathway and MBL in a mouse MI/R model.

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[3] Abbreviations used in this paper: MI/R, myocardial ischemia and reperfusion injury; MBL, mannose-binding lectin; WT, wild type; KO, knockout; LAD, left anterior descending artery; AAR, area at risk; rhMBL, recombinant human MBL; LV, left ventricle.

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Materials and Methods

Mice

All animals used in MI/R experiments were male mice aged 8–12 wk old. C57BL/6 (wild type (WT)), C1qa$^{-/-}$ (C1q knockout (KO)) (18), C2 and factor B$^{-/-}$ (C2/fB KO) (19) factor D$^{-/-}$ (fD KO) (20), and C1q/fD$^{-/-}$ (lectin or C1q/fD KO) mice were obtained from Charles River Laboratories. C1q KO, lectin, fD KO, and C2/fB KO mice were back-crossed 8–10 generations on the C57BL/6 background, whereas the MBL null mice are still on a mixed background with ~70–80% C57BL/6. MBL null animals (17) were obtained from Hybridon. All procedures were reviewed and conducted in accordance to the Institute's Animal Care and Use Committee (IACUC). Experiments were performed according to the standards and principles set forth in the National Institutes of Health (Guide for the Care and Use of Laboratory Animals—DHHS publication no. (NIH) 85-23, revised 1985).

Experimental MI/R

Mice were anesthetized with sodium pentobarbital (60 mg/kg) for intubation, then ventilated with positive pressure on a SAR small animal ventilator (CWE) and maintained under anesthesia with isoflurane (1–3 MAC). After creating a diagonal incision through the skin of the left chest, the overlying chest muscles were retracted using 5-0 black braided silk suture (Ethicon). The chest was opened within the third intercostal space, and the chest wall retracted using 5-0 black braided silk suture. A 8-0 black braided silk suture (U.S. Surgical) was passed underneath the left anterior descending branch (LAD) coronary artery ~2 mm from the tip of the left atrium. A 1- to 2-mm piece of 0-0 suture (Deknatel) was placed on the LAD, and the ligation tightened to occlude the artery. After 30 min of ischemia, the ligation was loosened and the 0-0 suture removed. The chest wall was closed using 5-0 black braided silk suture (Ethicon). The overlying chest muscles were allowed to retract, and the skin was sutured using 5-0 black braided silk suture (Ethicon). The animal was removed from the respirator and allowed to reperfuse for 3 h. EKG changes (e.g., ST segment) were monitored and used to establish ischemia and reperfusion.

Echocardiography

Mice underwent experimental MI/R as described above but reperfused for 3 or 6 h. Echocardiography was performed using a Hewlett Packard Sonos 5500 with a 12 MHz animal transducer (Agilent Technologies). Volume and percentage of ejection fraction were calculated from long axis area measurements of the left ventricle (LV) (21). As calculated by one-way ANOVA, there was no statistically significant difference in ejection fraction measured within each experimental MI/R group between the 3 and 6 h reperfusion time points. Thus, we pooled the analyses for the two time periods.

Measurement of infarct size and area at risk (AAR)

Following reperfusion, mice were anesthetized with sodium pentobarbital and 200 μl of heparin (100 U/ml, i.v.) injected. The chest cavity was opened and the LAD coronary artery ligation tightened. The right atrium was removed, the aorta was cannulated with polyethylene-10 tubing, and the heart was flushed with PBS (0.5–1 ml). The heart was then perfused with 100–200 μl of 1% Evan's blue (Acros). Hearts were excised and cross-sectioned from base to apex into 1-mm slices using a coronal acrylic matrix (Roboz). Sections were placed into 6-well plates (Costar) and incubated in 1% triphenyltetrazolium chloride (Acros) at 37°C for 15 min as we have described (16, 22). Following triphenyltetrazolium chloride staining, sections were fixed in 10% formalin (Sigma-Aldrich) at 4°C overnight. The apical side of each slice was imaged and measured using a Nikon SMZ800 stereoscopic zoom microscope and SPOT Imaging software (Diagnostic Instruments). The size of the infarction was determined by calculating total areas of the LV, infarct, nonischemic tissue, and ischemic AAR. As calculated by one-way ANOVA, there was no statistically significant difference in AAR (as a percentage of the LV) between experimental animals or groups. Infarct was expressed as a percentage of the LV or of the ischemic AAR.

Anti-C5, C2, MBL, and anti-MBL treatment

Anti-mouse C5 mAb BB5.1 was administered by penile vein injection at 50 mg/kg, 5 min before ischemia (7). Human C2 (Advanced Research Technologies) was administered by penile vein injection at a dose of 25 μg, 10 min before ischemia at a dose demonstrated to reconstitute functional classical complement pathway activity (23). Recombinant human MBL (rhMBL; a gift from NatImmune) was administered i.v. or i.p. 10 min before ischemia at 75 μg (17). Alternatively, rhMBL was combined with a humanized functionally inhibitory anti-human MBL mAb 3F8 (15) at ~1:1 (molar ratio), and injected (i.v.) 10 min before ischemia.

Microscopy

Following experimental MI/R, hearts were removed and placed in OCT and frozen at −20°C. Sections (5–6 μm) were stained for C3 and C1q using FITC-conjugated polyclonal goat anti-mouse C3 Ab (ICN Pharmaceuticals) and rat anti-mouse C1q mAb (Cell Sciences) using a secondary goat anti-rat IgG FITC (Jackson ImmunoResearch Laboratories). Images were taken on a Nikon Eclipse E600 fluorescence microscope, and analyzed using SPOT Imaging software (Diagnostic Instruments). Immunohistochemistry for MBL deposition following MI/R used Cy3-labeled rhMBL injected (75 μg, i.v.) 10 min before ischemia in MBL null mice (17). Colocalization of C3 was performed on cryosections analyzed for Cy3-MBL staining as described above.

Statistics

All statistical analysis of data was performed using SigmaStat software version 3.0 (SPSS). All data were evaluated using one-way ANOVA and post hoc analysis using the Student-Newman-Keuls method, then expressed as the mean ± SE.

Results

Infarct analysis was performed on hearts sectioned from base to apex as shown in Fig. 1*a* following MI/R. AAR (AAR/LV · 100) was 55 ± 4%, 50 ± 4%, 48 ± 4%, 53 ± 4%, 56 ± 7%, and 51 ± 8% for WT, C2/fB KO, C2/fB KO + C2, C1q KO, MBL null, and MBL null + rhMBL groups, respectively. Thus, the amount of LV undergoing ischemia between groups was not significantly different. Compared with WT mice, C2/fB KO mice are significantly protected from infarction following MI/R (Fig. 1, *b* and *c*, respectively). Restoration of C2 to C2/fB KO mice reconstitutes only the classical and lectin complement pathways but not the alternative pathway and significantly exacerbated MI/R-dependent tissue injury (Fig. 1*d*). This amount of C2 functionally restores the classical hemolytic pathway activity in these mice (23). A summary of infarct size analysis (Fig. 1*e*) demonstrated that inhibition of complement activation in the C2/fB KO mice prevented MI/R injury compared with WT mice. Thus, C2 is critically important for the generation of infarction.

C2 is necessary for the function and activation of both the classical and lectin complement pathways. The development of MBL null mice (yet have intact C1q-dependent classical pathway) and C1q KO mice (yet have an intact MBL complement pathway) enabled us to discriminate further between events initiating complement activation and tissue injury following MI/R. Importantly, we show that mice that lack C1q are not protected from infarction following MI/R compared with WT mice (Fig. 1*b* vs Fig. 2*a*). However, complement plays an important role in tissue injury in C1q KO mice following MI/R, because inhibition of C5 with BB5.1 (Alexion Pharmaceuticals) significantly attenuated myocardial infarction (Fig. 2, *b* and *e*). Because myocardial infarction was dependent on C2 (Fig. 1*e*) but C1q independent (Fig. 2*e*), we performed MI/R in MBL null mice. The absence of MBL afforded animals significant protection against infarction following MI/R (Fig. 2, *c* and *f*). MI/R injury was enhanced by restoring a functional MBL pathway with rhMBL (MBL null + rhMBL) before ischemia (17), resulting in a significant increase in infarction (Fig. 2, *d* and *f*).

Examination of ventricular function by echocardiography of experimental MI/R groups at the end of reperfusion periods mirrored our infarct analysis. Compared with sham-operated mice or C2/fB KO mice, WT and C1q KO mice demonstrated significant decreases in left ventricular ejection fractions following MI/R (Fig. 3*a*). However, MBL null mice showed significant retention of left ventricular function following MI/R (Fig. 3*b*). Left ventricular function was severely impaired following restoration of the MBL/

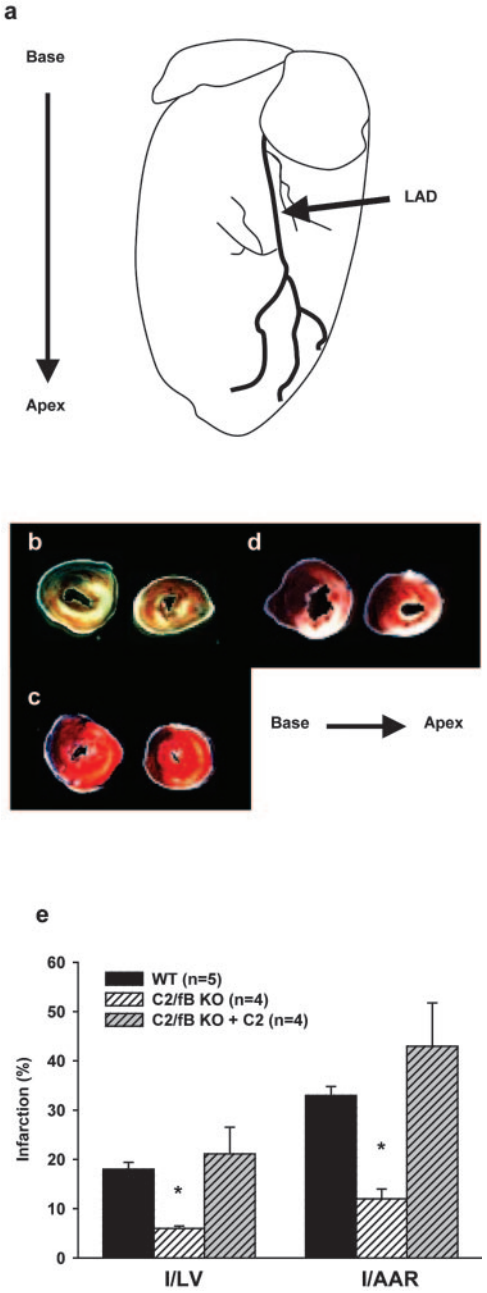

FIGURE 1. Myocardial infarction following MI/R in animals lacking C2 and factor B. *a*, Diagram outlining the direction of myocardial sections/slices, WT (*b*), C2/fB KO (*c*), C2/fB KO + C2 (*d*). Evans blue dye (blue) nonischemic area, whereas red and white demonstrate AAR. Within the AAR myocardium, red-stained tissue denotes non-infarcted area, whereas white denotes infarcted tissue. The percentage of infarction (*e*) was calculated from total weights of the LV, area of infarct (I), nonischemic tissue, and AAR. Infarct was expressed as a percentage of the LV (I/LV · 100) or of the AAR (I/AAR · 100). Bars and brackets represent mean ± SE. *, $p < 0.05$ compared with WT or C2/fB KO + C2.

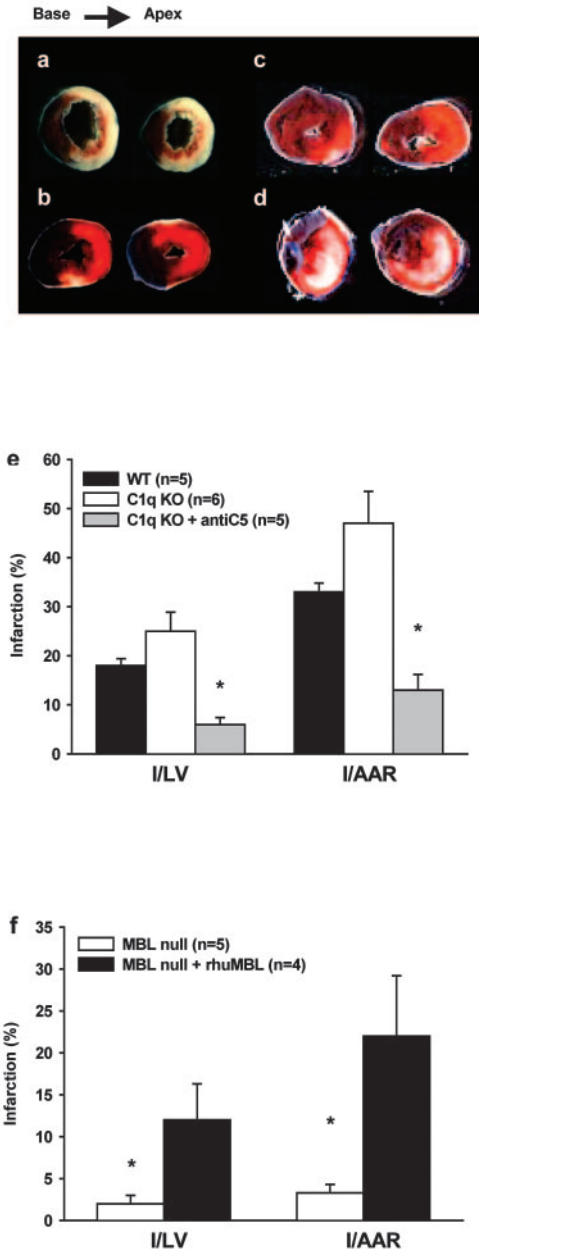

FIGURE 2. Infarction following MI/R in mice lacking the classical complement pathway or MBL/lectin pathway. Experimental MI/R was performed by LAD ligation for 30 min, followed by 3 h of reperfusion in the following animals: C1q KO (*a*), C1q KO + antiC5 (*b*), MBL null (*c*), MBL null + rhMBL (*d*). Data collected from experimental C1q KO and MBL null mice are summarized in *e* and *f*, respectively. Infarction was calculated as described above. Bars and brackets represent mean ± SE. *, $p < 0.05$ compared with C1q KO or WT (*e*) or MBL null + rhMBL (*f*).

lectin pathway with rhMBL (Fig. 3*b*). Furthermore, treatment with anti-human MBL mAb (humanized mAb 3F8) concomitant with administration of rhMBL attenuated ventricular dysfunction (Fig. 3*b*). Confirming our findings that the lectin pathway initiates injury following MI/R, mice lacking the alternative pathway of complement activation (fD KO; Fig. 3*a*) were not protected from left ventricular dysfunction following MI/R. Importantly, mice having only a functionally active lectin pathway (lectin mice lack both classic and alternative pathways of complement activation) demonstrated significant decreases in left ventricular ejection fractions compared with either sham-operated animals or MI/R in C2/fB KO mice (Fig. 3*a*).

C1q and C3 are deposited in myocardial infarcts. Compared with sham-operated mice (Fig. 4*a*), C1q was deposited in the myocardium following MI/R in WT mice (Fig. 4*a*), but C1q KO mice are not protected from MI/R injury. Furthermore, C3 was deposited on infarcted tissue and associated with border zone infarction/AAR myocardium of WT mice, a pattern similar to that observed in C1q KO mice (Fig. 4*b*). These results suggest C1q-independent

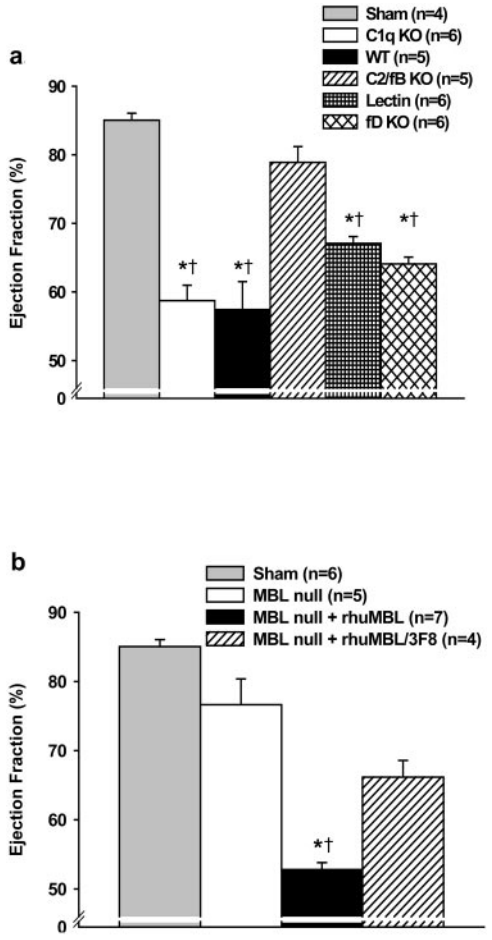

FIGURE 3. Left ventricular function following MI/R. Echocardiography was performed and ejection fraction (%) was calculated as described in *Materials and Methods*. *a*, Summary of ejection fraction data for WT, C1q KO, C2/fB KO, lectin, and fD KO mice (∗, $p \leq 0.001$ compared with sham; †, $p \leq 0.001$ compared with C2/fB KO). *b*, Summary of ejection fraction data for MBL null experimental groups (∗, $p \leq 0.001$ compared with sham and MBL null animals; †, $p \leq 0.001$ compared with MBL null + rhMBL/3F8). Bars and brackets represent the mean ± SE.

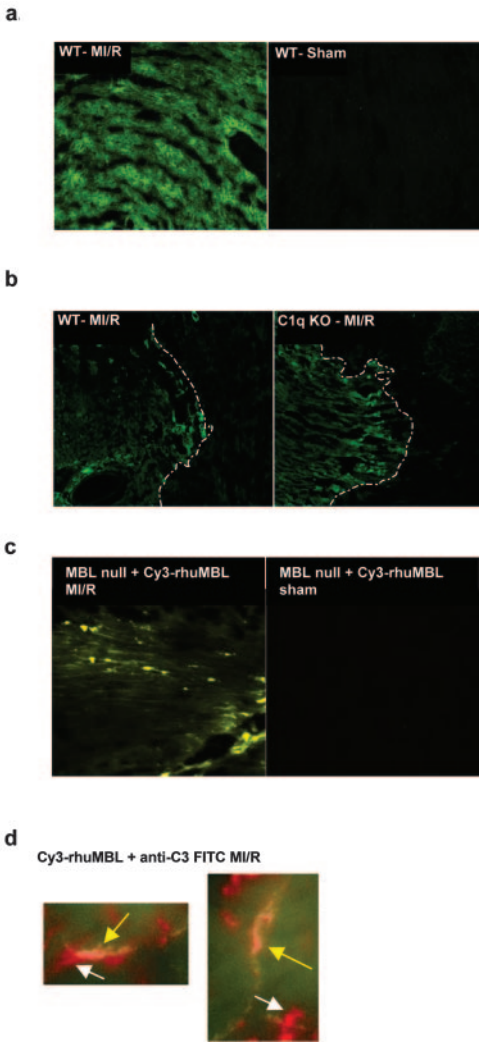

FIGURE 4. Complement activation and deposition. *a*, C1q deposited in the myocardium following MI/R. We observed increased C1q deposition in the myocardium of WT mice compared with sham operated mice. *b*, C3 deposited in the myocardium following MI/R. We observed C3 deposition in the AAR following MI/R in WT and C1q KO mice. The dotted white line in each figure demonstrates distinct C3 deposition in the AAR of ischemia (left side of the white line) compared with the adjacent nonischemic region (right hand side of each figure). *c*, MBL deposited in the myocardium following MI/R. MBL null mice were injected with Cy3-rhMBL before ischemia or sham operation procedure. We observed increased MBL staining within the AAR compared with the nonischemic area, and compared with little or no Cy3-rhMBL deposited in sham-operated myocardium. *d*, MBL colocalized with C3 deposition in the myocardium. Cy3-rhMBL (red/orange–white arrow) and C3 (green) colocalized (pink–yellow arrow) within the myocardium following MI/R.

complement activation following experimental MI/R. We surmised that C3 deposition is likely a result of MBL-dependent lectin complement pathway activation because MBL was deposited on infarcted tissue following experimental MI/R (Fig. 4*c*) thus confirming our previous results in rat MI/R (15, 16). Importantly, MBL and C3 colocalization was observed (Fig. 4*d*) suggesting MBL-dependent complement activation following experimental MI/R.

Discussion

Using mice deficient in key components of complement activation, and monoclonal Abs specific for MBL, we have attempted to define the relative contribution of the complement pathways in MI/R-induced injury. Initial examination of infarct size analysis demonstrated that inhibition of complement activation in C2/fB KO mice prevented MI/R injury. Thus, recognition of self Ag following MI/R injury is an early (e.g., above C3) event in complement pathway initiation. Additionally, the importance of complement and its downstream mediators like C5 in this process is demonstrated by significantly attenuated myocardial infarction by mAb inhibition of C5. It should be noted that cardiac function and infarct studies were performed while mice were under sustained isoflurane-based anesthesia, which has been shown to afford some protection against damage following MI/R (24–26). All groups received the same amount of anesthesia/isoflurane, and the AAR in all groups was not statistically different. Thus, complement plays an important role in the development of MI/R injury.

Mice that lack C1q appear to be afforded no protection from infarction following MI/R compared with WT mice. Given that myocardial infarction was dependent on C2 but C1q independent, we performed MI/R in MBL null mice. We showed that the absence of MBL imparts significant protection against infarction following MI/R, but injury was restored by addition of rhMBL (MBL null + rhMBL) before ischemia. Thus, MBL and/or the lectin complement pathway are responsible for initiating MI/R injury.

Echocardiographic analysis of cardiac function following MI/R confirmed our ex vivo assessment of infarct size. As expected, WT and C1q KO mice demonstrated significant decreases in left ventricular ejection fractions, but ejection fraction analysis in MBL null mice showed significant retention of left ventricular function following MI/R. However, left ventricular function was severely impaired following restoration of MBL-mediated lectin pathway activation with rhMBL. Treatment with anti-human MBL mAb (humanized mAb 3F8) concomitant with administration of rhMBL, attenuated ventricular dysfunction. Furthermore, C1q/fD deficient (lectin) mice having only the lectin pathway of complement activation (e.g., lacking both the classical and alternative complement pathways) demonstrated a significant decrease in left ventricular ejection fraction following MI/R. We also recognize that some gene-targeted strains may compensate for the deficiency of one pathway by strengthening the functional state of another activation pathway. Collectively, these findings demonstrate a significant contribution of MBL to tissue injury and left ventricular function, and the therapeutic potential of MBL blockade for the prevention of MI/R-associated ventricular damage.

Interestingly, fD KO mice lacking the alternative pathway of complement injury demonstrated significant decreases in LV ejection fractions compared with sham-operated or C2/fB KO mice following MI/R. This is in contrast to our previously reported findings in fD KO mice, which suggested that the alternative pathway likely amplified complement activation following gastrointestinal ischemia and reperfusion injury (8). We believe that the alternative pathway amplifies this sterile injury process following MI/R as we noted a tendency for decreased ejection fractions in those mice having lectin and alternative pathways (e.g., WT and C1q KO) compared with mice not having an alternative pathway (e.g., lectin and fD KO) but a functional lectin pathway (see Fig. 3*a*). Furthermore, similar to these findings, we recently demonstrated that the initiation of intestinal injury following I/R is also C1q-independent but MBL-dependent (23).

Our hypothesis is that MBL-dependent lectin pathway activation, but not the classical Ab-dependent pathway, plays a key role in myocardial reperfusion injury in this mouse model. A role for the classical pathway in MI/R relied largely on C1q and Ig deposition in the myocardium, together with inhibitor studies using C1-INH (27–29). However, C1-INH is not specific for the classical pathway and also inhibits mannose-associated serine protease 2, a key serine protease of the MBL complement pathway (30, 31). We, like others, confirm the deposition of C1q in damaged myocardium (32, 33); however, we did not observe cardioprotection in C1q KO mice following MI/R. C1q appears to play an important role in the clearance of cellular debris and apoptotic bodies (10, 11) and both C1q and MBL bind apoptotic cells (34, 35). Apoptosis following MI/R significantly contributes to myocardial injury (12, 22, 36, 37), perhaps via glycogen synthase kinase-3β regulation of mitochondrion permeability (38, 39). It may well be that C1q binding to apoptotic cells is anti-inflammatory and that MBL binding mediates proinflammatory signals in this setting. Further studies on the relative role of MBL and C1q under I/R conditions are warranted to more clearly define immunological responsiveness toward endogenous Ags following ischemia and reperfusion.

This study additionally implicates a beneficial biological consequence for low/moderate circulating MBL levels in disease. Functional inhibition of MBL in normal animals or absence of MBL in humans undergoing oxidative stress have also demonstrated the importance of this innate immune molecule to modulate inflammation and tissue injury (16, 40). MBL-deficient patients undergoing thoracic abdominal aortic aneurysm repair do not activate complement and express less proinflammatory markers following surgery (40). Restoration of MBL to normal levels in MBL-deficient patients induces complement activation and inflammation back to that observed in MBL-sufficient patients. Additionally, human biopsies of posttransplant acute renal failure show lectin complement pathway activation, and MBL deposition early in ischemically injured kidneys (41). Collectively these data demonstrate the potential pathophysiologic role of MBL during conditions of ischemia and reperfusion in a variety of vascular beds. Thus, specific blockade of MBL or inhibition of the lectin complement pathway may represent a therapeutically relevant strategy for the prevention of MI/R-associated cardiac damage.

The innate immune pattern recognition molecule MBL is important in pathogen clearance, and deficiencies of this opsonin complicates resolution of bacterial infections (17). However, the absolute requirement for MBL in initiation of complement activation and resulting injury in this model of MI/R is clear. We have yet to address the role of additional pattern recognition molecules, including ficolins, to the residual injury process in the absence and/or presence of MBL following MI/R. Our findings demonstrate that the innate immune system, in addition to foreign Ag recognition, recognizes altered "self" Ags under I/R conditions and contributes to tissue injury.

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Disclosures

The authors have no financial conflict of interest.

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