# Targeting of mannan-binding lectin-associated serine protease-2 confers protection from myocardial and gastrointestinal ischemia/reperfusion injury

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**Complement research experienced a renaissance with the discovery of a third activation route, the lectin pathway. We developed a unique model of total lectin pathway deficiency, a mouse strain lacking mannan-binding lectin-associated serine protease-2 (MASP-2), and analyzed the role of MASP-2 in two models of post-ischemic reperfusion injury (IRI). In a model of transient myocardial IRI, MASP-2–deficient mice had significantly smaller infarct volumes than their wild-type littermates. Mice deficient in the downstream complement component C4 were not protected, suggesting the existence of a previously undescribed lectin pathway-dependent C4-bypass. Lectin pathway-mediated activation of C3 in the absence of C4 was demonstrated in vitro and shown to require MASP-2, C2, and MASP-1/3. MASP-2 deficiency also protects mice from gastrointestinal IRI, as do mAb-based inhibitors of MASP-2. The therapeutic effects of MASP-2 inhibition in this experimental model suggest the utility of anti–MASP-2 antibody therapy in reperfusion injury and other lectin pathway-mediated disorders.**

The therapeutic benefit of using complement inhibitors to limit myocardial ischemia/reperfusion injury (MIRI) was convincingly demonstrated in an animal model two decades ago: Recombinant sCR1, a soluble truncated derivative of complement receptor type-1 (CR1), given intravenously to rats, reduced infarct volume by more than 40% (1). In a subsequent clinical trial, administration of sCR1 to patients with MI prevented contractile failure in the postischemic heart (2). The mechanism of complement activation in ischemic tissue has not, however, been defined, mainly because of the lack of appropriate experimental models, the limited understanding of the molecular processes that lead to complement activation on oxygen-deprived cells, and the cross-talk between the different complement activation pathways.

Three different pathways initiate the complement cascade: the classic, alternative, and lectin pathways. The classic pathway recognition subcomponent C1q binds to a variety of targets, most prominently immune complexes. Binding of C1q to immune complexes converts the associated C1r zymogen dimer to its active form, which cleaves and activates C1s. C1s converts C4 into C4a and C4b, and then cleaves C4b-bound C2 to form the C3 convertase C4b2a. This complex converts the abundant plasma component C3 into C3a and C3b. In the alternative pathway, spontaneous low-level hydrolysis of C3 results in deposition of protein fragments onto cell surfaces, triggering complement activation on foreign cells, but cell-associated regulatory proteins on host tissues avert activation, thus preventing self-damage. Activation of the lectin pathway is initiated by the binding of a multimolecular lectin pathway activation complex to pathogen-associated molecular patterns, mainly carbohydrate structures present on bacterial, fungal, or viral pathogens, or aberrant glycosylation patterns on apoptotic, necrotic, malignant, or oxygen-deprived cells (3–6). In man, the lectin activation pathway is driven by five different carbohydrate recognition subcomponents: MBL2 (mannan-binding lectin 2); three ficolins, namely L-ficolin, H-ficolin, and M-ficolin; and the recently discovered C-type lectin CL-11 (7). The recognition subcomponents form complexes with three serine proteases, MASP-1, -2, and -3 (MBL-associated serine proteases 1, 2, and 3), and MAp19, a nonenzymatic, truncated product of the *MASP2* gene, which may regulate lectin pathway activation by competing for the binding of MASPs to the carbohydrate recognition complexes (5–12). Two forms of MBL, MBL-A and MBL-C, as well as ficolin A and CL-11, form lectin activation pathway complexes with MASPs in mouse plasma (5, 7, 11). There is strong evidence that only MASP-2 is required to translate binding of the lectin pathway recognition complexes into complement activation (8, 13–15). This conclusion is supported by the phenotype of mice deficient in MASP-1 and -3 that, apart from a delay in the onset of lectin pathway-mediated complement activation in vitro, have normal lectin pathway activity. Reconstitution of MASP-1 and -3–deficient serum with recombinant MASP-1 overcomes the delay in lectin pathway activation, implying that MASP-1 may facilitate MASP-2 activation (16). A recent study has shown that MASP-1 (and probably also MASP-3) are required to convert the alternative pathway activation enzyme Factor D from its zymogen form into its enzymatically active form (17). The physiological importance of this process is underlined by the absence of alternative pathway functional activity in plasma of MASP-1/3–deficient mice.

The recently generated mouse strains with combined targeted deficiencies of the lectin pathway carbohydrate recognition subcomponents MBL-A and MBL-C may still initiate lectin pathway activation via the remaining murine lectin pathway recognition subcomponents ficolin A (18) and CL-11 (7). The absence of any residual lectin pathway functional activity in MASP-2–deficient

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mice delivers a conclusive model to study the role of this effector arm of innate humoral immunity in health and disease.

The availability of C4 and MASP-2–deficient mouse strains allowed us to define a unique MASP-2–dependent, C4-independent route of C3 activation. The essential contribution of this unique lectin pathway-mediated C4-bypass to postischemic tissue loss is underlined by the fact that MASP-2–deficient mice are protected from MIRI, whereas C4-deficient mice are not.

## Results

**Generation of MASP-2–Deficient Mice.** To examine the role of MASP-2 in lectin pathway activation in vitro and in vivo, we generated a MASP-2–deficient mouse line using a conventional gene-targeting vector and confirmed replacement of exons 11 and 12 (encoding the C-terminal part of the CCPII-domain and the serine protease domain) with the neomycin cassette in the targeted murine *Masp2* allele. The absence of MASP-2 mRNA and protein was confirmed by Northern blotting, qRT-PCR, ELISA, and Western blotting. MAp19 mRNA was up-regulated twofold, but levels of other complement components were unaffected (*SI Materials and Methods*).

**MASP-2 Is Essential for Lectin Pathway Functional Activity.** In vitro analysis of MASP-$2^{-/-}$ plasma showed a total absence of lectin pathway-dependent C4 cleavage on mannan- and zymosan-coated surfaces (Fig. 1*A*). C4 cleavage was gradually restored by reconstituting MASP-$2^{-/-}$ plasma with recombinant mouse MASP-2 (rMASP-2). Addition of iMASP-2 (in which the active serine residue is substituted by alanine to disrupt proteolytic activity) had no effect (Fig. 1*B*). No C3 deposition was observed on mannan-coated surfaces using MASP-2–deficient plasma. In C1q-depleted plasma, C3 deposition was slightly lower than in WT plasma ($LP_{50}$ ~0.25% for WT plasma, ~0.4% for C1q-depleted plasma), consistent with a 25 to 30% consumption of C4 that accompanies the depletion process (Fig. 1*C*). The total deficiency of lectin pathway activity, however, leaves the other complement pathways intact: MASP-$2^{-/-}$ plasma can still activate complement via the classical (Fig. 1*D*) and the alternative pathway (Fig. 1*E*). The somewhat lower classic pathway activation in MASP$2^{-/-}$ plasma may be because of the loss of a minor contribution of the lectin pathway to C3 activation on immune complex coated ELISA plates.

**Critical Contribution of the Lectin Pathway to Inflammatory Tissue Loss in MIRI.** To study the contribution of the lectin pathway to MIRI, we compared MASP-$2^{-/-}$ mice and WT littermate controls in a model of MIRI using transient ligation and reperfusion of the left anterior descending branch of the coronary artery (LAD). A typical result is shown in Fig. 2*A*. In the horizontal sections of the infarcted hearts, the nonperfused infarct areas (colorless) are significantly smaller in hearts of MASP-$2^{-/-}$ mice than in WT controls. The measurements shown in Fig. 2 *B* and *C* demonstrate that MASP-$2^{-/-}$ mice have a significant reduction in MIRI-induced myocardial damage. Fig. 2*B* depicts the infarct sizes and the area at risk (AAR) (as percentages of total myocardial volume of the left ventricle) in MASP-$2^{+/+}$ and MASP-$2^{-/-}$ mice and Fig. 2*C* shows these values plotted against one another for individual mice, clearly demonstrating that MASP-2–deficient animals are protected ($P < 0.01$). To exclude the possibility that the difference in infarct size is caused by a lower susceptibility of the myocardial tissue to ischemic damage in MASP-$2^{-/-}$ mice, we prepared and reperfused hearts of MASP-$2^{-/-}$ and MASP-$2^{+/+}$ mice in the Langendorff mode (19). In contrast to the plasma-perfused hearts shown in Fig. 2*C*, results for the buffer perfused hearts show no difference in infarct sizes between the two groups (Fig. 2 *D* and *E*).

**Absence of C4 Has No Impact on Inflammatory Tissue Loss in MIRI.** Using the same procedure, we assessed the impact of C4 deficiency on infarct sizes following experimental MIRI. As shown in Fig. 2 *F* and *G*, identical infarct sizes were observed in C4-deficient mice and their WT littermates. This observation was unexpected, as it

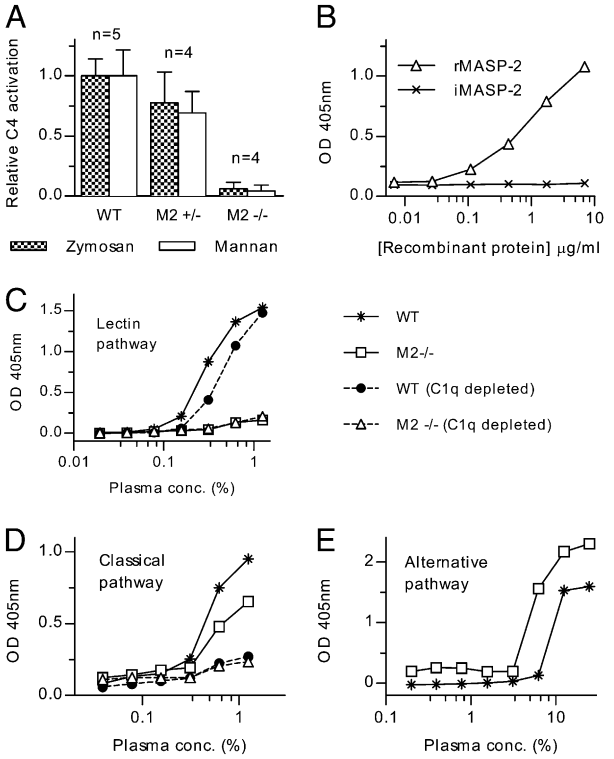


**Fig. 1.** MASP-2 is essential for lectin pathway functional activity. (*A*) Lectin pathway-dependent C4 activation: plasma from WT, heterozygous (M2$^{+/-}$), and homozygous (M2$^{-/-}$) MASP-2–deficient mice were assayed for C4 convertase activity on microtiter plates coated with mannan or zymosan. Serial dilutions of pooled WT plasma were included on each plate, standard curves plotted, and C4 deposition calculated as a proportion of that in the pooled WT serum. Results are means (± SD). (*B*) Reconstitution of plasma with recombinant MASP-2. Plasma (0.5%) pooled from four MASP-$2^{-/-}$ mice was preincubated with recombinant murine MASP-2 (rMASP-2) or inactive recombinant MASP-2 (iMASP-2). C4 convertase activity was then assayed on microtiter plates coated with mannan. (*C*) The classic pathway makes no significant contribution to C3 deposition on mannan: diluted mouse sera were added to microtiter plates coated with mannan and C3b deposition assayed. (*D*) MASP-$2^{-/-}$ mice retain a functional classical pathway: C3b deposition was assayed on microtiter plates coated with immune complexes (generated in situ by coating with BSA then adding goat anti-BSA IgG). (*E*) MASP-2–deficient mice retain a functional alternative pathway: C3b deposition was assayed on zymosan coated microtiter plates under conditions that permit only alternative pathway activation (buffer containing $Mg^{2+}$ and EGTA). Results in *B* to *E* are means of duplicates and are typical of three independent experiments.

conflicts with the widely accepted view that the major C4 activation fragment, C4b, is an essential component of the classic and the lectin pathway C3 convertase, C4b2a. We therefore assessed whether a residual lectin pathway-specific activation of complement C3 can be detected in C4-deficient plasma.

**Lectin Pathway Can Activate Complement C3 in the Absence of C4 via a Unique MASP-2–Dependent C4-Bypass.** Encouraged by historical reports indicating the existence of a C4-bypass activation route in C4-deficient guinea pig serum (20), we analyzed whether C4-deficient mice have residual classic or lectin pathway activity by monitoring activation of C3 under pathway-specific conditions that exclude contributions of the alternative pathway. A residual C3 cleavage activity was observed in C4-deficient plasma when initiating complement activation via the lectin pathway. Addition of excess fluid-phase mannose blocked C3 activation in C4-deficient plasma completely. No lectin pathway-dependent C3 activation was detectable in MASP-$2^{-/-}$ plasma assayed in paral-

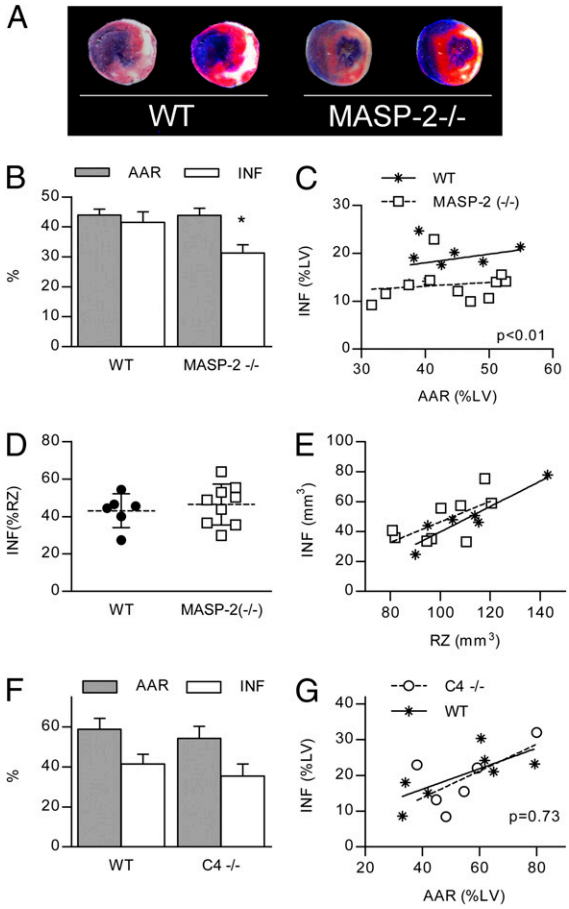


**Fig. 2.** MASP-2 deficiency significantly reduces MIRI-induced tissue loss. (*A*) MIRI-induced tissue loss following LAD ligation and reperfusion in MASP-2$^{-/-}$ mice and WT littermate controls. Hearts were subjected to 30 min of focal ischemia by occluding the LAD, followed by 120 min reperfusion. At the end of the experiments, the LAD was reoccluded and 5% Evans blue injected into the left ventricle (LV) to determine the risk zone (RZ). Hearts were excised, sectioned, and incubated at 37 °C for 30 min in 3% TTC solution, which is a metabolic stain. Nonperfused infarcted tissue remains colorless, nonperfused and noninfracted tissue (RZ) stains red, and normal perfused myocardium stains blue. Right panels are image-enhanced. (*B*) Risk volume as a percentage of total myocardial volume. The infarct size (INF), calculated as a percentage of the AAR, is significantly reduced in MASP-2$^{-/-}$ mice compared with their WT littermates. (*$P$ = 0.035, two-tailed Student $t$ test.) (*C*) Plots INF against AAR (both as percentages of total LV volume). MASP-2$^{-/-}$ mice show a highly significantly reduction in infarct size, regardless of the extent of the ischemic insult (AAR). (*D* and *E*) Hearts from MASP-2–deficient mice are not protected from reperfusion injury when myocardial ischemia is induced ex vivo in buffer-perfused hearts (Langendorff mode heart preparations), which demonstrates that the differences shown in *B* and *C* are caused by a plasma factor, not by a lower susceptibility of the myocardium of MASP-2$^{-/-}$ mice to ischemic damage. LAD ligations and reperfusion were conducted as in *B* and *C*, but in absence of plasma ($n$ = 10 per group in *B* to *E*). (*F* and *G*) C4$^{-/-}$ mice are not protected from MIRI-induced tissue loss. (*F*) INF and AAR in C4$^{-/-}$ mice ($n$ = 6) and WT littermate controls ($n$ = 7). (*G*) INF as a function of AAR, showing that C4$^{-/-}$ mice are as susceptible to MIRI as their WT littermates. (Error bars in *B*, *D*, and *F* show means ± SD; *C*, *E*, and *G* analyzed using ANCOVA).

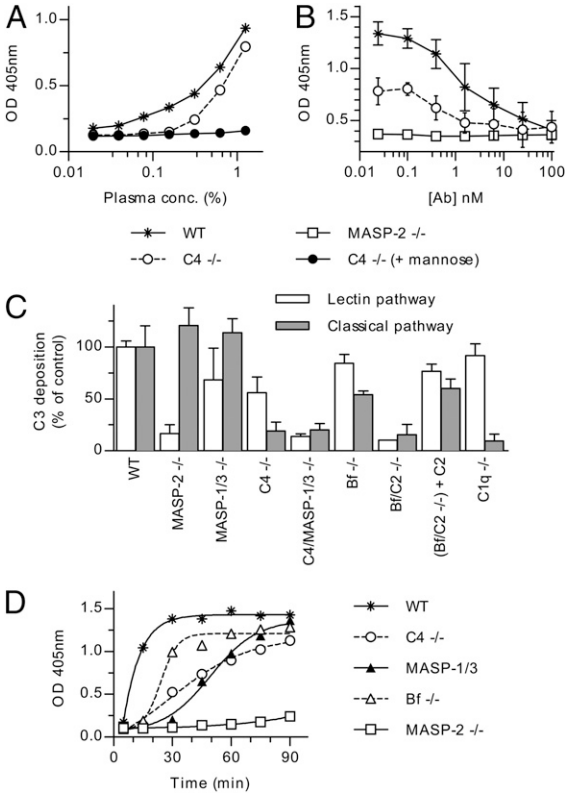


**Fig. 3.** Lectin pathway-dependent activation of C3 in the absence of C4. (*A*) The lectin pathway activates C3 in the absence of C4. C3b deposition was assayed on mannan-coated microtiter plates using recalcified WT and C4$^{-/-}$ plasma at concentrations prohibitive for alternative pathway activation (1.25% and below). Preincubating C4$^{-/-}$ plasma with excess (1 mg/mL) fluid-phase mannose before the assay completely inhibits C3 deposition. Results are typical of three independent experiments. (*B*) Inhibition of C3 deposition using anti–MASP-2 mAb. Plasma (1%) was mixed with various concentrations of anti-rat MASP-2 mAb AbyD 04211 (abscissa) and C3b deposition assayed on mannan-coated plates. Results are means (± SD) of four assays. (*C*) A comparative analysis of C3 convertase activity in plasma from complement deficient mouse strains tested under lectin or classic activation pathway-specific conditions. WT mice ($n$ = 6), MASP-2$^{-/-}$ mice ($n$ = 4), MASP-1/3$^{-/-}$ mice ($n$ = 2), C4$^{-/-}$ mice ($n$ = 8), C4/MASP-1/3$^{-/-}$ mice ($n$ = 8), Bf/C2$^{-/-}$ ($n$ = 2), and C1q$^{-/-}$ mice ($n$ = 2) were tested in parallel. Reconstitution of Bf/C2$^{-/-}$ plasma with 2.5 μg/mL recombinant rat C2 (Bf/C2$^{-/-}$ + C2) restored C3b deposition. Serial dilutions of pooled WT plasma were included on each plate, standard curves plotted, and C3 deposition calculated as a proportion of that seen in the pooled WT serum. Individual samples were assayed in duplicated at a concentration of 1% plasma. Results are means ± SD **$P$ < 0.01 (two-tailed $t$ test). (*D*) Kinetics of C3 convertase activity in plasma from various complement deficient mouse strains tested under lectin activation pathway-specific assay conditions (1% plasma, results are typical of three independent experiments).

lel, implying that this C4-bypass activation route of C3 is MASP-2–dependent (Fig. 3*A*).

To further corroborate these findings, we established a series of recombinant inhibitory mAbs isolated from phage display antibody libraries by affinity screening against recombinant human and rat MASP-2A. These tools allowed us to confirm the MASP-2 dependency of this unique lectin pathway-specific C4-bypass. As shown in Fig. 3*B*, AbyD 04211, an inhibitory monoclonal antibody, which selectively binds to mouse and rat MASP-2, inhibited activation of C3 in C4-deficient and WT mouse plasma in a concentration-dependent fashion, with similar $IC_{50}$ values.

An analogous lectin pathway-specific C4-bypass was discovered in plasma from a human donor with an inherited deficiency of both C4 genes (C4A and C4B), resulting in total absence of C4 (21). The MASP-2 dependency of C3 activation in human C4-deficient plasma was demonstrated using AbDH3, a monoclonal antibody that specifically binds to human MASP-2 and ablates MASP-2 functional activity. AbDH3 inhibited the deposition of C3b in both C4-sufficient and C4-deficient human plasma with comparable potency (*SI Results*).

To assess the role of other complement components in the C4-bypass activation of C3, we tested plasma from MASP-1/3$^{-/-}$ and Bf/C2$^{-/-}$ mice alongside MASP-2$^{-/-}$, C4$^{-/-}$, and C1q$^{-/-}$ plasma

(as controls) under both lectin and classic pathway-specific assay conditions. Substantial C3 deposition is seen in C4$^{-/-}$ plasma tested under lectin pathway-specific conditions, but not under classic pathway-specific conditions (Fig. 3*C*). In MASP-1/3$^{-/-}$ plasma, C3 deposition occurred in both lectin and classic pathway assays. Neither pathway was activated using plasma with a combined deficiency of C4 and MASP-1/3, or using C2/Bf$^{-/-}$ plasma. Reconstitution of C2/Bf$^{-/-}$ mouse plasma with recombinant C2, however, restored both lectin pathway and classic pathway-mediated C3 cleavage.

The kinetics of C3 activation under lectin pathway-specific conditions are shown in Fig. 3*D*. No C3 cleavage was seen in MASP-2$^{-/-}$ plasma. Factor B$^{-/-}$ plasma cleaved C3 at half the rate of WT plasma ($T_{1/2}$ = 24 min for fB$^{-/-}$ plasma, 12 min for WT), perhaps because of the loss of the amplification loop. A significant delay in the lectin pathway-dependent conversion of C3 to C3b was seen in C4$^{-/-}$ ($T_{1/2}$ = 33 min) as well as in MASP-1/3–deficient plasma ($T_{1/2}$ = 49 min). This delay of C3 activation in MASP-1/3$^{-/-}$ plasma was recently shown to be MASP-1–, rather than MASP-3–dependent (16).

These results strongly suggest that MASP-2 functional activity is essential for the activation of C3 via the lectin pathway both in presence and absence of C4. Furthermore, C2 and MASP-1 are required for this unique lectin pathway-specific C4-bypass activation route of C3 to work.

**Absence of MASP-2 Results in a Significant Degree of Protection from Gastrointestinal Ischemia/Reperfusion Injury.** We explored the role of MASP-2 in gastrointestinal ischemia/reperfusion injury (GIRI) using an established murine model (22, 23). MASP-2$^{-/-}$ mice and WT controls were subjected to acute intestinal ischemia by surgically clamping of the superior mesenteric artery for 40 min, followed by reperfusion for 3 h. Intestinal injury was assessed by semiquantitative pathology scoring of 200 to 400 villi in a defined area of the jejunum. MASP-2$^{-/-}$ mice had a significant reduction of I/R tissue damage compared with WT littermates (pathology scores of MASP-2$^{-/-}$ I/R group: 4 ± 1, $n$ = 6; pathology scores of MASP-2$^{+/+}$ I/R group: 11 ± 3, $n$ = 7; $P < 0.05$) (Fig. 4*A*).

To assess whether a transient inhibition of MASP-2 functional activity can be achieved by applying selective antibody-based MASP-2 inhibitors in vivo, we assessed the degree and duration of lectin pathway inhibition by the murine-specific MASP-2 inhibitor, AbyD 04211. Following intraperitoneal injection at a dose of 0.6 mg/kg body weight, blood was collected by cardiac puncture for up to 17 d, and plasma assayed for lectin pathway-mediated C4 activation. As shown in Fig. 4*B*, lectin pathway activity is abolished within 6 h, is completely absent for up to 48 h, and does not recover significantly (less than 10% of the activity levels before antibody treatment) for 7 d.

To test whether a therapeutic depletion of MASP-2 functional activity can protect animals from GIRI, WT mice (male C57BL/6J, 8–10 wk old) were injected with AbyD 04211 (i.p., 1 mg/kg body weight), or an identical dose of an irrelevant isotype control antibody or saline 18 h before the intestinal I/R or sham surgery. As shown in Fig. 4 *C* and *D*, when saline-treated mice were subjected to intestinal I/R surgery, they had significant tissue damage compared with sham-operated controls (25 ± 7, $n$ = 10; vs. 1 ± 0, $n$ = 5, $P < 0.01$). Pretreatment with the isotype control antibody gave no protection from I/R injury compared with saline control (17 ± 2 vs. 25 ± 7, $n$ = 10 per group, $P > 0.05$). In contrast, pretreatment with AbyD 04211 reduced tissue I/R damage more than twofold compared with mice treated with the isotype control antibody (8 ± 2 vs. 17 ± 2, $n$ = 10 per group, $P < 0.01$). The ischemic intestinal injury in the GIRI group treated with anti–MASP-2 mAb was not reduced down to the baseline levels seen in the sham control group (8 ± 2, $n$ = 10 vs. 2 ± 1, $n$ = 5, $P < 0.01$), but a significant sparing of tissue damage was evident in both MASP-2$^{-/-}$ and anti–MASP-2 mAb treated animals. The anti–MASP-2 mAb results further validate the deleterious role the lectin pathway plays in ischemia reperfusion injury.

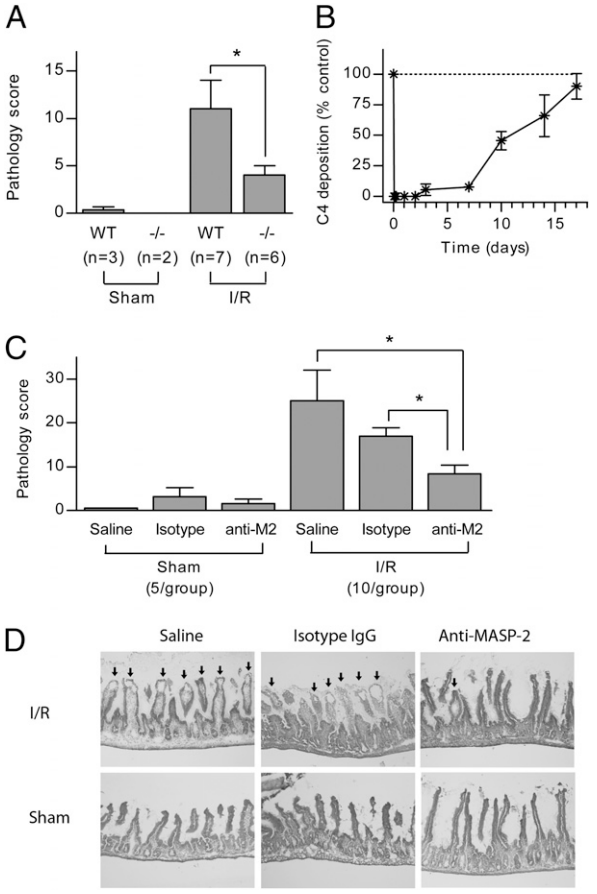


**Fig. 4.** The absence of MASP-2 protects mice from GIRI-mediated injury. (*A*) MASP-2$^{-/-}$ mice show a significant degree of protection from severe GIRI damage following transient (40 min) occlusion of the mesenteric artery and reperfusion (3 h) of ischemic gut tissue. *$P < 0.05$ as determined by Student $t$ test (two-tailed). (*B*) In vivo ablation of lectin pathway activity following a single intraperitoneal injection of recombinant antimurine MASP-2 antibody AbyD 04211 (0.6 mg/kg body weight). At each time point, three mice were killed and serum assayed for lectin pathway-dependent C4 activation. Lectin pathway functional activity was normalized to lectin pathway activity in pooled sera from naive mice measured either in the absence (100%) or in the presence of 100-nM blocking antibody (0%). Results are means (± SEM). (*C*) Effect of anti–MASP-2 mAb treatment on the severity of GIRI: mice were dosed with 1 mg/kg of AbyD 04211, or an irrelevant isotype control antibody, or injected with saline 18 h before being subjected to 40 min gastrointestinal ischemia and 3 h of reperfusion ($n$ = 10 per group). (*$P < 0.05$ when comparing animals treated with either the MASP-2 inhibitory antibody AbyD 04211 or an isotype control). Sham animals ($n$ = 5 per group) were treated in an identical fashion, except that no clamp was applied to the mesenteric artery. (*D*) Histological presentation of GIRI-mediated pathology in the small intestine of WT C57BL/6 mice pretreated with a single intraperitoneal injection of either isotonic saline, an isotype control antibody (1 mg/kg body weight), or recombinant antimurine MASP-2 antibody AbyD 04211 (1 mg/kg body weight) 12 h before the induction of GIRI, and their respective sham controls. Arrowheads indicate subepithelial spaces in the luminal part of the villi (characterized by the lack of cellular content beneath the continuous epithelial layer) as typical features of GIRI pathology. (Magnification, 100×.)

## Discussion

Many recent reports aimed to clarify the mechanisms leading to complement activation on oxygen-deprived cells. The involvement of IgM antibodies in complement-dependent GIRI is well established (22, 23). With IgM being a potent activator of the classic pathway, it was assumed that mice deficient of the classic pathway (such as C1qa$^{-/-}$ mice) would be protected from complement-dependent GIRI and MIRI. Surprisingly, two recent studies show

that C1qa$^{-/-}$ mice are not protected, but mice deficient of the lectin pathway recognition molecules MBL-A and MBL-C are (24, 25). These findings were confirmed in two subsequent GIRI studies, which showed that the critical proinflammatory contribution of IgM occurred in the absence of classic pathway activation, using the lectin pathway through direct interactions between autoreactive IgM and MBL (26, 27). In contrast, the same MBL-null strain showed only a moderate degree of protection in a model of renal IRI (28). Taken together, these studies suggest that protection of MBL-null mice may vary between experimental models of IRI, as the role of the remaining lectin pathway recognition molecules ficolin A and CL-11 in mediating IRI is not yet understood. We have recently shown that human plasma MBL is rapidly consumed in the reperfusion phase following surgically induced ischemia during abdominal aneurism repair, implying that MBL drives activation of the lectin pathway during reperfusion (29). However, the situation may even be more complex as—in addition to MBL—three different ficolins may serve as lectin pathway recognition subcomponents.

Using MASP-2$^{-/-}$ mice in a model of MIRI, we have demonstrated that lectin pathway activity is an essential component of the inflammatory process leading to loss of myocardial tissue. MASP-2$^{-/-}$ mice still activate complement through both the classic and alternative pathways, but are devoid of any residual lectin pathway activity, although retaining all four lectin pathway pattern-recognition molecules. Moreover, MASP-2 was shown to be essential for postischemic inflammatory pathology in a model of GIRI. Our results unequivocally show that neither the classic nor the alternative pathway is sufficient to initiate the inflammatory pathology of postischemic tissue injury in the absence of lectin pathway activity. It is, nevertheless, plausible that the alternative pathway may augment complement activation in other tissues. This theory would explain why the deficiency of factor B ameliorates postischemic inflammatory tissue loss in a model of ischemic acute renal failure (30).

Our findings, together with previous reports showing deposition of downstream complement components on postischemic tissue (25), suggest that MASP-2 mediates tissue damage by activating the lectin pathway. However, a pathophysiological role for MASP-2 outside the complement system cannot be excluded. For example, a recent report indicates that MASP-2 cross-links the complement and coagulation systems, MASP-2 activation leading to the formation of a fibrin clot (31).

A second major finding of the current study is the discovery of a previously unrecognized C4-independent, MASP-2–dependent route of complement activation. Although other groups have previously suggested the existence of an MBL-dependent C4 bypass (32–35), the detailed molecular composition and sequence of activation events remain to be elucidated. Our results imply that the C4-bypass additionally requires the presence of C2 as well as MASP-1. The loss of lectin pathway-mediated C3 cleavage activity in plasma of mice with combined C4 and MASP-1/3 deficiency may be explained by the ability of MASP-1 to enhance MASP-2–dependent complement activation through direct cleavage and activation of MASP-2 (16). Likewise, MASP-1 may aid MASP-2 activity through its ability to cleave C2 (36). The inability of C2/fB$^{-/-}$ plasma to activate C3 via the lectin pathway was shown to be C2-dependent, as the addition of recombinant rat C2 to C2/fB$^{-/-}$ plasma restored the ability of the reconstituted plasma to activate C3.

The finding that C4 deficiency specifically disrupts the classic pathway, but that the lectin pathway retains a physiologically critical level of C3 convertase activity, calls for a reassessment of the role of the lectin pathway in various disease models, including experimental *Streptococcus pneumoniae* infection (37), experimental allergic encephalomyelitis (38), and models of C3-dependent murine liver regeneration (35). The latter group demonstrated that C4-deficient mice can activate C3 in an alternative pathway-independent fashion, as in vivo inhibition factor B did not effect C3 cleavage-dependent liver regeneration in C4$^{-/-}$ mice (35). The lectin pathway mediated C4-bypass may also explain the lack of a protective phenotype of C4 deficiency in our model of MIRI, as well as in a previously described model of renal allograft rejection (39). In contrast, we have recently demonstrated a significant protective phenotype of MASP-2$^{-/-}$ mice in models of renal transplantation (40). The MASP-2–dependent C4-bypass provides a physiologically relevant mechanism that may be important under conditions where availability of C4 limits C3 activation, for example on the surface of pathogens that sequester complement control proteins (e.g., C4 binding protein) to avoid complement attack.

Finally, our results demonstrate that transient and sustained blockage of the lectin pathway can be achieved in vivo by systemic application of inhibitory MASP-2–specific monoclonal antibodies. Inhibiting MASP-2 using relatively low doses of inhibitory antibody in vivo may be therapeutically viable because of the relatively low abundance of MASP-2 in plasma [260–330 ng/mL in mouse plasma (*SI Materials and Methods*) and 170–1,196 ng/mL in human plasma (41)] and the strict absence of any extrahepatic MASP-2 biosynthesis (11).

A transient inhibition of MASP-2 functional activity may therefore provide an attractive therapeutic approach in treating a wide range of ischemia-induced inflammatory pathologies.

## Materials and Methods

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. Recombinant murine MASP-2 and inactive MASP-2 (iMASP-2) were expressed in *Drosophila* (12, 42). Recombinant rat C2 was prepared as described (43). Recombinant antibodies against MASP-2 (AbDH3 and AbD04211) were isolated from Human Combinatorial Antibody Libraries (Serotec; AbD) (44) using recombinant human and rat MASP-2A as antigens (43). An anti-rat Fab2 fragment that potently inhibited lectin pathway-mediated activation of C4 and C3 in mouse plasma ($IC_{50}$~1 nM) was converted to a full-length IgG2a antibody. Polyclonal anti-murine MASP-2A antiserum was raised in rats.

C1q-depleted mouse plasma was prepared as described (45) and assayed before and after depletion for C3, C4, and alternative pathway activation, to ensure that components essential for the other pathways were not codepleted. C1q$^{-/-}$, Factor B$^{-/-}$, and C2/Factor B$^{-/-}$ plasma was provided by Marina Botto (Imperial College London, United Kingdom); the C4$^{-/-}$ mouse line provided by Michael C. Carroll (Harvard Medical School, Boston, MA) (46). The human plasma deficient of both C4A and C4B has been characterized by Yang et al. (21).

**Complement Assays.** Recalicified plasma was used for all assays. Lectin pathway-dependent C4 activation was quantified as previously described (45).

To measure C3 activation, microtiter plates were coated with mannan (1 μg/well), zymosan (1 μg/well) in coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$) or immune complexes, generated in situ by coating with 1% human serum albumin (HSA) in coating buffer then adding sheep anti-HSA serum (2 μg/mL) in TBS (10 mM Tris, 140 mM NaCl, pH 7.4) with 0.05% Tween20 and 5 mM $Ca^{2+}$. Plates were blocked with 0.1% HSA in TBS and washed three times with TBS/Tween20/$Ca^{2+}$. Plasma samples were diluted in 4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, added to the plates and incubated for 1.5 h at 37 °C. After washing, bound C3b was detected using rabbit anti-human C3c (Dako), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG and *p*-nitrophenyl phosphate. Alternative pathway activity was measured in plasma diluted in BBS containing 5 mM $MgCl_2$ and 8 mM EGTA.

**Murine Model of MIRI.** All animal experiments were approved by the ethics committee of King's College London and licensed by the United Kingdom Home Office. Ten- to 12-wk-old female MASP-2$^{-/-}$ mice and their WT littermates were used in a double-blinded study. Ischemia was induced by ligation of the LAD (47). Thirty minutes of LAD occlusion were followed by 120 min of reperfusion. MIRI was verified by observing color changes in the AAR.

Infarct size (INF) and risk zone (RZ) were determined by planometry (48). Perfused tissue was stained by intravenous delivery of 300 μL of Evan's blue (5% wt/vol). Hearts were explanted, cooled on ice, cut into 750-μm transverse slices, and incubated with 3% 2,3,5-triphenyl tetrazolium chloride (TCC) in 0.1 M $Na_2HPO_4/NaH_2PO_4$ (pH 7.4), staining viable tissue red.

To model MIRI in a serum-free system, isolated hearts were mounted in a Langendorff apparatus, perfused with modified Krebs-Henselit buffer for 30 min, and subjected to 30 min of global ischemia (clamping aortic supply), followed by 2 h of reperfusion. Nonischemic tissue was stained with TTC and the volume of the TTC-negative infarct in the left ventricle determined as the percentage of left ventricular volume (49).

**Induction of I/R Injury in the Gastrointestinal Tract.** The surgical protocol for GIRI was performed as previously described (21). Following anesthesia, a laparotomy was performed and a surgical microclip applied to the superior mesenteric artery. After 40 min of ischemia, the microclip was removed and the ischemic tissue allowed to reperfuse for 3 h. Sham controls underwent laparotomy without clamping the superior mesenteric artery. Following reperfusion, animals were killed and corresponding segments of the distal jejunum harvested. Cryostat sections were stained with H&E, blind-coded, and examined under light microscopy. The pathology score was assessed as previously described (21). The first set of experiments assessed GIRI in 8-wk-old female MASP-2$^{-/-}$ and their MASP-2$^{+/+}$ littermate controls. In the second set of experiments, six groups of 8-wk-old female WT C57BL/6 mice were studied: sham-operated mice and I/R-operated mice pretreated with either saline, or isotype control antibody, or anti–MASP-2 antibody AbyD 04211. The antibodies (each dosed at 1 mg/kg) or the saline were injected intraperitoneally 18 h before surgery.

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1. Weisman HF, et al. (1990) Soluble human complement receptor type 1: In vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* 249:146–151.
2. Shandelya SM, Kuppusamy P, Weisfeldt ML, Zweier JL (1993) Evaluation of the role of polymorphonuclear leukocytes on contractile function in myocardial reperfusion injury. Evidence for plasma-mediated leukocyte activation. *Circulation* 87:536–546.
3. Collard CD, et al. (2000) Complement activation after oxidative stress: Role of the lectin complement pathway. *Am J Pathol* 156:1549–1556.
4. Walport MJ (2001) Complement. First of two parts. *N Engl J Med* 344:1058–1066.
5. Schwaeble W, Dahl MR, Thiel S, Stover C, Jensenius JC (2002) The mannan-binding lectin-associated serine proteases (MASPs) and MAp19: Four components of the lectin pathway activation complex encoded by two genes. *Immunobiology* 205:455–466.
6. Fujita T (2002) Evolution of the lectin-complement pathway and its role in innate immunity. *Nat Rev Immunol* 2:346–353.
7. Hansen S, et al. (2010) Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. *J Immunol* 185:6096–6104.
8. Thiel S, et al. (1997) A second serine protease associated with mannan-binding lectin that activates complement. *Nature* 386:506–510.
9. Stover CM, et al. (1999) Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. *J Immunol* 162:3481–3490.
10. Takahashi M, Endo Y, Fujita T, Matsushita M (1999) A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. *Int Immunol* 11: 859–863.
11. Stover CM, Thiel S, Lynch NJ, Schwaeble WJ (1999) The rat and mouse homologues of MASP-2 and MAp19, components of the lectin activation pathway of complement. *J Immunol* 163:6848–6859.
12. Iwaki D, et al. (2006) Small mannose-binding lectin-associated protein plays a regulatory role in the lectin complement pathway. *J Immunol* 177:8626–8632.
13. Vorup-Jensen T, et al. (2000) Distinct pathways of mannan-binding lectin (MBL)- and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. *J Immunol* 165:2093–2100.
14. Thiel S, et al. (2000) Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein MAp19. *J Immunol* 165:878–887.
15. Rossi V, et al. (2001) Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and -2. *J Biol Chem* 276:40880–40887.
16. Takahashi M, et al. (2008) Mannose-binding lectin (MBL)-associated serine protease (MASP)-1 contributes to activation of the lectin complement pathway. *J Immunol* 180: 6132–6138.
17. Takahashi M, et al. (2010) Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D. *J Exp Med* 207:29–37.
18. Takahashi K, et al. (2002) Lack of mannose-binding lectin-A enhances survival in a mouse model of acute septic peritonitis. *Microbes Infect* 4:773–784.
19. Tanno M, et al. (2003) Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: Evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. *Circ Res* 93: 254–261.
20. May JE, Frank MM (1973) Hemolysis of sheep erythrocytes in guinea pig serum deficient in the fourth component of complement. I. Antibody and serum requirements. *J Immunol* 111:1671–1677.
21. Yang Y, et al. (2004) Complete complement components C4A and C4B deficiencies in human kidney diseases and systemic lupus erythematosus. *J Immunol* 173:2803–2814.
22. Zhang M, et al. (2004) Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury. *Proc Natl Acad Sci USA* 101:3886–3891.
23. Zhang M, et al. (2006) Identification of the target self-antigens in reperfusion injury. *J Exp Med* 203:141–152.
24. Hart ML, et al. (2005) Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. *J Immunol* 174:6373–6380.
25. Walsh MC, et al. (2005) Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* 175:541–546.
26. Zhang M, et al. (2006) Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury. *J Immunol* 177:4727–4734.
27. McMullen ME, et al. (2006) Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. *Immunobiology* 211:759–766.
28. Møller-Kristensen M, et al. (2005) Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 61:426–434.
29. Norwood MG, et al. (2006) Consumption of mannan-binding lectin during abdominal aortic aneurysm repair. *Eur J Vasc Endovasc Surg* 31:239–243.
30. Thurman JM, Ljubanovic D, Edelstein CL, Gilkeson GS, Holers VM (2003) Lack of a functional alternative complement pathway ameliorates ischemic acute renal failure in mice. *J Immunol* 170:1517–1523.
31. Gulla KC, et al. (2010) Activation of mannan-binding lectin-associated serine proteases leads to generation of a fibrin clot. *Immunology* 129:482–495.
32. Schweinle JE, Ezekowitz RA, Tenner AJ, Kuhlman M, Joiner KA (1989) Human mannose-binding protein activates the alternative complement pathway and enhances serum bactericidal activity on a mannose-rich isolate of *Salmonella*. *J Clin Invest* 84:1821–1829.
33. Selander B, et al. (2006) Mannan-binding lectin activates C3 and the alternative complement pathway without involvement of C2. *J Clin Invest* 116:1425–1434.
34. Atkinson JP, Frank MM (2006) Bypassing complement: Evolutionary lessons and future implications. *J Clin Invest* 116:1215–1218.
35. Clark A, et al. (2008) Evidence for non-traditional activation of complement factor C3 during murine liver regeneration. *Mol Immunol* 45:3125–3132.
36. Møller-Kristensen M, Thiel S, Sjöholm A, Matsushita M, Jensenius JC (2007) Cooperation between MASP-1 and MASP-2 in the generation of C3 convertase through the MBL pathway. *Int Immunol* 19:141–149.
37. Brown JS, et al. (2002) The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *Proc Natl Acad Sci USA* 99:16969–16974.
38. Boos LA, Szalai AJ, Barnum SR (2005) Murine complement C4 is not required for experimental autoimmune encephalomyelitis. *Glia* 49:158–160.
39. Lin T, et al. (2006) Deficiency of C4 from donor or recipient mouse fails to prevent renal allograft rejection. *Am J Pathol* 168:1241–1248.
40. Farrar CA, et al. (2009) Mannan binding lectin associated serine protease-2 (MASP-2) is a critical player in the pathophysiology of renal ischaemia reperfusion (I/R) injury and mediates tissue injury in absence of complement C4. *Mol Immunol* 46:2832.
41. Møller-Kristensen M, et al. (2003) Levels of mannan-binding lectin-associated serine protease-2 in healthy individuals. *J Immunol Methods* 282:159–167.
42. Iwaki D, Fujita T (2005) Production and purification of recombinants of mouse MASP-2 and sMAP. *J Endotoxin Res* 11:47–50.
43. Chen CB, Wallis R (2004) Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases. *J Biol Chem* 279:26058–26065.
44. Knappik A, et al. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* 296:57–86.
45. Lynch NJ, et al. (2004) L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement. *J Immunol* 172:1198–1202.
46. Fischer MB, et al. (1996) Regulation of the B cell response to T-dependent antigens by classical pathway complement. *J Immunol* 157:549–556.
47. Eckle T, et al. (2006) Systematic evaluation of a novel model for cardiac ischemic preconditioning in mice. *Am J Physiol Heart Circ Physiol* 291:H2533–H2540.
48. Clark JE, Kottam A, Motterlini R, Marber MS (2009) Measuring left ventricular function in the normal, infarcted and CORM-3-preconditioned mouse heart using complex admittance-derived pressure volume loops. *J Pharmacol Toxicol Methods* 59: 94–99.
49. Sicard P, et al. (2010) The activation of p38 alpha, and not p38 beta, mitogen-activated protein kinase is required for ischemic preconditioning. *J Mol Cell Cardiol* 48:1324–1328.