

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

Wilhelm J. Schwaeble^{a,1}, Nicholas J. Lynch^a, James E. Clark^b, Michael Marber^b, Nilesh J. Samani^c, Youssif Mohammed Ali^{a,d}, Thomas Dudler^e, Brian Parent^e, Karl Lhotta^f, Russell Wallis^a, Conrad A. Farrar^g, Steven Sacks^g, Haekyung Lee^h, Ming Zhang^h, Daisuke Iwakiⁱ, Minoru Takahashiⁱ, Teizo Fujitaⁱ, Clark E. Tedford^e, and Cordula M. Stover^a

Departments of ^aInfection, Immunity, and Inflammation and ^cCardiovascular Sciences, University of Leicester, Leicester LE1 9HN, United Kingdom; ^bBritish Heart Foundation Centre and ^gMedical Research Council Centre for Transplantation and National Institute for Health Research Biomedical Research Centre at Guy's and St. Thomas' National Health Service Foundation Trust, King's College London, London SE1 9RT, United Kingdom; ^dFaculty of Pharmacy, Department of Microbiology, University of Mansoura, Mansoura 35516, Egypt; ^eOmeros Corporation, Seattle, WA 98101; ^fLandeskrankenhaus Feldkirch, 6807 Feldkirch, Austria; ^hDepartment of Anesthesiology, State University of New York-Downstate Medical Center, New York, NY 11203; and ⁱDepartment of Immunology, Fukushima Medical University, Fukushima 960-1295, Japan

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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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¹To whom correspondence should be addressed. E-mail: ws5@le.ac.uk.

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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 postschismic 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 CCP2-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. MASP-2 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. 1A). 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. 1B). 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 (LP₅₀ ~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. 1C). 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. 1D) and the alternative pathway (Fig. 1E). 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. 2A. 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. 2B and C demonstrate that MASP-2^{-/-} mice have a significant reduction in MIRI-induced myocardial damage. Fig. 2B 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. 2C 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 reperused hearts of MASP-2^{-/-} and MASP-2^{+/+} mice in the Langendorff mode (19). In contrast to the plasma-perfused hearts shown in Fig. 2C, results for the buffer perfused hearts show no difference in infarct sizes between the two groups (Fig. 2D 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. 2F and G, identical infarct sizes were observed in C4-deficient mice and their WT littermates. This observation was unexpected, as it

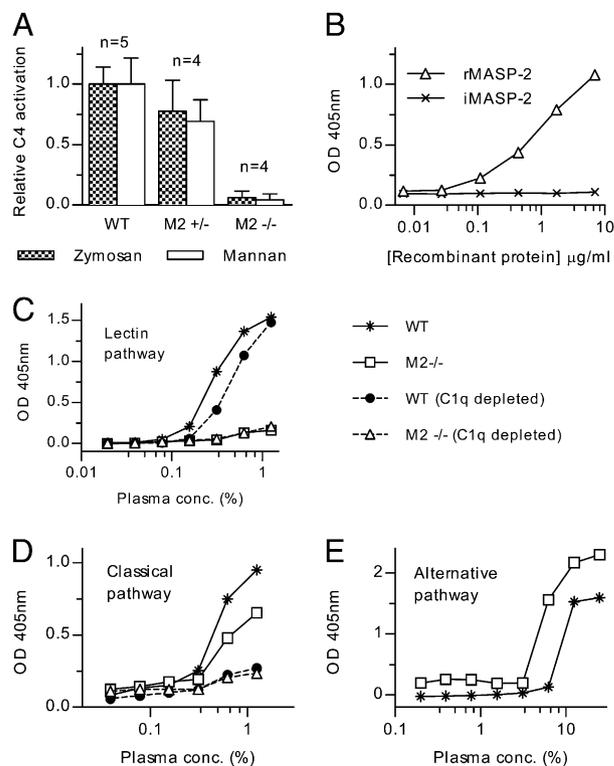


Fig. 1. MASP-2 is essential for lectin pathway functional activity. (A) Lectin pathway-dependent C4 activation: plasma from WT, heterozygous (M2^{+/-}), and homozygous (M2^{-/-}) MASP-2-deficient mice were assayed for C4 convertase activity on microtiter plates coated with mannan or zymosan. Serial dilutions 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 Mg²⁺ 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-

(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. 3C). 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. 3D. 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. 4A).

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. 4B, 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.

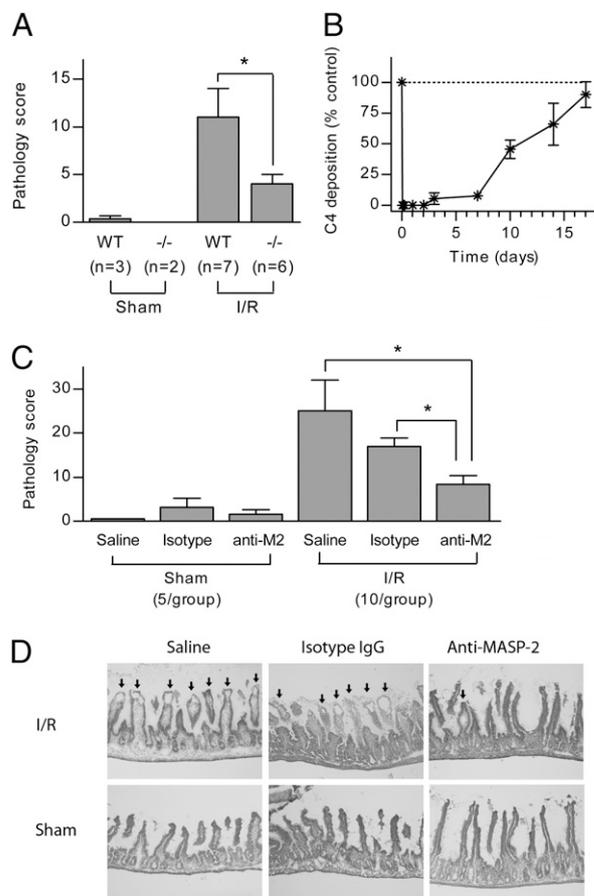


Fig. 4. The absence of $MASP-2$ protects mice from GIRI-mediated injury. (A) $MASP-2^{-/-}$ mice show a significant degree of protection from severe GIRI damage following transient (40 min) occlusion of the mesenteric artery and reperfusion (3 h) of ischemic gut tissue. $*P < 0.05$ as determined by Student 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 (\pm 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 \times .)

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 C1q^{-/-} 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 potentially inhibited lectin pathway-mediated activation of C4 and C3 in mouse plasma (IC₅₀ ~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. Recalcified 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 Na₂CO₃, 35 mM NaHCO₃) 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 Ca²⁺. Plates were blocked with 0.1% HSA in TBS and washed three times with TBS/Tween20/Ca²⁺. Plasma samples were diluted in 4 mM barbital, 145 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 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 MgCl₂ 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 Na₂HPO₄/NaH₂PO₄ (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-Hensleit 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 de-

scribed (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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