Pathogenic role of complement in renal ischemia/reperfusion injury

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CHAPTER 5

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Mannan-binding lectin mediates renal ischemia/reperfusion injury independent of complement activation

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ABSTRACT

Ischemia/reperfusion injury (IRI) remains a major problem in renal transplantation. Clinical studies have identified that high serum levels of Mannan-binding lectin (MBL), the initiator of the lectin pathway of complement activation, are associated with inferior renal allograft survival. Using a rat model, we identified an entirely novel role for MBL in mediating renal IRI. Therapeutic inhibition of MBL was protective against kidney dysfunction, tubular damage, neutrophil and macrophage accumulation, and expression of proinflammatory cytokines and chemokines. Following reperfusion, exposure of tubular epithelial cells to circulation-derived MBL resulted in internalization of MBL followed by the rapid induction of tubular epithelial cell death. Interestingly, this MBL-mediated tubular injury was completely independent of complement activation since attenuation of complement activation was not protective against renal IRI. Our identification that MBL-mediated cell death precedes complement activation strongly suggests that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury. In addition, also human tubular epithelial cells *in vitro* were shown to be susceptible to the cytotoxic effect of human MBL. Taken together, these data reveal a crucial role for MBL in the early pathophysiology of renal IRI and identify MBL as a novel therapeutic target in kidney transplantation.

INTRODUCTION

Ischemia/reperfusion injury (IRI) is a key event in clinical conditions such as infarction, sepsis and organ transplantation. Restoration of blood flow to ischemic tissue paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events including release of pro-inflammatory cytokines and chemokines, recruitment of leukocytes and activation of the complement system (1-3). Numerous clinical and experimental studies have shown that renal IRI profoundly impacts short-and long-term graft survival following kidney transplantation, and is strongly associated with delayed graft function (DGF) and clinical morbidity and mortality (3-5). DGF results in oliguria, increased allograft immunogenicity and risk of acute rejection episodes (5) and is associated with extensive loss of the cortico-medullary proximal tubular epithelial cells (PTEC), often referred to as acute tubular necrosis (ATN). To date, no effective therapy or treatment for renal IRI in the clinic exists.

Renal ischemia is accompanied by a reduction of membrane-bound complement regulators on PTEC, rendering these cells susceptible to complement activation

following reperfusion (6). Activation of the complement system has been documented as one of the hallmarks of renal IRI (6-8). The complement system is a central component of innate immunity and consists of three pathways of activation, namely the classical, alternative and lectin pathway. The complement cascade is activated by the binding of recognition molecules, such as C1q and MBL, to their respective target, e.g. pathogens, apoptotic or necrotic cells (9). All three pathways converge at the level of complement factor C3, which is cleaved and subsequently deposits as C3b on target cells resulting in opsonization and clearance by phagocytic cells. In absence of effective clearance, further activation of the complement cascade leads to generation of the membrane attack complex (MAC) C5b-9 and lysis of target cells.

MBL is the major recognition molecule of the lectin pathway of complement activation. Single-nucleotide polymorphisms in both structural and regulatory parts of the MBL gene have been found to lead to large interindividual variations in the concentration of functional MBL in serum (0-4 μg/ml) (10). Previously, we have shown that low pre-transplantation levels of MBL are associated with better graft survival after deceased-donor kidney transplantation. These findings identify MBL as a potential risk factor for graft and patient survival in renal transplantation (10;11). In rodents, MBL exists in two distinct isoforms, namely MBL-A and –C (12). Nevertheless, specific inhibition of MBL-A in myocardial infarction in rats has been shown to be effective in reducing post ischemic damage (13). Modulation of the complement system has been recognized as a promising strategy in drug discovery, and a large number of therapeutic modalities have been developed (14;15), suggesting that therapeutic strategies could be employed to improve clinical outcomes following renal IRI. In the present study we examined the role of MBL in the pathophysiology of renal IRI and explored the therapeutic targeting of MBL in a rat model of renal IRI. We identified an entirely novel role for MBL in mediating reperfusion-induced kidney injury following ischemia which is completely independent of complement activation.

METHODS

Animals

The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Eight-week-old male Lewis rats (200–250 gram) purchased from Harlan (Horst, The Netherlands), were housed in standard laboratory cages and were allowed free access to food and water throughout the experiments. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 minutes using a bulldog clamp (Fine Science Tools, Heidelberg,

Germany). During clamping the contralateral kidney was removed. Sham-treated rats had identical surgical procedures except for clamping of the left kidney but including removal of the contra-lateral kidney. Before induction of ischemia, rats were treated with anti-rat MBL-A mAb (IgG1; P7E4; 1 mg/kg), anti-rat C5 mAb (IgG2b; 18A; 20 mg/kg) or as a control anti-human fibronectin mAb (IgG1; 1 mg/kg or 20 mg/kg respectively) (13;16). To deplete rats (n=5 per group) of C3, cobra venom factor (CVF, 2 mg/kg; Sigma-Aldrich, Zwijndrecht, The Netherlands) or PBS as a control, was administered intraperitoneally 19 hours before induction of ischemia. To prevent CVF-induced C5 activation, rats were pretreated with anti-rat C5 mAb (18A; 20 mg/kg).

Assessment of kidney function

Renal function was determined by measuring creatinine and urea in serum samples using standard autoanalyzer methods by our hospital research services.

Renal histology

Histological evaluations were performed in a blinded manner by a single pathologist. Formalin-fixed kidneys embedded in paraffin were sectioned and stained with silver by standard methods.

Renal immunostaining

Rat kidney sections (4 μm) of snap-frozen kidneys were air dried and acetone-fixed. Neutrophil (PMN) and macrophage (Mph) accumulation was assessed using digitonin (DIG)-conjugated mAbs against CD43 (W3/13) and CD68 (ED-1) respectively, followed by horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany). The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Rat C3 deposition was assessed by direct staining using a fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal to rat C3 (made in-house). Quantification of immunohistochemistry was performed in a blinded manner by assessing 10 consecutive high power fields (HPFs; magnification ×200) of the outer medulla and cortico-medullary junction on each section. Using image J software, the positive area in each image (expressed in pixels) was quantified. Deposition of rat or human MBL was assessed using anti-rat MBL (mAb 14C3) or anti-human MBL (mAb 3E7; Hycult Biotech, Uden, The Netherlands) respectively, followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc). The staining was visualized using tyramide labeled with tetramethylrhodamine-5-(and 6)-isothiocyanate (Sigma-Aldrich). Nuclei were stained using Hoechst (Molecular Probes, Leiden, The Netherlands). Fluorescent micrographs were made using confocal laser scanning microscopy (LSM 510, Zeiss, Germany).

RNA isolation and Real-Time PCR

Rat kidney RNA was extracted from snap frozen renal tissue and purified using an RNeasy Mini isolation Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Specific rat primers for Kidney injury molecule (KIM)-1, Neutrophil gelatinase-associated lipocali (NGAL), Macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2 and ribosomal protein S-15 (RPS-15, housekeeping gene), were designed using Oligo Explorer and obtained from Biolegio (Nijmegen, The Netherlands). The primer sequences were as follows: KIM-1 (forward, 5'- TACCAACATCAACCAGAGTCTC -3'; reverse, 5'-ACATAGAAGCCCTTAGTCGG-3'), NGAL (forward, 5'-CGATGAACTGAAGGAGCGATTC -3'; reverse, 5'- TGGCAACAGGAAAGATGGAG - 3'), MCP-1 (forward, 5'-GCCCAGAAACCAGCCAAC -3'; reverse, 5'- GCCAGTGAATGAGTAGCAGC - 3'), MIP-2 (forward, 5'-TCAATGCCTGACGACCCTAC-3'; reverse, 5'-GAGCCCATGTTCTTCCTTCC - 3') and RPS-15 (housekeeping gene): forward 5' CGTCACCCGTAATCCACC-3' and reverse 5'-CAGCTTCGCGTATGCCAC -3'). PCR was performed using SYBR Green PCR mastermix (Biorad). Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (Biorad). Expression of each gene was normalized against mRNA expression of the housekeeping gene Rsp-15. RT PCRs were performed in duplicate.

Immunohistochemistry

Functional activity of the lectin pathway in rat serum was assessed by detection of C3 deposition on mannan-coated plates as previously described (17). Hemolytic activity of rat serum was assessed in an AP50 hemolytic assay as previously described (18).

Cell culture

Immortalized human PTEC (HK-2) (18) were grown as previously described (19). Primary PTEC from pre-transplant biopsies (20) and human umbilical vein endothelial cell (HUVEC) (21) were isolated and cultured as described. Primary cells were used at passage 3 or less.

Flow cytometry

Cells were fixed using 1% paraformaldehyde (Pharmacy) and permeabilized using 0.1% saponin (Sigma-Aldrich). Internalized MBL was detected using anti-human MBL (mAb 3E7; Hycult Biotech) followed by RPE-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, USA). MBL staining was assessed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). MBL binding and subsequent internalization was inhibited using 10 mM EDTA (Sigma Aldrich).

Fluorescence microscopy

Cells were fixed using cold methanol (Pharmacy) and stained with anti-human MBL (mAb 3E7; Hycult Biotech) and rabbit polyclonal anti-human protein disulfide isomerase (PDI, Cell Signaling Technology, Danvers, MA, USA) followed by alexa-488 conjugated goat anti-mouse IgG (Molecular Probes) and alexa-568 conjugated goat anti-rabbit IgG (Molecular Probes), respectively.

Viability assays

Cell viability was assessed by FACS analysis using propidium iodide (PI; Molecular Probes, Leiden, The Netherlands) and Annexin V-FITC (VPS Diagnostics, Hoeven, The Netherlands). Cell viability was also assessed by counting Hoechst-positive, but PI-negative cells using fluorescence microscopy. Inhibition studies were done using D- or L-mannose (Sigma-Aldrich) or a blocking antibody to MBL (3F8).

Preparation of human MBL

MBL was purified from human serum as previously described (22).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and subjected to nonparametric statistical analysis using one- and two-factor ANOVA or Mann-Whitney test using GraphPad Prism software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of MBL preserves renal function after ischemia/reperfusion

To therapeutically target MBL in renal IRI we used a mAb to MBL-A (P7E4) which previously has been shown to effectively reduce post-ischemic damage following myocardial infarction in rats (13). Five minutes before induction of ischemia rats were treated with anti-MBL-A resulting in a transient inhibition of lectin pathway activity that normalized 24 hours afterwards (Fig 1A), while classical pathway activity was unaffected (data not shown). The lectin pathway activity in rats receiving the isotype-matched control antibody was unaffected (Fig 1A). Induction of ischemia for 45 minutes followed by reperfusion resulted in a marked impairment of renal function in control treated rats as measured by serum levels of creatinine (Fig 1B) and urea (Fig 1C) with levels rising within 24 hours and remaining high until 72 hours after reperfusion. In contrast, rats receiving the blocking antibody to MBL-A were protected against renal dysfunction, with serum levels of creatinine and urea close to the normal range (Fig 1B and C).

Furthermore, renal dysfunction in control-treated rats was accompanied by a significant decrease in weight at 72 hours after reperfusion, compared to sham or anti-MBL treated rats (Fig 1D). Despite the transient inhibition of MBL-A, the protection against renal IRI was almost complete, indicating that MBL-A exerts its harmful effects relatively short after reperfusion.

Anti-MBL treatment reduces tubular injury and leukocyte infiltration

Ischemia/reperfusion resulted in extensive ATN (Fig 2A) leading to tubular cast formation and obstruction of tubules (Fig 2E). These events were significantly reduced by inhibition of MBL-A (Fig 2B,F), with a reduction of 70% in ATN (Fig

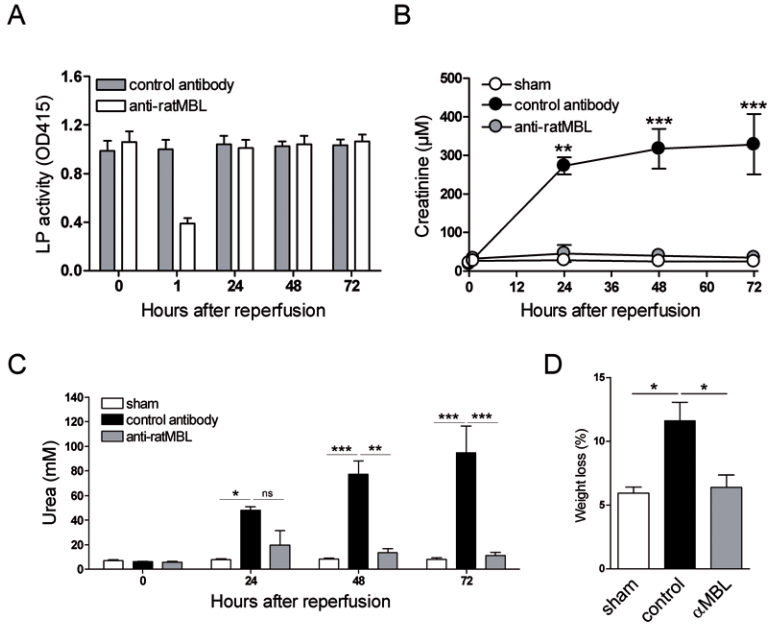

Figure 1. Inhibition of MBL-A protects against renal ischemia/reperfusion injury. Before induction of unilateral ischemia and removal of the contralateral kidney, rats received anti-MBL-A or an isotype-matched control mAb (1 mg/kg). Before and following reperfusion blood samples were drawn at consecutive time points to determine lectin pathway activity (A). Renal function was assessed by measuring serum creatinine (B) and urea (C) levels. After 72 hours of reperfusion rats were weighted and compared to pre-IRI weight to calculate weight loss (D). Data are shown as mean ± SEM, n= 6 per group. The comparison between groups is indicated by asterisks. * P < 0.05; ** P<0.01;***P<0.001.

2C) and 66% in tubular cast formation (Fig 2G). Assessment of renal mRNA levels of KIM-1 and NGAL, both markers of tubular injury, revealed significantly lower KIM-1 expression (Fig 2D) in the anti-MBL treated group. NGAL expression had a tendency to decrease (Fig 2H).

Tubular injury after reperfusion was accompanied by extensive infiltration of PMN (Fig 3A) and Mph (Fig 3E) in the outer medullary zone. Inhibition of MBL-A significantly reduced accumulation of PMN (Fig 3B, C) and Mph (Fig 3F, G), and chemokine expression of MIP-2 (Fig 3D) and MCP-1 (Fig 3H).

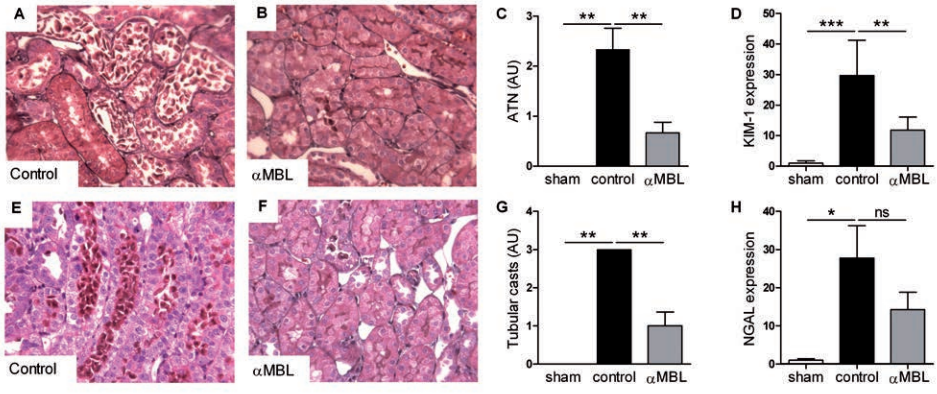

Figure 2. Tubular injury is significantly reduced by inhibition of MBL-A. Silver staining of renal sections from control (A, E) and anti-MBL (B, F) treated rats 72 hours following reperfusion showing ATN (A) and tubular cast formation (E) (original magnification x200). ATN (C) and tubular cast formation (G) were analyzed semi-quantitatively. Renal mRNA expression of injury markers KIM-1 (D) and NGAL (H) in renal tissue at 72 hours following reperfusion was assessed. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. * P < 0.05; ** P<0.01;***P<0.001.

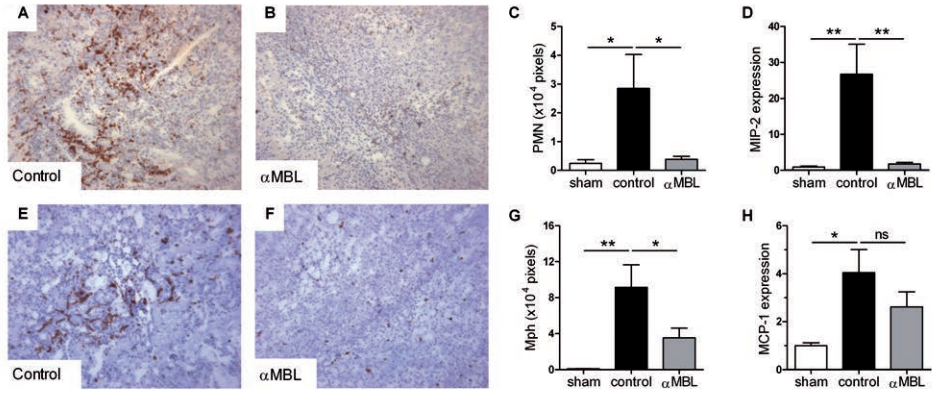

Figure 3. Macrophage and neutrophil accumulation was significantly reduced by inhibition of MBL-A. Renal sections from control (A, E) and anti-MBL (B, F) treated rats 72 hours following reperfusion were stained for PMN (A, B) and Mph (E, F) (original magnification x200). Infiltrates of PMN (C) and Mph (G) were quantified using digital image analysis. Renal mRNA expression of the chemokines MIP-2 (D) and MCP-1 (H) in renal tissue at 72 hours following reperfusion was assessed. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. * P < 0.05; ** P<0.01.

Induction of reperfusion injury is independent of complement activation

In mice it has been shown that the MAC (C5b-9) plays an important role in the induction of tubular injury following renal IRI (6). To dissect the role of C5 (which is downstream of MBL and C3) in our renal IRI model, we applied an anti-rat C5 antibody (mAb 18A), which was previously shown to significantly reduce injury in a rat myocardial IRI model (16). Injection of anti-rat C5 before induction of ischemia resulted in complete inhibition of the terminal pathway of complement for at least 24-48 hours, whereas the control antibody had no effect (Fig 4A). However, despite this relative long-term inhibition of C5, rats were not protected against renal dysfunction, showing equally high creatinine levels as compared to control-treated rats (Fig 4B). Furthermore, there was no significant difference in tubular injury (Fig 4C) and cast formation (Fig 4D) suggesting that the tubular injury is not induced by C5 activation in this model. Moreover, assessment of Mph and PMN influx revealed an extensive influx of inflammatory cells in both the anti-C5 and control-treated group (Fig 4E, F).

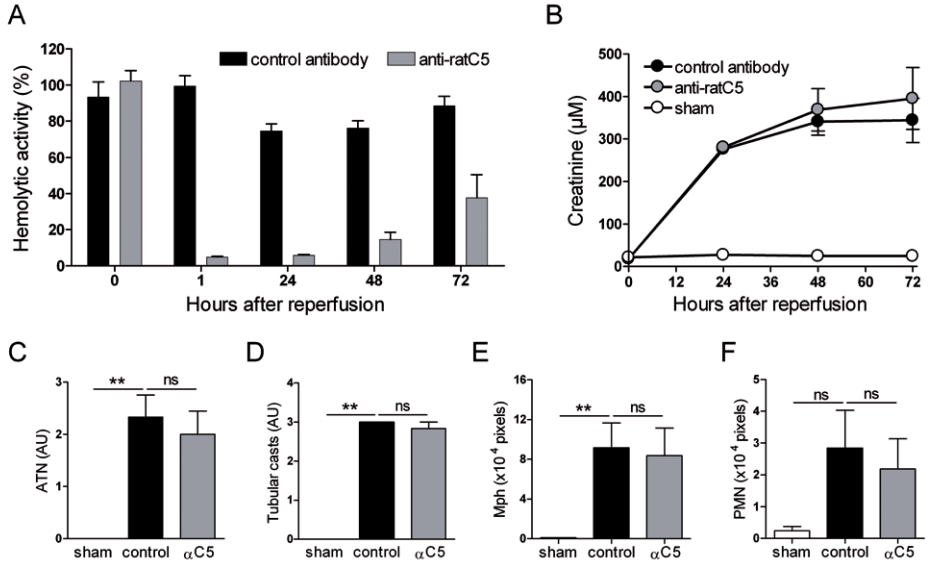

Figure 4. Inhibition of C5 does not ameliorate renal ischemia/reperfusion injury. Before induction of unilateral ischemia and removal of the contralateral kidney rats received anti-rat C5 or a control mAb (20 mg/kg). Before and after reperfusion blood samples were drawn at consecutive time points to determine the inhibitory capacity of anti-rat C5 mAb by measuring serum hemolytic activity in an AP50 hemolytic assay (A). Renal function was assessed by measuring serum creatinine levels (B). Using silver staining, ATN (C) and tubular cast formation (D) was assessed semi-quantitatively at 72 hours after reperfusion. Renal sections were stained for Mph (E) and PMN (F) infiltrate and analyzed quantitatively. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. ** P<0.01.

Since inhibition of MBL-A was shown to be very effective in reducing renal IRI, whereas inhibition of C5 was not, we set out to dissect the role of C3. Depletion of C3 was achieved using cobra venom factor (CVF) and was preceded by administration of anti-C5 to prevent excessive C5 cleavage and generation of C5a. Injection of anti-C5 abrogated terminal complement activity (Fig 5A) and subsequent administration of CVF completely depleted the pool of C3 for at least 48 hours after reperfusion (Fig 5B). Despite depletion of C3 and inhibition of C5, this did not result in protection against renal IRI but even tended to an increased

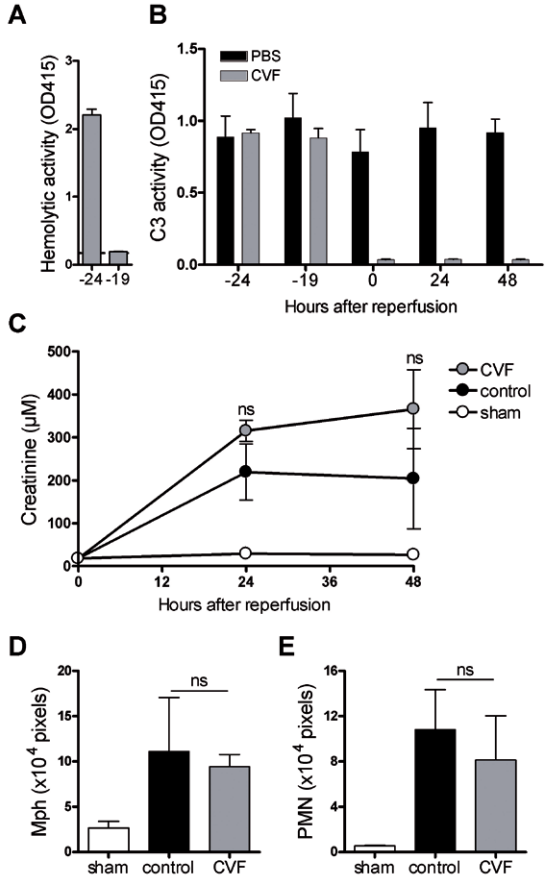

Figure 5. Depletion of C3 does not protect against renal ischemia/reperfusion injury. Twenty-four hours before induction of ischemia, all groups received anti-rat C5 (20 mg/kg). Five hours afterwards and 19 hours before induction of ischemia, rats received CVF to deplete C3. Sham-operated rats also received CVF, while the control group received PBS. Blood samples were drawn at consecutive time points to determine hemolytic activity in an AP50 assay (A) and C3 activation in a lectin pathway activation assay (B). Renal function was assessed by measuring serum creatinine levels (C). Renal sections following 48 hours of reperfusion were stained for Mph (D) and PMN (E) infiltrate and analyzed quantitatively. Data are shown as mean ± SEM (n= 5 per group).

impairment of renal function (Fig 5C). CVF did not affect kidney function, since sham operated rats which also received CVF and were uninephrectomized, showed stable serum creatinine levels (Fig 5C). Also the influx of Mph (Fig 5D) and PMN (Fig 5E) at 48 hours was not reduced, indicating that not solely C3a or C5a were responsible for the influx of inflammatory cells into the renal tissue.

Complement is activated in a late phase following renal ischemia/reperfusion and is preceded by deposition of MBL and tubular injury

Taken together, these findings led us to question the contribution of different complement components in the early induction of renal IRI. Therefore, we further investigated the kinetics of tubular injury and complement activation in our model. Signs of ATN, including loss of tight junctions and epithelial sloughing, were early events (within 2-5 hours after reperfusion; Fig 6A) and were accompanied by deposition of MBL (Fig 6B). At this stage however, deposition of C3 (Fig 6C) and C5b-9 (Fig S1) was completely absent, implying that tubular injury could not be caused by activation of complement and generation of the lytic C5b-9 complex. Only from 24 hours onwards, C3 deposition was observed on injured MBL-positive tubular cells (Fig 6D). This delayed appearance of C3 could not be explained by a change in local expression, as C3 expression even decreased from 5 hours onwards (Fig S2). Together, these findings suggest a differential role for MBL and complement activation in renal IRI.

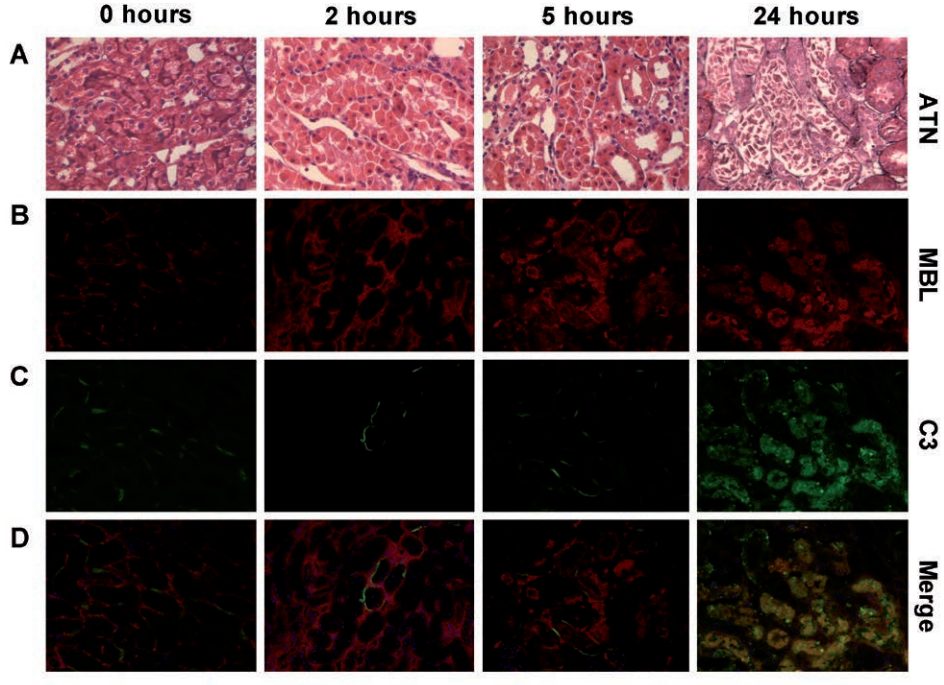

Figure 6. Complement is activated in a late phase following reperfusion and is preceded by deposition of MBL and tubular injury. Silver staining (A) of renal sections from rats (n=6 per group) sacrificed prior to, 2, 5 or 24 hours after reperfusion showing early loss of tight junctions and cell adherence at 2 and 5 hours followed by ATN at 24 hours. Renal sections were triple stained (D) for MBL (B; red), C3 (C; green) and Hoechst (blue) (original magnification x200).

Upon reperfusion MBL leaks from circulation and is internalized by tubular epithelial cells

This prompted us to seek for alternative effector functions than activation of complement by which MBL potentially could injure tubular cells. First we studied the localization and deposition of MBL following reperfusion in more detail. Staining of kidneys for rat MBL-A, showed minor staining in the peritubular capillaries of sham operated rats (Fig 7A), reflecting circulating MBL. In contrast, 2 hours after reperfusion we observed an interstitial staining for MBL, surrounding the basolateral side of the tubuli (Fig 7B). Even more, also an intracellular staining of MBL-A in tubular epithelial cells was observed (Fig 7C), suggesting that MBL

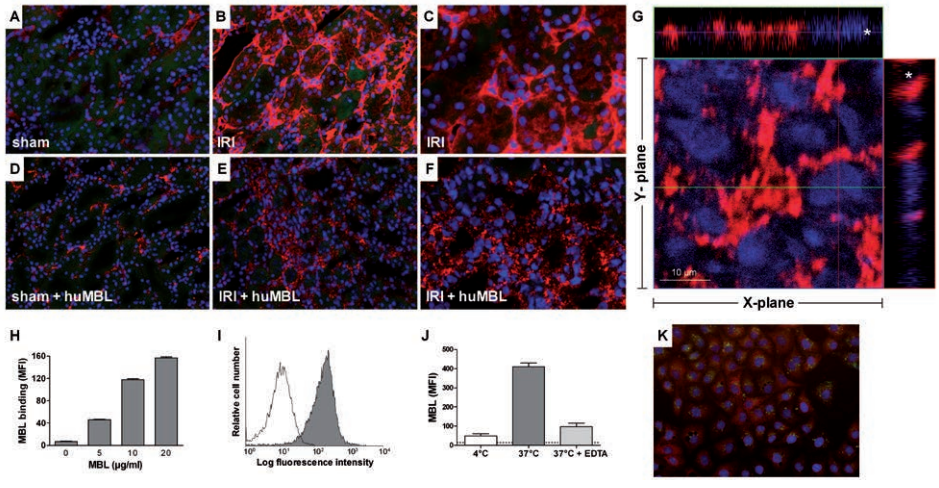

Figure 7. Following reperfusion MBL leaks from circulation and is internalized by tubular epithelial cells. Renal sections from sham operated (A) and ischemic rats (B, C) 2 hours following reperfusion, were stained for rat MBL (red). Nuclei (blue) were stained using Hoechst. To discriminate between locally expressed or circulation-derived MBL, rats were injected with human MBL and sacrificed 3 hours after reperfusion. Sections from the right control kidney (D) or left ischemic kidney (E, F) were stained for human MBL (red). Presence of intracellular human MBL (red) was confirmed using confocal scanning laser microscopy (G), showing MBL staining in the same Z-plane (marked by asterisks) near the nuclei (blue). To study the interaction of human MBL with human PTEC *in vitro*, HK-2 cells were incubated with purified human MBL (0-20 µg/ml) for 30 minutes at 4°C, followed by flow cytometry analysis (H, I). To study internalization of MBL, PTEC were incubated with 10 µg/ml human MBL at 4 or 37°C and intracellular MBL staining was assessed using flow cytometry (J). Values shown are mean ± SEM of triplicate determinations from a single experiment and are representative of 3 similar experiments. Dotted line represents background staining. Furthermore, internalization of MBL by cultured human PTEC was assessed using fluorescence microscopy (K). Human PTEC (HK-2) were incubated with 10 µg/ml human MBL for 60 min at 37°C and stained (n=5) for intracellular MBL (green), ER-marker PDI (red) and nuclei (blue) (original magnification x200 for A, B, D, E; x400 for C, F, K).

from circulation had been internalized. However, assessment of MBL-A mRNA revealed local MBL-A expression in the kidney (Fig S2), leaving the option that intracellular MBL might be the result of local production.
To discriminate between locally expressed or circulation-derived MBL, we intravenously administered human MBL (50 µg) shortly after reperfusion resulting in a serum concentration of 2 µg/ml human MBL (data not shown). Specific staining for human MBL of the reperfused kidney 3 hours following reperfusion revealed an intense staining surrounding (Fig 7E) and inside tubular cells (Fig 7F), which was absent in the contralateral control kidney (Fig 7D). Intracellular MBL was further demonstrated using confocal microscopy (Fig 7G), showing MBL staining (red) in the same plane (Z-plane) in between the nuclei (blue). This confirms that upon reperfusion of the ischemic kidney, MBL from circulation leaks into the interstitium where it is internalized by tubular cells. This staining was most pronounced in the cortico-medullary border, the region mostly damaged.
To further study whether MBL is able to interact with tubular cells, we cultured and exposed human PTEC to human purified MBL *in vitro*, which resulted in dose-dependent binding of MBL as determined by flow cytometry (Fig 7H, I). To study internalization of MBL by tubular cells as observed *in vivo* following reperfusion, human PTEC were incubated with MBL at 4 or 37°C followed by an intracellular FACS staining (Fig 7J). We observed an increased staining at 37°C, indicating that MBL was indeed internalized. Incubation of MBL in presence of EDTA, which chelates calcium needed for binding, largely inhibited binding and subsequent internalization of MBL. These data were confirmed by immunofluorescence using an intracellular staining method, showing a perinuclear staining pattern of human MBL (Fig 7K), indicating that binding of MBL is followed by internalization. Together these data show that MBL specifically binds to PTEC which is followed by internalization.

MBL induces tubular epithelial cell death

To study the functional consequence of MBL binding and internalization, human PTEC were incubated with different concentrations of purified MBL (0-10 µg/ml) for different time points. Strikingly, already within 5 hours of MBL incubation (10 µg/ml) alterations in cell morphology were observed. Tubular epithelial cells start shrinking and rounding, followed by detachment from the plate within 24 hours of incubation with MBL (Fig 8A), resembling the situation observed *in vivo* upon IRI. FACS staining of PTEC for PI and Annexin V after 24 hours of incubation with MBL (10 µg/ml) revealed that most cells were in an early (25%) or late apoptotic (44%) phase (Fig 8C), whereas untreated cells (90%) remained viable (Fig 8B). This cytotoxic effect was time- and dose-dependent and was

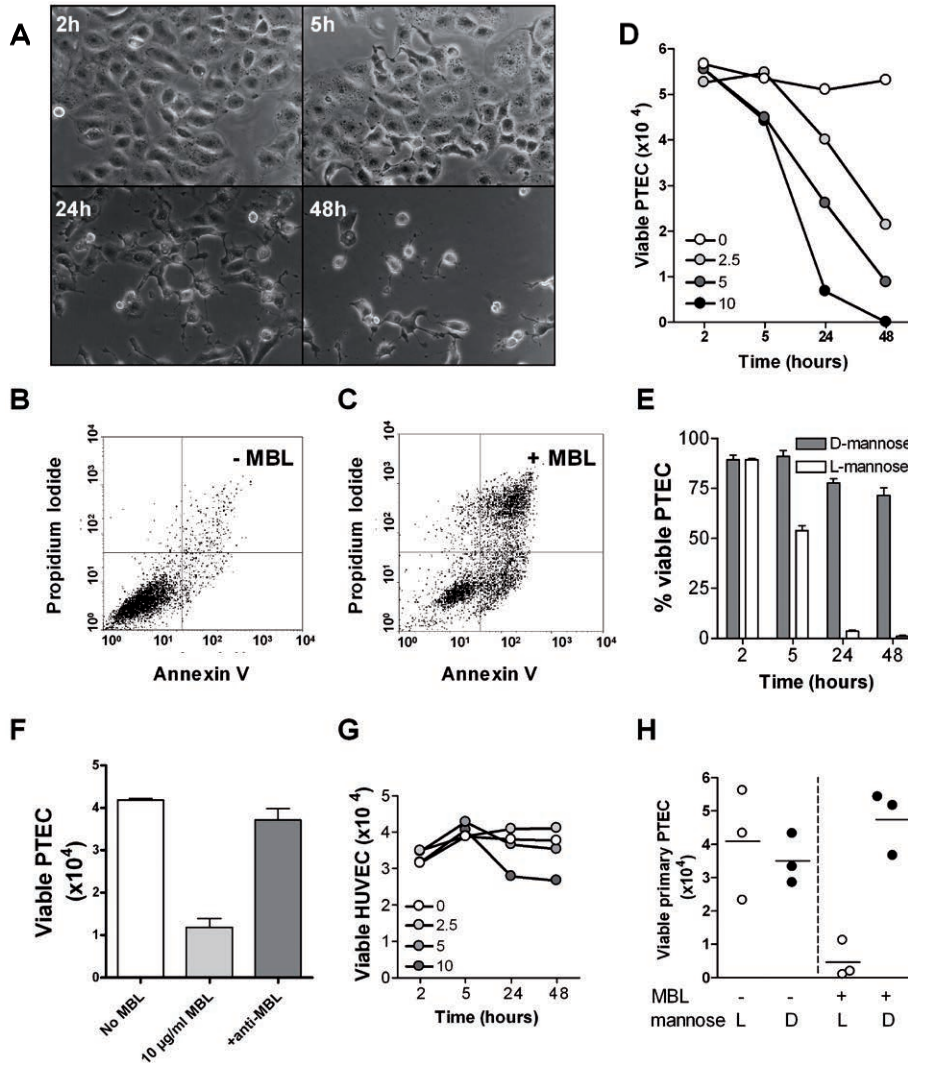

Figure 8. MBL induces tubular epithelial cell death. Human PTEC (HK-2) were incubated with 2.5, 5 or 10 (A) µg/ml human MBL for 2-48 hours. Wells were photographed (A, magnification ×200) and cell viability was assessed by counting Hoechst-positive, but PI-negative cells in the well using fluorescence microscopy (D). Cell viability at 24 hours incubation with 0 (B) or 10 (C) µg/ml MBL was assessed by FACS analysis using propidium iodide (PI) and Annexin V-FITC. HK-2 cells were incubated with 10 µg/ml MBL in the presence of D- or L-mannose (200 mM) for 2-48 hours (E) and cell viability was determined using PI and Annexin V. HK-2 cells were incubated with 10 µg/ml MBL in the absence or presence of an inhibitory mAb to MBL (25 µg/ml; 3F8) for 24 hours (F). Primary HUVEC were incubated with 2.5, 5 or 10 µg/ml human MBL for 2-48 hours (G). Primary PTEC were incubated with 10 µg/ml MBL in the presence of D- or L-mannose (200 mM) for 24 hours (H). Cell viability was assessed by counting Hoechst-positive, but PI-negative cells (F-H).

accelerated at higher doses of MBL (Fig 8D), and even at a physiologic MBL concentration of 2.5 μg/ml this effect was clearly present. To show specificity, MBL was preincubated with D-mannose (a ligand for MBL) which resulted in a very effective inhibition of MBL-induced cell death, as assessed with Annexin V and PI (Fig 8E). In contrast, incubation with L-mannose, which does not bind MBL, showed no inhibition indicating that binding through the lectin domain of MBL is involved in exerting this cytotoxic effect. Prolonged incubation with MBL in the presence of D-mannose still resulted in complete inhibition of cell death, showing that D-mannose not only delayed, but actually prevents MBL-induced cytotoxicity. These data were confirmed using a blocking antibody to MBL (3F8) recognizing an epitope on the carbohydrate recognition domain resulting in an almost complete inhibition of MBL-induced cell death (Fig 8F). The cytotoxic effect was specific for epithelial cells, since HUVEC were not affected by MBL (Fig 8G). All PTEC experiments were performed using a PTEC cell line (HK-2). Therefore, we confirmed these observations by exposing primary human PTEC to MBL, which again resulted in MBL-induced cell death. Also here, this cell death could be prevented by the addition of D-, but not L-mannose (Fig 8H).

DISCUSSION

In the present study we identified a novel role for MBL in the pathogenesis of renal IRI. In a rat model of renal IRI, we demonstrated that therapeutic inhibition of MBL is protective against renal IRI. Following reperfusion, exposure of tubular epithelial cells to circulation-derived MBL resulted in internalization of MBL followed by the rapid induction of tubular epithelial cell death. This MBL-mediated tubular injury was completely independent of complement activation since interference with C3 or C5 was not protective against renal IRI. MBL-mediated cell death preceded complement activation strongly suggesting that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury, and not the lytic C5b-9 complex.

Several studies, mainly performed in mice, have shown an important role for complement in the induction of renal IRI. A study using C3, C5 and C6 deficient mice showed a predominant role for C5b-9 in renal IRI (6), and also inhibition of C5 with monoclonal antibodies was protective (23). In contrast to these data, others have shown that gene knock-out (24), inhibition (24) or depletion of C3 (25) was not protective against renal IRI. In our model deposition of C3 and C5b-9 following reperfusion was observed, although this was in a relatively late phase (24 hours onwards). A similar late kinetics of C5b-9 deposition has also been shown in a mouse IRI model (23), although this model was characterized

by an early increase in deposition of C3 (2 hours). We did not observe early C3 deposition, indicating possible differences in kinetics and pathogenesis of renal IRI among species. Differences in organ size and metabolism between species could play a role in the observed differences, given that in mice clamping times of usually 20-25 minutes are needed to induce renal injury, while in rats 45-60 minutes of clamping time is needed to induce substantial IRI. Also, difference in relative strength of complement activation pathways could be of influence. In this respect, it has been shown that serum complement activity even among rat strains are varying (26), explaining why in certain rat strains complement activation could be more involved.

Despite the fact that complement activation was not involved in the induction of renal IRI in our model, we did find a pivotal role for MBL in the pathogenesis of renal IRI, since transient inhibition of MBL-A in the early phase following reperfusion was completely protective. Studies in MBL-deficient mice, which are protected against renal IRI, confirm these findings (27). It is tempting to speculate that these mechanisms might contribute to our previous observation that genetically determined high levels of MBL were an important risk factor for renal graft loss following kidney or simultaneous pancreas kidney transplantation (10;11).

Deposition of MBL has been observed in human biopsies early after transplantation (28) and in pigs following renal IRI (14), and is classically linked to activation of the lectin pathway of complement. Interaction of MBL with meprins expressed on tubular epithelial cells was shown to be the initial step for complement activation (29;30). However, for the first time we here show a differential role for MBL in renal IRI, independent of its capacity to activate the complement system. MBL is able to directly induce renal tubular epithelial cell death in the complete absence of complement activation.

Recently, in intestinal IRI in man it was shown that MBL null alleles were associated with preserved epithelial cell integrity (31). Interestingly, also here no signs of complement activation were observed, suggesting that also in intestinal IRI, MBL might have a cytotoxic effect on epithelial cells.

In oncology, MBL has been shown to bear anti-tumor activity which has been termed MBL-dependent cell-mediated cytotoxicity (32). However, to the best of our knowledge, our studies are the first to identify a direct cytotoxic effect of MBL on non-malignant cells. Recently, an intracellular form of MBL has been described which possibly functions as a cargo transport lectin facilitating ER-to-Golgi traffic in glycoprotein quality control (33). Exogenous MBL internalized by tubular epithelial cells potentially could overload and dysregulate this system leading to induction of epithelial cell death, however more research is required to unravel the exact mechanism involved in MBL-induced tubular cell death.

In summary, our results document the important role of MBL in the early pathogenesis of renal IRI and suggest that therapeutic intervention at the level of MBL could significantly alter the extent of kidney damage following ischemia/reperfusion.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

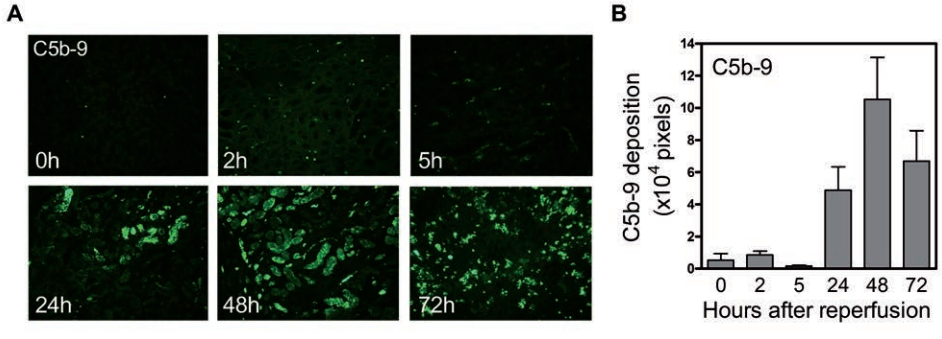

Figure S1. C5b-9 deposits in a late phase following renal ischemia/reperfusion. Deposition of C5b-9 in the cortico-medullary region at consecutive time points after reperfusion (A). C5b-9 deposition was assessed using a mAb to a neoepitope on rat C5b-9 (2A1, Hycult Biotechnology, Uden, The Netherlands) followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The staining was visualized using tyramide labeled with fluorescein (tyramide-FITC, Sigma-Aldrich-Aldrich). C5b-9 deposition was quantified using digital image analysis (B).

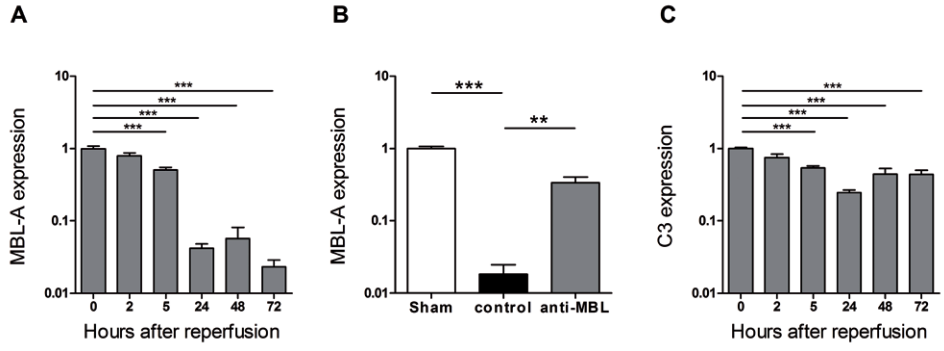

Figure S2. Renal ischemia/reperfusion affects local MBL-A and C3 expression. Renal mRNA expression of MBL-A (A, B) and C3 (C) at consecutive time points after reperfusion in untreated rats (A, C) and rats treated with anti-MBL or control antibody at 72 hours after reperfusion (B) was assessed. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. * $P < 0.05$; ** $P<0.01$. The primer sequences were as follows: MBL-A (forward, 5'- GGAAACCCTGAAGACTTGC -3'; reverse, 5'- CTGCCTCCATATTTGCCAG - 3') and C3 (forward, 5'- GCCAGCAGCTCTACAATGTG -3'; reverse, 5'- GACTGCCACTTTCCCATAGC- 3').

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