CHAPTER 6

IN PREPARATION

Mannan-binding lectin mediates endoplasmic reticulum stress and affects mitochondrial homeostasis in tubular cells following renal ischemia/ reperfusion through GRP78/BIP

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ABSTRACT

Ischemia/reperfusion injury (IRI) is a key event in kidney transplantation and is characterized by tubular epithelial cell injury. Recently, we demonstrated a crucial role for Mannan-binding lectin (MBL) in the pathogenesis of renal IRI. MBL, activator of the lectin pathway of complement, was shown to be directly cytotoxic to tubular cells independent of complement activation.

Here, we demonstrate that exposure of tubular cells to MBL *in vitro* mediates endoplasmic reticulum (ER)-stress. Assessment of ER-stress markers sXBP-1 and CHOP revealed a potent ER-stress response with a twenty- and tenfold induction respectively, accompanied by a strong IL-6 expression. Strikingly, ER-stress was followed by an excessive ATP production, loss of the mitochondrial membrane potential and production of mitochondrial superoxide. Confocal microscopy revealed a strong colocalization of internalized MBL with ER-stress sensor GRP78 and mitochondria, suggesting that interaction of MBL with GRP78 and mitochondria is detrimental to cellular homeostasis.

Assessment of sXBP-1 following renal ischemia/reperfusion in rats revealed an extensive induction of ER-stress within 2 hours, accompanied by an elevated IL-6 expression. Interestingly, assessment of GRP78 protein expression revealed a complete loss of GRP78 in the cortico-medullary region already 2 hours after reperfusion followed by tubular injury and abundant KIM-1 expression within 24 hours. Importantly, therapeutic inhibition of MBL completely prevented loss of GRP78, diminished IL-6 expression and protected against tubular injury.

In conclusion, we demonstrated that MBL mediates ER-stress and affects mitochondrial homeostasis in tubular cells. Interaction with GRP78 might be the mechanism by which MBL mediates tubular injury following renal ischemia/reperfusion.

INTRODUCTION

In kidney transplantation, ischemia/reperfusion (I/R) is an inevitable event and associated with extensive injury to the renal proximal tubular epithelial cells (PTEC). 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 and clinical morbidity and mortality (1-3). To date, no effective therapy or treatment for renal IRI in the clinic exists.

Hypoxia/reoxygenation during renal ischemia/reperfusion (I/R) is a complex stress, characterized by interconnected cellular processes such as ATP depletion,

protein misfolding, generation of reactive oxygen species (ROS), and elevation of intracellular calcium. PTEC are highly metabolic active and equipped with a vast amount of mitochondria, and are therefore particularly sensitive to these processes.

The ER is the major intracellular calcium storage site and high lumenal calcium is essential for normal ER function. ATP shortage during ischemia results in a calcium leak from the ER, which impairs the activity of molecular chaperones and maturation of native proteins. These events result in accumulation of mis- and unfolded proteins in the lumen of the ER leading to ER-stress (4-6).

Calcium flux from the ER into the cytosol is buffered to some degree by mitochondrial calcium uptake stimulating mitochondrial ATP production. However, once a continuous increase in cytosolic calcium exceeds the buffering capacity, mitochondria become dysfunctional and mitochondria-mediated apoptotic pathways are activated leading to cell death (7;8). Various adaptive responses to maintain homeostasis and survival are therefore employed, including the activation of the ER-stress response.

The glucose-regulated proteins (GRPs), a family of molecular chaperones and calcium-binding proteins located in ER are induced by ER-stress. Induction of GRPs by ER-stress protects cells against oxidative stress and ischemia-related processes. The concept of ischemic preconditioning is based on this concept where a mild insult is sufficient to induce GRP expression and renders cells tolerant to a subsequent lethal insult (9;10). GRP78, a molecular chaperone also known as BiP or immunoglobulin heavy chain binding protein, is the master modulator of the ER-stress response and under physiologic conditions is associated with three resident ER transmembrane proteins, Upon dissociation and binding of GRP78 to misfolded proteins, these transmembane proteins are activated which leads to a variety of cellular responses to restore normal ER function (4-6).

Recently, we demonstrated a crucial role for Mannan-binding lectin (MBL) in the pathogenesis of renal IRI (11). MBL, an innate immune protein and activator of the lectin pathway of complement, was shown to be directly cytotoxic to tubular epithelial cells independent of complement activation. Upon reperfusion of the ischemic kidney, vascular leakage exposes tubular epithelial cells to circulation-derived MBL, which contributed to tubular injury. Considering the important protective role of GRPs in renal IRI, we hypothesize that MBL might interfere with GRPs leading to tubular cell death.

Here, we demonstrate that renal I/R is accompanied by ER-stress and loss of GRP78 in the most affected cortico-medullary region of kidney. Therapeutic targeting of MBL *in vivo* protects tubular cells located in this region from loss of vital GRP78 and consequent tubular injury. *In vitro*, we demonstrated that

basolateral internalization of MBL by tubular cells induces ER-stress accompanied by excessive ATP production, loss of the mitochondrial membrane potential and production of mitochondrial superoxide followed by tubular cell death.

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) (11) or as a control anti-human fibronectin mAb (IgG1; 1 mg/kg). Rats were sacrificed 2, 5 or 24 h following reperfusion.

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 on formalin-fixed kidneys embedded in paraffin, which 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. Kidney sections were stained with digitonin (DIG)-conjugated mAb against CD43 (W3/13; macrophages), goat pAb against rat Kidney-injury molecule (KIM)-1 (R&D systems, Abingdon, UK) or rabbit pAb against rat GRP78 (Abcam), followed by horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany), rabbit anti-goat (DAKO, Glostrup, Denmark) or goat anti-rabbit pAb (DAKO), respectively. The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Quantification of immunohistochemistry was performed in a blinded manner by assessing high power fields (HPFs; original magnification ×200) of the complete rat kidney using

a slide scanning microscope (Leica, Rijswijk, The Netherlands). Using imageJ software, the positive area in the outer medulla and cortico-medullary junction (expressed in pixels) was quantified.

RNA isolation and real-time PCR

RNA was extracted from snap frozen renal rat tissue or cultured human PTEC (HK-2) and purified using an RNeasy Mini isolation Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Specific rat primers for GRP78, CHOP and ribosomal protein S-15 (RPS-15, housekeeping gene), and specific human primers for spliced XBP-1 (12) and CHOP were designed using Oligo Explorer and obtained from Biolegio (Nijmegen, The Netherlands). The primer sequences were as follows: rat KIM-1 (forward, 5'-TACCAACATCAACCAGAGTCTC-3'; reverse, 5'-ACATAGAAGCCCTTAGTCGG-3'), rat CHOP (forward, 5'-GAGTCTAATACTCGATCATACC-3'; reverse, 5'-TTGATTCTTCCTCTTCGTTCC-3'), rat GRP78 (forward, 5'-GAGATTGTTCTGGTTGGCGGATCTACTC-3'; reverse, 5'-CCATATGCTACAGCCTCATCTGGGTT-3'), rat RPS-15 (housekeeping gene: forward 5'-CGTCACCCGTAATCCACC-3' and reverse 5'- CAGCTTCGCGTATGCCAC-3'), human CHOP (forward, 5'-GGAGCATCAGTCCCCACTT-3'; reverse, 5'-TGTGGGATTGAGGGTCACATC-3'), human sXBP-1 (forward, 5'-TGCTGAGTCCGCAGCAGGTG-3'; reverse, 5'-GCTGGCAGGCTCTGGGGAAG-3'), human IL-6 (forward, 5'- AAGCCAGAGCTGTGCAGATGAGTA-3'; reverse, 5'-AACAACAATCTGAGGTGCCCATGC-3') and human GAPDH (housekeeping gene: forward 5'-TTCCAGGAGCGAGATCCCT-3' and reverse 5'-CACCCATGACGAACATGGG-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 rat RPS-15 or human GAPDH. RT PCRs were performed in duplicate.

XBP1 PCR splicing analysis

ER-stress-induced processing of rat XBP1 mRNA was evaluated by PCR and restriction site analysis. Primer for rat XBP-1, encompassing the IRE1 cleavage site of XBP1, was amplified using the rat XBP-1 primer (forward, 5'- AAACAGAGTAGCAGCGCAGACTGC-3'; reverse, 5'-GGATCTCTAAAACTAGAGGCTTGGTG-3') resulting in a 601-bp PCR product. PCR products were subsequently incubated with PstI at 37°C for 5 h followed by separation on 2% agarose gels. sXBP-1 lacks the PstI restriction site, which results in a larger PCR product. Upper bands representing spliced XBP-1 were quantified using image J software.

Cell culture

Immortalized human PTEC (HK-2) were grown as previously described (13). Cells were grown and exposed to MBL in 48-wells culture plates. To study basolateral exposure of MBL, cells were grown to 95% confluency in a transwell system (0,4 µM polyester membrane 12 mm inserts; Corning Life Sciences, New York, USA)

IL-6 ELISA

Secreted IL-6 in culture supernatants was measured using a commercial IL-6 sandwich ELISA (Sanquin, Amsterdam, Netherlands) according to the manufacturer's instructions.

Fluorescence microscopy

HK-2 cells were fixed using cold methanol or 1% paraformaldehyde (both from Pharmacy) and stained with mAb against human MBL (mAb 3E7; Hycult Biotech) and a rabbit pAb against human golgin-97 (Invitrogen, Carlsbad, USA), mitofilin (Abcam, Cambridge, UK) or GRP78 (Abcam) followed by alexa-488 conjugated goat anti-mouse IgG (Molecular Probes) and alexa-568 conjugated goat anti-rabbit IgG (Molecular Probes), respectively.

Mitochondrial assays

Mitochondrial membrane activity and mitochondrial superoxide production were assessed using MitoTracker Red and MitoSOX Red (both from Invitrogen), respectively. PTEC were exposed to MBL for consecutive time points followed by 15 minutes incubation with Mitotracker Red, MitoSOX Red was incubated during MBL exposure. Consequently, red fluorescence was assessed using fluorescence microscopy.

ATP measurement

Intracellular ATP was measured using a commericial ATP bioluminescent assay kit (Sigma Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's instructions.

Viability assays

HK-2 cells were washed and permeabilized using cold methanol and nuclei were stained using Hoechst. Viability was assessed by counting Hoechst-positive cells using fluorescence microscopy.

Preparation of human MBL

MBL was purified from human recalcified plasma as previously described (14;15).

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

Basolateral interaction with MBL is cytotoxic for human tubular cells

Recently, we demonstrated direct cytotoxicity of MBL towards tubular cells following renal I/R (11). In the present study we set out to dissect the mechanism by which MBL mediates tubular cell death. First, the *in vivo* condition following renal I/R was mimicked by exposing human PTEC (HK-2) to purified human MBL. Exposure to MBL at 4°C for 1 h resulted in extensive binding of MBL as assessed by fluorescence imaging and FACS analysis (Fig 1A). Incubation at 37°C, which allows internalization, revealed that MBL was internalized within minutes and trafficked to a perinuclear region (Fig 1B). Prolonged incubation with MBL resulted in extensive vacuolization after 8 h followed by rounding and detachment of cells within 24 h (Fig 1C). Vascular leakage following reperfusion results in exposure of tubular cells to MBL from the basolateral side, from where MBL should be subsequently internalized (11). To mimic this process in our *in vitro* model, we made use of a transwell system and exposed PTEC from the upper (apical) or lower (basolateral) compartment. Only basolateral exposure of PTEC to human MBL induced cell death, whereas apical exposure only had minimal effect (Fig 1D). This suggests that a basolateral membrane protein or internalization process is involved in this MBL-mediated cell death.

Internalized MBL colocalizes with the endoplasmic reticulum and mitochondria

Subsequently, intracellular trafficking of MBL was studied. No colocalization of MBL with the Golgi was found, whereas staining for GRP78 (Fig 2), an ER-residing protein, revealed a strong colocalization suggesting trafficking of MBL to the ER. GRP78 is a stress-inducible-ER chaperone, which serves as a master modulator of the ER-stress response. Recently, it was shown that GRP78 under stress conditions also traffics to mitochondria (16). Staining for MBL and a mitochondrial marker (mitofilin) indeed revealed a partial colocalization, suggesting that MBL might interacts with GRP78-positive mitochondria. Colocalization of MBL with GRP78 or mitofilin was subsequently confirmed by confocal microscopy.

MBL induces ER-stress in human tubular cells

GRP78 has been shown to prevent oxidative stress, calcium disturbances and cell death in PTEC and is indispensable for tubular cell homeostasis (17-19). To study the functional consequences of MBL internalization and interaction with GRP78, several ER-stress markers were assessed following exposure to MBL. Splicing of mRNA sXBP-1 by IRE1, expression of CHOP as a consequence of PERK and eIF2a phosphorylation, and IL-6 expression induced by JNK-pathway were assessed as markers for ER-stress and revealed a twenty- and tenfold induction of sXBP-1 (Fig 3A) and CHOP (Fig 3B) respectively. sXBP-1 and CHOP expression occurred within 30 minutes indicating that ER-stress mediated by MBL is one of

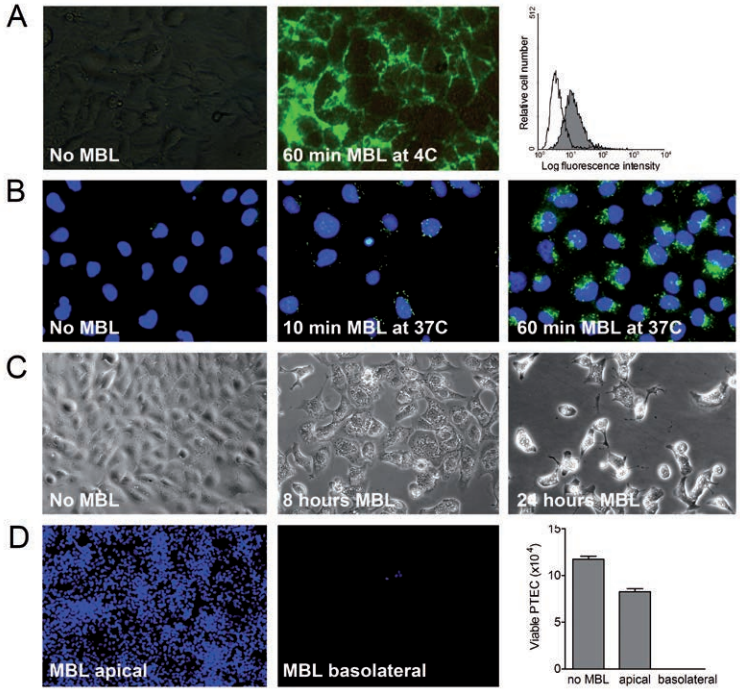

Figure 1. Basolateral interaction with MBL is cytotoxic for human tubular cells. Human PTEC (HK-2) were incubated with 10 µg/mL MBL for 1 h at 4°C (A) or 10 and 60 min at 37°C (B). Cells were stained extracellular (A) or intracellular (B) for MBL (green) and cells were photographed using a fluorescence microscope (original magnification ×200) or analysed by flow cytometry (A). Nuclei (blue) were stained using Hoechst (B). Cells were incubated with 10 µg/mL human MBL for 8 or 24 h at 37°C (C) and cells were photographed (original magnification ×200). To study apical or basolateral exposure to MBL (D), cells were cultured in a transwell system and exposed to 10 µg/mL MBL from the upper (apical) or lower (basolateral) compartment for 24 h. Cell viability was assessed by counting Hoechst-positive, but PI-negative cells in the well using fluorescence microscopy (original magnification ×100). Values shown are mean ± SEM of triplicate determinations from a single experiment and are representative of two similar transwell experiments.

the first consequences of MBL exposure. ER-stress following MBL exposure was accompanied by a strong and dose-dependent IL-6 protein expression (Fig 3C). Exposure to 10 or 20 µg/mL MBL induced a rapid cell death within 12 h, which might explain the lower levels of IL-6 in these conditions.

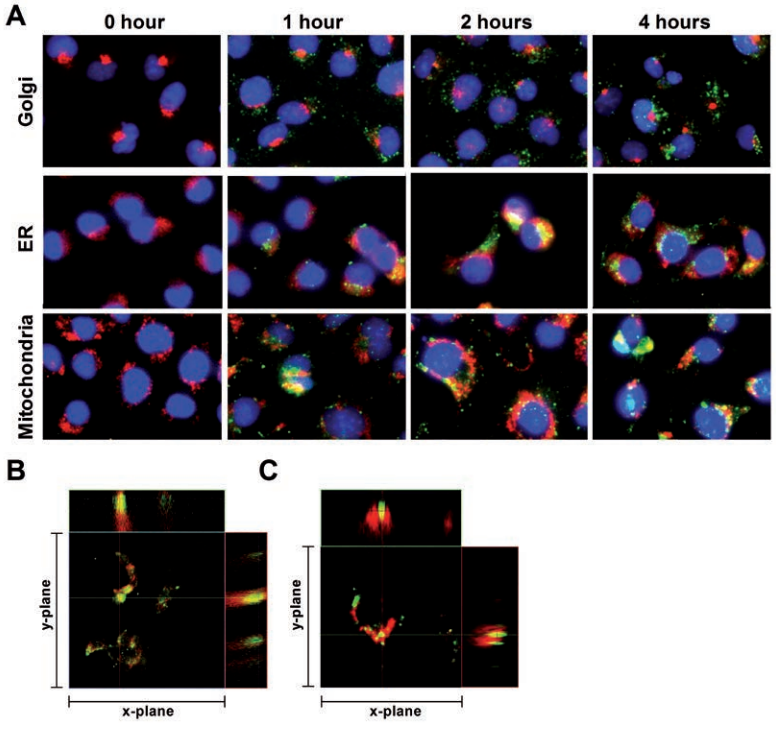

Figure 2. Intracellular trafficking of internalized human MBL. PTEC (HK-2) were exposed to MBL for 1, 2 or 4 h at 37°C, fixed and stained (A) for MBL (green) and a marker (red) for the golgi (golgin), endoplasmic reticulum (GRP78) or mitochondria (mitofilin). Nuclei (blue) were stained using Hoechst. Double staining (yellow) was found for MBL with GRP78 or mitochondria, but not with golgin. Cells were photographed using fluorescence microscopy (original magnification x600). PTEC (HK-2) were exposed to MBL for 4 h at 37°C, fixed and stained for MBL (green) and a marker (red) for the endoplasmic reticulum (B, GRP78) or mitochondria (C, mitofilin). Nuclei (blue) were stained using Hoechst. Colocalization (yellow) was assessed by confocal microscopy.

MBL affects mitochondrial homeostasis

ER-stress results in calcium flux from the ER into the cytosol, which is buffered to some degree by mitochondrial calcium uptake. Increased mitochondrial calcium stimulates oxidative metabolism and ATP production. This prompted us to study whether MBL-mediated ER-stress might influence mitochondrial homeostasis. Assessment of cellular ATP levels revealed that ER-stress was followed by an

excessive ATP production in the first hours of MBL exposure. Ablation of ATP production at 24 h was accompanied by tubular cell death. (Fig 4A).

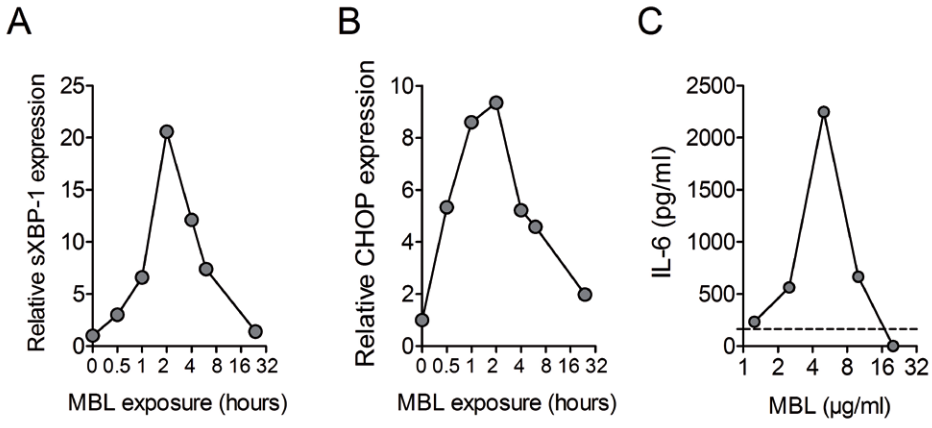

Figure 3. MBL induces ER-stress in human PTEC. PTEC (HK-2) were incubated with 10 µg/mL MBL for consecutive time points. Subsequently, mRNA was harvested and expression of spliced (s)XBP1 (A) and CHOP (B) was assessed by RT-PCR. PTEC were incubated with MBL (0-10 µg/mL) for 24 h. Culture supernatants were collected and secreted IL-6 content was measured by an IL-6 sandwich ELISA (C).

Prolonged ER-stress might lead to mitochondrial injury induced by excessive calcium influx into the mitochondria. Strong calcium entry activates mitochondrial superoxide generation and collapse of the mitochondrial membrane potential. In addition, mitochondrial GRP78 has been shown to stabilize Raf-1 to maintain mitochondrial permeability and to protect cells from ER-stress-induced apoptosis (20). This prompted us to more in-depth study mitochondrial homeostasis.

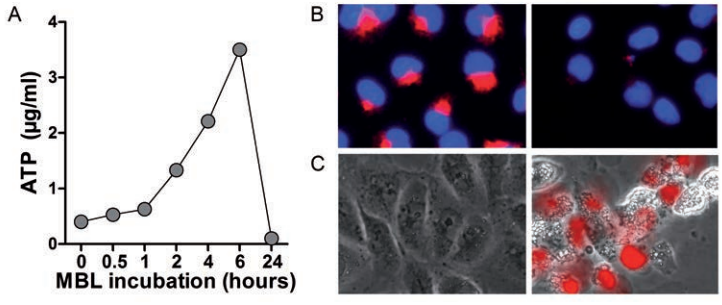

Figure 4. MBL affects mitochondrial homeostasis. PTEC (HK-2) were exposed to 10 µg/mL MBL for 0-24 h, after which intracellular ATP content was measured (A). PTEC (HK-2) were exposed to 10 µg/mL MBL for 4 h (B, C). To assess the mitochondrial membrane potential (B), cells were incubated with MitoTracker Red for 15 min after MBL exposure. Cells were fixed and nuclei (blue) were stained using Hoechst. To assess superoxide production (C), cells were incubated with MitoSOX Red during 4h MBL exposure. Cells were photographed using a fluorescence microscope (original magnification x600).

Active mitochondria were stained using MitoTracker Red, which accumulation in mitochondria is dependent on the mitochondrial membrane potential. Normal PTEC showed active mitochondria, however exposure to MBL for 4 h dramatically reduced the mitochondrial membrane potential (Fig 4B). Loss of membrane potential was accompanied by mitochondrial superoxide production, as assessed by MitoSOX Red which specifically is targeted to active mitochondria and upon oxidation by mitochondrial superoxide produces red fluorescence (Fig 4C). The massive increase in ATP production, loss of mitochondrial membrane potential and ROS formation were preceded by a period of extensive ER-stress (Fig 3), suggesting that mitochondrial alterations are the result of the initial ER-stress induced by MBL.

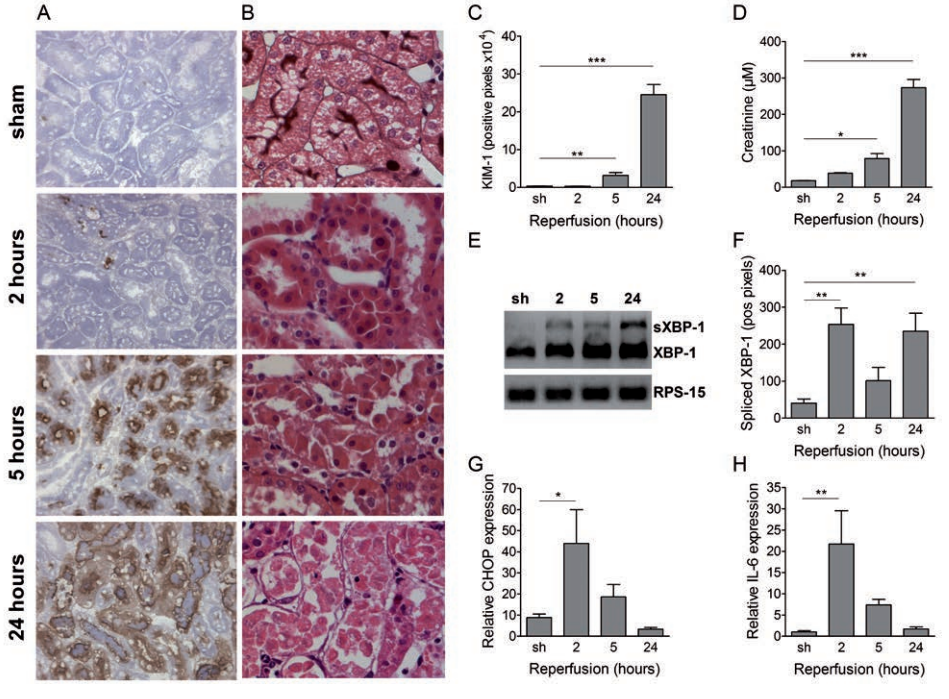

Figure 5. Renal I/R is accompanied by ER-stress and tubular injury. Lewis rats (n=6 per group) underwent unilateral ischemia for 45 min and removal of the contralateral kidney. Rats (n=6 per group) were sacrificed at consecutive time points and renal sections were stained for Kidney-injury molecule (KIM)-1 (A, original magnification x200) or processed for silver staining (B, original magnification x400). KIM-1 was quantified using digital image analysis (C). Following reperfusion or in sham (sh) operated rats, blood samples were drawn at consecutive time points to assess renal function by measuring serum creatinine (D). Renal mRNA expression of rat spliced (s)XBP-1 (E,F). CHOP (G) and IL-6 (H) was assessed in renal tissue at consecutive time points following reperfusion or in sham (sh) operated rats. 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.

Renal I/R is accompanied by ER-stress and tubular injury

To study the *in vivo* relevance of these findings, we assessed the role of MBL in ER-stress in a rat model of renal IRI. Firstly, tubular injury and occurrence of ER-stress was assessed. Clamping of the renal pedicle for 45 minutes and removal of the contralateral kidney resulted in severe renal dysfunction (Fig 5D) within 24 h, which was accompanied by extensive protein expression of Kidney-injury molecule (KIM)-1 within 5 h upon reperfusion (Fig 5A, C). A silver staining revealed that tubular alterations and loss of tight junctions already occurred within 2 h upon reperfusion (Fig 5B). Interestingly, this injury was accompanied by a significant splicing of XBP-1 (Fig 5E, F), expression of CHOP (Fig 5G) and IL-6 (Fig 5H) 2 h after reperfusion.

Renal I/R is accompanied by loss of ER-stress sensor GRP78

Staining for KIM-1 revealed that mainly the tubuli in the corti-medullary transition were injured and extensively expressed KIM-1 at 24 h after reperfusion (Fig 6A). Strikingly, staining for GRP78 revealed that particularly in this region, tubular GRP78 protein expression was lost (Fig 6B) already 2 h after reperfusion. While some tubuli normally expressed GRP78, others completely lost expression (Fig

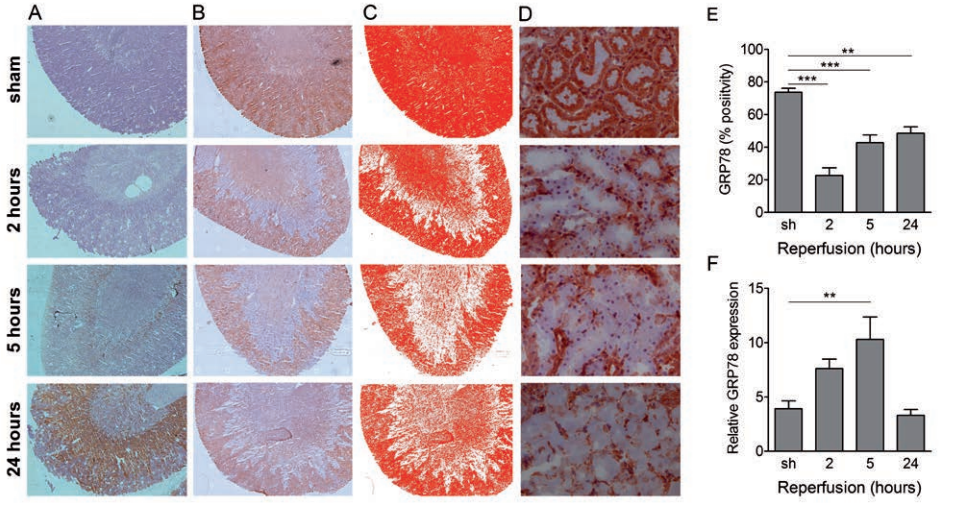

Figure 6. Renal I/R is accompanied by loss of ER-stress sensor GRP78 in the cortico-medullary tubular cells. Renal sections (n=6 per group) from sham operated rats or rats sacrificed 2, 5 or 24 h after reperfusion were stained for KIM-1 (A) or GRP78 (B). Sections were photographed and photographs were digitally stitched and analyzed (C) using digital image. GRP78 staining in the cortico-medullary region (D, original magnification x400) was digitally quantified (E). GRP78 mRNA expression (F) was assessed in total renal tissue at consecutive time points following reperfusion or in sham (sh) operated rats. 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.

6D), which might result in extensive ER-stress in these cells. Quantification (Fig 6C, E) showed that most staining was significantly lost within 2 h upon reperfusion which was accompanied by an increased mRNA expression of GRP78.

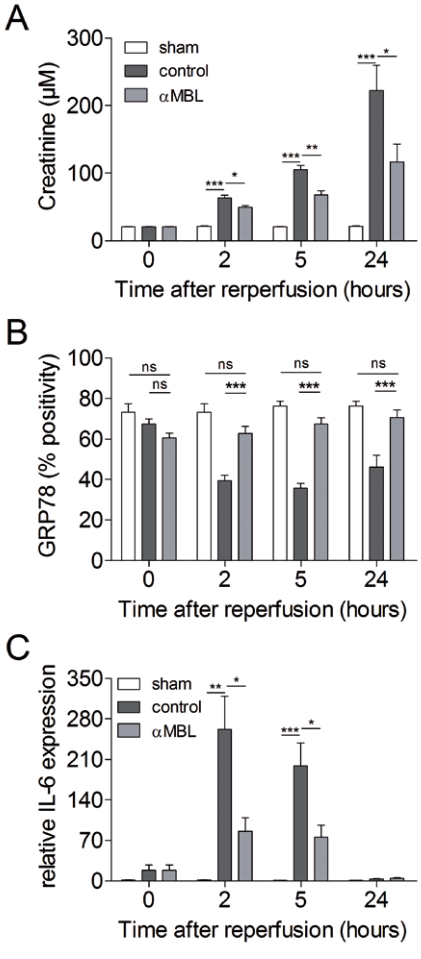

Figure 7. Inhibition of MBL prevents loss of ER-stress sensor GRP78 and subsequent IL-6 expression. Before induction of 45 min of unilateral ischemia and removal of the contralateral kidney, rats received anti-MBL-A or an isotype-matched control mAb (1 mg/kg). Before and following reperfusion blood samples were drawn at consecutive time points to assess renal function by measuring serum creatinine (A) Renal sections (n=6 per group) from sham operated, control-treated or anti-MBL treated rats sacrificed 2, 5 or 24 h after reperfusion were stained for GRP78. Staining in the cortico-medullary region was subsequently digitally quantified (B). IL-6 mRNA expression (C) was assessed in renal tissue at consecutive time points. 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.

Inhibition of MBL prevents loss of ER-stress sensor GRP78

To assess the role of MBL in this, we therapeutically target MBL using the protective and blocking mAb (P7E4) to MBL (11). Inhibition of MBL following I/R significantly protected against renal dysfunction (Fig 7A). Importantly, inhibition of MBL completely prevented the loss of tubular GRP78 early after reperfusion, whereas control-treated animals lost their GRP78 protein expression in the cortico-medullary tubular cells (Fig 7B). Loss of GRP78 was accompanied by an extensive expression of IL-6, which might be related to ER-stress and loss of GRP78. In addition, anti-MBL treatment also significantly reduced IL-6 expression

early after reperfusion (Fig 7D), suggesting that MBL plays an important role in early tubular ER-stress following reperfusion. These findings await further studies on the role of MBL in tubular mitochondrial homeostasis *in vivo* which might be closely related to this process.

DISCUSSION

Recently we demonstrated a pivotal role for MBL in the pathogenesis of renal IRI (11). MBL has cytotoxic effects towards PTEC independent of complement activation contributing to tubular injury following reperfusion. Here, we demonstrate that MBL interacts with GRP78 in PTEC, mediates ER-stress and affects mitochondrial homeostasis. Therapeutic inhibition of MBL *in vivo* protected tubular cells from loss of vital GRP78 and consequent tubular injury

GRP78 is a constitutively expressed molecular chaperone, however expression is enhanced under a variety of stressful conditions including hypoxia, glucose deprivation, alterations in intracellular calcium and oxidative stress (21), events that take place during renal I/R. Induction of GRP78 is critical for maintaining viability of cells that are subjected to such stresses and loss of GRP78 would therefore be detrimental. Here, we show that renal I/R is accompanied by ER-stress and loss of GRP78 in the cortico-medullary region of kidney, the region that is mostly affected. Importantly, therapeutic inhibition of MBL protected tubular cells located in this region from loss of vital GRP78 and consequent tubular injury, indicating that MBL might be injurious to tubular cells by interfering with GRP78. Expression of proinflammatory IL-6, a consequence of GRP78 degradation (22) and ER-stress (23;24), was also significantly reduced by inhibition of MBL following reperfusion.

GRP78 has been shown to prevent oxidative stress, calcium disturbances and cell death in renal epithelial cells (17;25). Expression of antisense RNA, targeted to GRP78 sensitized tubular cells to oxidant-induced cell death (19), indicating that GRP78 is indispensable in tubular cells under oxidative stress.

Internalization of circulation-derived MBL *in vivo* was shown to be the process by which tubular epithelial cells following renal I/R might be injured (11). By exposing tubular cells *in vitro* to MBL, we here demonstrated that basolateral internalization of MBL by tubular cells induced a potent ER-stress response which was accompanied by an excessive ATP production, a subsequent loss of the mitochondrial membrane potential, production of mitochondrial superoxide followed by induction of cell death. Internalized MBL colocalized with mitochondria and GRP78. Although GRP78 is mostly known from its function as molecular chaperone in the ER, GRP78 has also been shown to be

present, although in low amounts, on mitochondria, in the cytoplasm, on the cell membrane and as a secreted form (16;26-30). Membrane-bound GRP78 might be a possible candidate as a cellular receptor by which MBL is internalized. Indeed, it has been shown that MBL is able to interact with GRP78 in hepatic cells (31), suggesting that a detrimental interaction with GRP78 in tubular cells might be possible, however the mechanism by which MBL is internalized is still subject of investigation. Interestingly, overexpression of calreticulin, another ER chaperone also protected tubular cells against oxidative stress (18). Calreticulin has been described as the MBL receptor on phagocytosing cells (32;33), making this also a possible candidate by which MBL might be internalized.

The mechanism by which MBL affects mitochondrial homeostasis and ER-stress is still speculative since both processes are tightly intertwined. Depletion of cellular ATP as a consequence of ischemia or oxidative stress is known to induce ER-stress, since most GRP78 uses the energy from ATP hydrolysis to promote folding and prevent aggregation of proteins within the ER (34). Vice versa, ER-stress enhances mitochondrial calcium to generate more ATP (35;36). However, excessive calcium flux from the ER to the mitochondria during prolonged ER-stress will lead to mitochondrial injury and subsequent ATP depletion (7;37). GRP78 has been shown to be involved in both processes. First of all, GRP78 prevents calcium flux from the ER (36), probably by interacting with the translocon calcium channel in the ER-membrane (8). In addition, GRP78 on mitochondria has been shown to stabilize the mitochondrial membrane potential by associating with raf-1 and cooperatively confer resistance to ER-stress induced cell death (20). Interference of MBL with GRP78 might affect both processes. Our data suggest that initially ER-stress is induced following MBL exposure, followed by disturbances in mitochondrial homeostasis. Release of calcium from ER stores might be the main cause of MBL-mediated ER-stress. An increase in cytosolic calcium in tubular cells then would lead to enhanced calcium influx into mitochondria, disrupting mitochondrial metabolism eventually leading to cell death.

Recently is has been shown that ligation of cell-surface GRP78 on cancer cells also causes release of calcium from ER stores mediated via phospholipase C, thereby increasing cytosolic calcium (38). In line with this, it is known that especially tumor cells have increased levels of GRP78 on the cell surface. Importantly, is has been shown that MBL bears antitumor effects, which has been termed MBL-dependent cell-mediated cytotoxicity (39). Since MBL is able to interact with GRP78, we speculate that, next to killing of tumor cells, the cytotoxic effect of MBL on tubular cells might also be mediated by signaling through cell surface GRP78.

Under physiologic conditions GRP78 is hardly present on normal cells, however

induction of ER-stress has been shown to promote GRP78 localization on the surface of kidney cells (30). ER-stress induced by glucose deprivation during renal ischemia might be the initial trigger for GRP78 to traffic to the basolateral membrane of the tubular cells. Especially tubular cells in the cortico-medullary border are subjected to low oxygen and glucose levels during I/R, making these cells particular sensitive to ER-stress. It is therefore tempting to speculate that vascular leakage following reperfusion enables circulation-derived MBL to enter the interstium and bind to GRP78 on the cell surface of the tubular cells. Signaling to GRP78 might then have detrimental effects. Next to signaling, internalization of MBL might also be mediated by GRP78, since evidence has emerged that GRP78 also serves as a receptor for viral entry into host cells (40).

In this study we show that loss of GRP78 *in vivo* is tightly connected to MBL, because inhibition of MBL completely preserved GRP78 protein expression in the cortico-medullary tubular cells. However, the mechanism by which GRP78 is degraded is still unknown. Recently, it was shown in macrophages that the mycotoxin deoxynivalenol induced ER-stress, which was accompanied by loss of GRP78 and high expression of IL-6(22). The authors suggested that the loss of GRP78 was related to an autophagic pathway. The process of autopahgy has also been described in renal IRI and might be involved in degradation of GRP78. In addition, it has been shown that cytosolic GRP78 might be secreted(26), however the mechanism involved requires further investigation.

In summary, our results document the important role of MBL in renal IRI. We demonstrated that inhibition of MBL *in vivo* protected tubular cells from loss of vital GRP78 and consequent tubular injury. Basolateral internalization of MBL by tubular cells *in vitro* induced ER-stress accompanied by excessive ATP production, loss of mitochondrial membrane potential and production of mitochondrial superoxide followed by tubular cell death. These results indicate that therapeutic intervention at the level of MBL might be a promising target in kidney transplantation.

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