Cardiovascular, Pulmonary and Renal Pathology

Myeloperoxidase Is Critically Involved in the Induction of Organ Damage after Renal Ischemia Reperfusion

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**In this study the role of myeloperoxidase (MPO) in a
murine (C57BL/6) model of ischemia and reperfusion
(I/R)-induced renal failure was investigated. The renal
function after I/R was analyzed in MPO-deficient
($Mpo^{-/-}$) mice and compared with wild-type (WT)
controls. A significant reduction in renal function
loss (blood urea nitrogen) was observed after 24
hours of reperfusion of ischemically damaged kid-
neys in $Mpo^{-/-}$ mice compared with I/R WT controls
(I/R $Mpo^{-/-}$ = 31.3 ± 1.7 mmol/L versus I/R WT =
42.8 ± 2.1 mmol/L, sham = 7.0 ± 0.5 mmol/L; P =
0.003). The early reperfusion phase (2 hours of reper-
fusion) was characterized by a substantial increase in
apoptosis and early complement activation, surpris-
ingly similar in $Mpo^{-/-}$ and WT mice. Improved renal
function in $Mpo^{-/-}$ mice after extended reperfusion
was accompanied by a reduced neutrophil influx (P =
0.017) compared with WT controls. Activation and
deposition of complement was not significantly re-
duced in $Mpo^{-/-}$ mice compared with WT controls
after 24 hours of reperfusion, indicating no specific
in vivo role for MPO in activating complement after
renal I/R. Taken together, these results demonstrated
an important contribution of MPO in the induction of
organ damage after renal I/R by influencing critical
factors such as neutrophil extravasation but not com-
plement activation.** *(Am J Pathol 2007, 171:1743–1752;
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In clinical medicine, complications arising from organ
ischemia and reperfusion (I/R) are common phenomena,
complicating the treatment of severely injured or ill pa-
tients and influencing the outcome of various clinical
conditions. Throughout the past decades studies into the
pathophysiology of I/R-induced organ damage have
identified several critical factors—cellular injury, endo-
thelial dysfunction, microcirculatory collapse, neutrophil
activation and extravasation, and complement activation,
all contributing to the development of organ dysfunction.
Our limited understanding of exact pathophysiological
mechanisms has so far impaired the development of new
and effective therapies.

Cellular injury, induced by ischemia and aggravated
on reperfusion, forms a potent trigger for activation of an
extensive inflammatory response, illustrated by the pro-
duction of various cytokines such as tumor necrosis fac-
tor-α, interferon-γ, and the interleukins 6, 10, 12, and
18,[1,2] the activation and sequestration of polymorphonu-
clear neutrophils (PMNs) in the affected area,[3] as well as
the expression and deposition of various components of
the innate immune response, such as complement fac-
tors.[4–6] Under healthy conditions, cells and proteins of
the innate immune system protect the organism by or-

chestrating a well mounted attack on invading microorganisms, but when faced with extensive I/R injury, sufficient means of control seem absent. Already during the 1980s a detrimental role for PMNs was shown during hypoperfusion and ischemia followed by reperfusion.[7,8] These early studies proposed a role for activated neutrophils in the frequently observed no-reflow phenomenon[9] and the generation of harmful reactive oxygen species after reperfusion of ischemically damaged tissue.[10]

Myeloperoxidase (MPO) is a 140-kDa heme protein that is predominantly stored in the lysosomes of monocytes and in the azurophilic granules of PMNs.[11] It is one of the most abundant enzymes released on neutrophil activation. The capacity of MPO to catalyze the formation of hypochlorite (HOCl) from hydrogen peroxide (H_2O_2) and chloride ions makes it a powerful tool in the bactericidal armament of these cells. However, there are clinical studies indicating a potentially harmful effect of MPO in immune-mediated inflammatory syndromes, such as multiple sclerosis,[12] acute coronary syndrome,[13,14] and renal disease.[15] In addition, a considerable line of research indicates that MPO and MPO-derived oxidants are involved in the pathogenesis of atherosclerosis,[16–18] organ damage after myocardial I/R and infarction,[19] as well as complement activation *in vitro*.[20] Furthermore, MPO contributes to the dysfunction of the local vasculature during acute inflammation by modifying local NO production and availability.[21]

To study the *in vivo* contribution of MPO in the development of I/R-induced injury, the role of MPO in a mouse model of renal I/R injury was investigated. Disease severity was compared between MPO-deficient ($Mpo^{-/-}$) and wild-type (WT) mice with respect to renal function, complement activation, neutrophil activation and extravasation, renal morphology, and apoptosis. Our results show that MPO plays a detrimental role in the pathogenic mechanisms involved in this model and is in part responsible for the development of renal damage resulting from I/R without influencing complement activation *in vivo*.

Materials and Methods

Mice

$Mpo^{-/-}$ mice, backcrossed to a C57BL/6 background six times, were genotyped using polymerase chain reaction (PCR)-amplified DNA from tail clippings.[22] WT male C57BL/6 control mice (11 weeks of age) were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Mice were kept according to University of Maastricht animal facility regulations, and all experiments were approved by the local animal ethical committee.

Experimental Procedures

Experiments were performed as previously described, with minor modifications.[6] At the start of the experiments, mice ($n = 6$ in each group) were anesthetized with sodium pentobarbital (100 mg/kg i.p.). Body temperature was maintained at 37°C by a heating pad until animals

recovered from anesthesia. Under aseptic conditions a 1.0-cm-long midline abdominal incision was made, and ischemia was induced by applying a nontraumatic vascular clamp to the left renal pedicle for 40 minutes. Subsequently, the wound was covered with cotton soaked in sterile phosphate-buffered saline (PBS). After removal of the clamp, the left kidney was inspected for restoration of blood flow and the contralateral kidney was removed and stored for analysis. The wound was closed in two layers and in mice that were to be sacrificed after 24 hours, 0.25% bupivacaine was applied topically to the wound for postoperative pain management. The animals were sacrificed 2 or 24 hours after reperfusion. At the time of sacrifice, plasma was collected and the left kidney was harvested for morphological, immunohistochemical, and immunofluorescent analyses. Macroscopic evaluation of the ischemic kidneys during the procedure resulted in the exclusion of one mouse, because its kidney appeared to have been insufficiently ischemic during the experiment. One WT frozen tissue sample to be used for preparation of frozen sections was lost during work-up.

Plasma Measurements of Blood Urea Nitrogen (BUN) and MPO

In mice sacrificed after 24 hours, BUN levels in the plasma were determined by an enzymatic degradation assay on a Synchron LX20 PRO chemistry analyzer (Beckman Coulter Inc., Fullerton, CA). Plasma MPO levels were determined by in-house catching enzyme-linked immunosorbent assay as described previously.[23] Briefly, microtiter plates were coated (1 μg/ml) with Fcγ fragment-specific goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), incubated for 48 hours at 4°C, and blocked with 1% bovine serum albumin in PBS. Plates were then incubated with an anti-murine MPO-specific monoclonal antibody (mAb) (clone 8F4; Hbt, Uden, The Netherlands), followed by incubation with appropriately diluted plasma samples. Next, the plates were incubated with polyclonal rabbit anti-human MPO (DakoCytomation, Glostrup, Denmark) and alkaline phosphatase-labeled polyclonal goat anti-rabbit IgG as primary and secondary detection antibody, respectively. 4-Nitrophenyl phosphate (pNPP) was used as substrate, and results were analyzed spectrophotometrically at 405 nm. Concentrations were calculated from a standard curve of purified murine MPO (range, 2.5 to 100 ng/ml).

Immunofluorescence and Immunohistochemistry

Kidneys were snap-frozen in OCT compound. Five-μm sections were cut with a cryostat, dried, and stored at -70°C. The slides were fixed in -20°C cold acetone and stained for neutrophil influx using a rat anti-mouse neutrophil antibody (clone NIMP-R14, Hbt).[24] After incubation of the primary antibody, endogenous peroxidase activity was blocked using 0.05% H_2O_2 in PBS, and rabbit anti-rat IgG and goat anti-rabbit IgG-PO (both Dako-
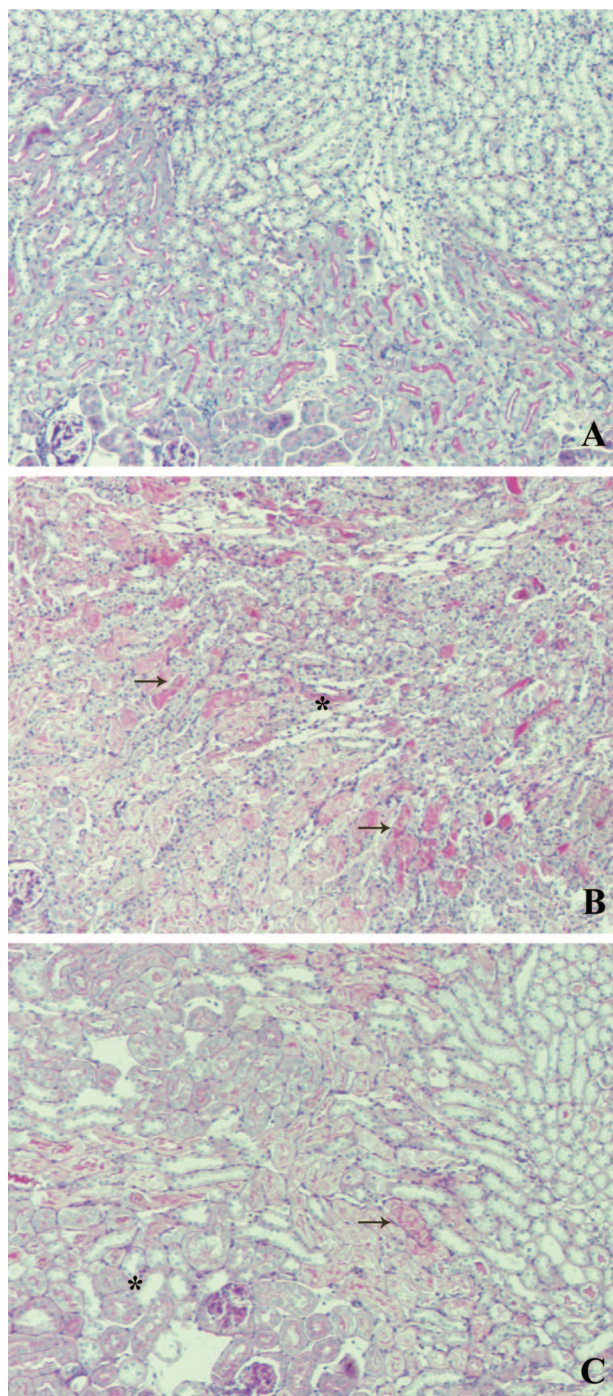
Figure 1. Similar renal morphology of ischemically damaged WT and KO kidneys after 24 hours of reperfusion. Microphotographs showing periodic acid-Schiff staining of kidney sections of sham-treated (**A**) as well as WT (**B**) and *Mpo*$^{-/-}$ (**C**) mice subjected to 40 minutes of ischemia followed by 24 hours of reperfusion. Shown are representative microphotographs of all groups. Original magnifications, ×100.

Cytomation) were then used as secondary and tertiary antibodies, respectively. Antibody binding was visualized using 3-amino-9-ethylcarbazole with H$_2$O$_2$ as substrate. Sections were counterstained with hematoxylin, and juxtamedullary neutrophil influx was quantified by counting the average number of NIMP-R14-positive cells in 10 high-power fields of four tissue sections per kidney in WT

and *Mpo*$^{-/-}$ mice (*n* = 5 per group), at 24 hours after reperfusion.

To determine co-localization of PMNs with renal MPO deposits, renal cross-sections were double-stained with a biotinylated mouse mAb specific for murine MPO (clone 8F4, Hbt)[23] and rat anti-mouse neutrophil mAb NIMP-R14 (Hbt), using Alexa 488-labeled streptavidin and Alexa 568-labeled goat anti-rat IgG (both Invitrogen Molecular Probes, Leiden, The Netherlands), respectively, as conjugates.

Murine complement factors MBL-A, MBL-C, C3, C6, and C9 were determined by using rat monoclonal (MBL-A, clone 8G6; MBL-C, clone 14D12; iC3b, clone 2/11; Hbt) or rabbit polyclonal anti-mouse C6 (kindly provided by Dr. A. Tenner, University of California, Irvine, CA) and rabbit anti-mouse C9 (in-house made by M.R.D.) primary antibodies. Specific binding was detected by using peroxidase-conjugated secondary antibodies to rat and rabbit IgG, respectively (Jackson ImmunoResearch). Staining was visualized by 3-amino-9-ethylcarbazole followed by hematoxylin. No significant staining was detected in slides incubated with control rat IgG (for NIMP-R14, MBL-A, MBL-C, and C3), mouse IgG (for MPO), and rabbit IgG (for C6 and C9). After immunohistochemical staining of kidneys (*n* = 5 per group), renal deposition of complement factors was scored arbitrarily as negative (−), slightly positive (+), moderately positive (++), and intensively positive (+++).

Western Blot

Western blot analyses of C6 deposition in sham-treated or reperfused ischemic knockout (KO) and WT kidneys was performed as described before, with minor modifications.[25] Renal tissue samples from WT and KO I/R or sham-treated animals were homogenized in lysis buffer (200 mmol/L NaCl, 10 mmol/L Tris base, 5 mmol/L ethylenediaminetetraacetic acid, 10% glycerin, 1 mmol/L phenylmethyl sulfonyl fluoride, 0.1 U/ml aprotinin, and 1 µg/ml leupeptin). Tissue homogenates were centrifuged at 300 rpm for 10 minutes, after which the collected supernatants were centrifuged again at 10,000 rpm for 3 minutes. The protein concentration of the different lysates was determined using Bradford analyses. Aliquots containing equal amounts (10 µg) of total protein or normal mouse serum as positive control were heated to 100°C for 5 minutes in sodium dodecyl sulfate-sample buffer containing 5% β-mercaptoethanol (Sigma, Chicago, IL), transferred to a 8% sodium dodecyl sulfate-polyacrylamide gel and blotted on an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blotting of the proteins, the blocking and antibody incubation steps were performed in phosphate-buffered saline containing 5% bovine serum albumin and 0.1% Tween 20 (Sigma). C6 was detected by incubating polyvinylidene difluoride membranes overnight at 4°C in buffer containing properly diluted rabbit anti-mouse C6 (Hbt). Binding of the primary antibody was detected with a peroxidase-conjugated secondary antibody to rabbit IgG (Jackson ImmunoResearch). After washing positive bands were

Table 1.   Immunohistochemical and Histological Analysis of Renal Complement Deposition and Damage

| Ischemia (minutes) | Reperfusion (hours) | Lectin pathway | | | | Alternative Pathway C3 | | Common pathway C6 / C9 | | Histology PAS | |
| | | MBL-A | | MBL-C | | | | | | | |
		WT	$Mpo^{-/-}$	WT	$Mpo^{-/-}$	WT	$Mpo^{-/-}$	WT	$Mpo^{-/-}$	WT	$Mpo^{-/-}$
−	−	−	−	−	−	−	−	−	−	0	0
40	2	−	−	+	+	+	+	−	−	−	−
40	24	+ +	+ +	+ +	+ +	+	+	+ +	+ +	29.2	27.4*

*Average score from 10 randomly chosen ×200 fields. I/R WT = 29.2 ± 2.5 versus I/R $Mpo^{-/-}$ = 27.4 ± 3.3, P = 0.68.

visualized using chemiluminescence (Supersignal; Pierce, Rockford, IL).

Apoptosis Assay

The presence of internucleosomal DNA cleavage in kidneys was established with a commercial ligase-mediated-PCR assay kit (Apoalert; Clontech, Palo Alto, CA), enabling semiquantitative measurement of the extent of apoptosis.[26]

Renal Morphology

Paraffin-embedded sections from the 24-hour reperfusion group were prepared and stained using periodic acid-Schiff staining. Morphological changes resulting from I/R injury were graded using the scoring system described by Leemans and colleagues.[27] Tubules, cast deposition, brush border loss, and necrosis were identified in at least 10 randomly chosen ×200 fields in the cortico-medullary region of three sections per kidney. Total scores were calculated for each kidney.

Statistical Analysis

Data are expressed as means ± SEM and were analyzed by unpaired two-tailed Student's *t*-test, using Graphpad Prism 4.01 for Windows (Graphpad Software, San Diego, CA). A *P* value ≤0.05 was considered statistically significant.

Results

Renal Histology

To directly assess tissue damage induced by 40 minutes of ischemia followed by 24 hours of reperfusion, paraffin sections were stained using periodic acid-Schiff staining (Figure 1). Moderate to severe damage involving ~25% of the cortex was similarly observed in both $Mpo^{-/-}$ and WT kidneys (Table 1), using the histopathological scoring system developed by Leemans and colleagues[27] to assess the renal damage. No damage was seen in sham-treated animals.

MPO Deficiency Reduces Renal Dysfunction

Renal dysfunction was reflected by an increase in BUN levels after 24 hours of reperfusion (Figure 2). Forty minutes of unilateral ischemia followed by 24 hours of reperfusion caused an elevation of BUN levels in $Mpo^{-/-}$ and WT mice. However, $Mpo^{-/-}$ mice displayed a markedly less pronounced increase in renal failure compared with WT mice (I/R $Mpo^{-/-}$ = 31.3 ± 1.7 mmol/L versus I/R WT = 42.8 ± 2.1 mmol/L, P = 0.003, sham = 7.0 ± 0.5 mmol/L). These findings illustrate a contribution of MPO to the development of organ failure of reperfused ischemic kidneys.

MPO Deficiency Fails to Abrogate Apoptosis and Mannose-Binding Lectin Deposition during Early Reperfusion

Previously, it was demonstrated that apoptosis plays an important role in the development of organ damage induced by the reperfusion of ischemic kidneys.[28] Because some studies show that MPO induces apoptosis by directly mediating caspase activation, the hypothesis that MPO deficiency preserves renal function by inhibition of apoptosis was tested. Apoptosis after 2 hours of reperfusion, analyzed by typical DNA cleavage, is depicted in Figure 3. Clearly shown is the I/R-induced increase in apoptosis, when comparing experimental and
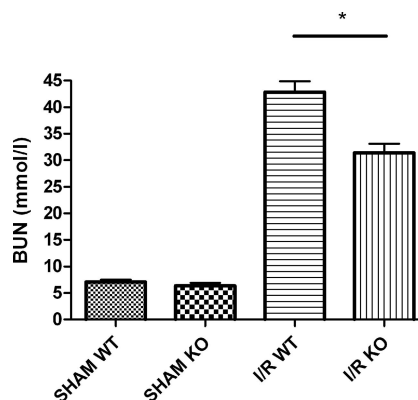
Figure 2. MPO deficiency significantly reduces renal function loss after renal I/R. Renal function after 24 hours of reperfusion as reflected by BUN concentration. Statistical significance of renal function in $Mpo^{-/-}$ mice as compared with WT animals was denoted at P = 0.003 (*).
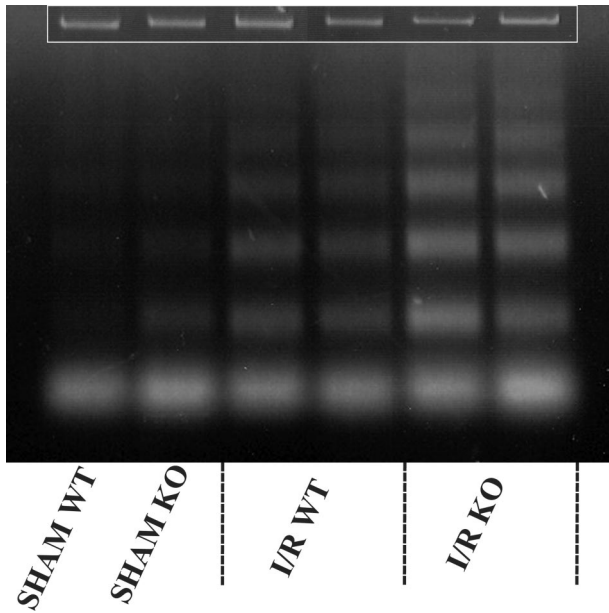
Figure 3. MPO deficiency does not affect I/R-induced early apoptosis. Shown are two sham-treated animals as well as two representatives of each experimental group. Loading controls are given in the **inset**.

sham-treated animals. $Mpo^{-/-}$ and WT animals showed a similar increase in apoptosis. The slightly larger amount of apoptosis that was observed in $Mpo^{-/-}$ as compared with WT mice after 2 hours of reperfusion, was similarly observed in sham-treated $Mpo^{-/-}$ and WT mice. This indicates that the rise in apoptosis in $Mpo^{-/-}$ and WT experimental animals was in fact similar and certainly not reduced in $Mpo^{-/-}$ animals.

It has been shown that bound complement components are critical in the induction and propagation of I/R-induced organ damage after initial cellular damage and apoptosis.[29,30] Predominantly the murine MBL variant C is one of the first inflammatory factors observed to bind during early reperfusion.[31] Therefore, immunohistochemical staining was performed on early 2-hour reperfusion samples, to study the deposition of activated MBL-C. No differences were detected in the amount of MBL-C present in the cortico-medullary region on peritubular capillaries and in the interstitium of reperfused ischemic kidneys from either $Mpo^{-/-}$ or WT mice (Figure 4), suggesting similar levels of complement activation as a result of comparable ischemia-induced cellular damage and apoptosis in $Mpo^{-/-}$ and WT animals.

PMN Influx and MPO Release

Next the influx and activation of inflammatory mediators present during the subsequent progression phase of reperfusion was investigated. Neutrophils invading the damaged tissue contribute to the local inflammatory response in part by releasing their lysosomal constituents, including MPO. MPO released by the activated PMN has been shown to be important in the activation and adhesion of other neutrophils.[32,33] WT mice had high levels of
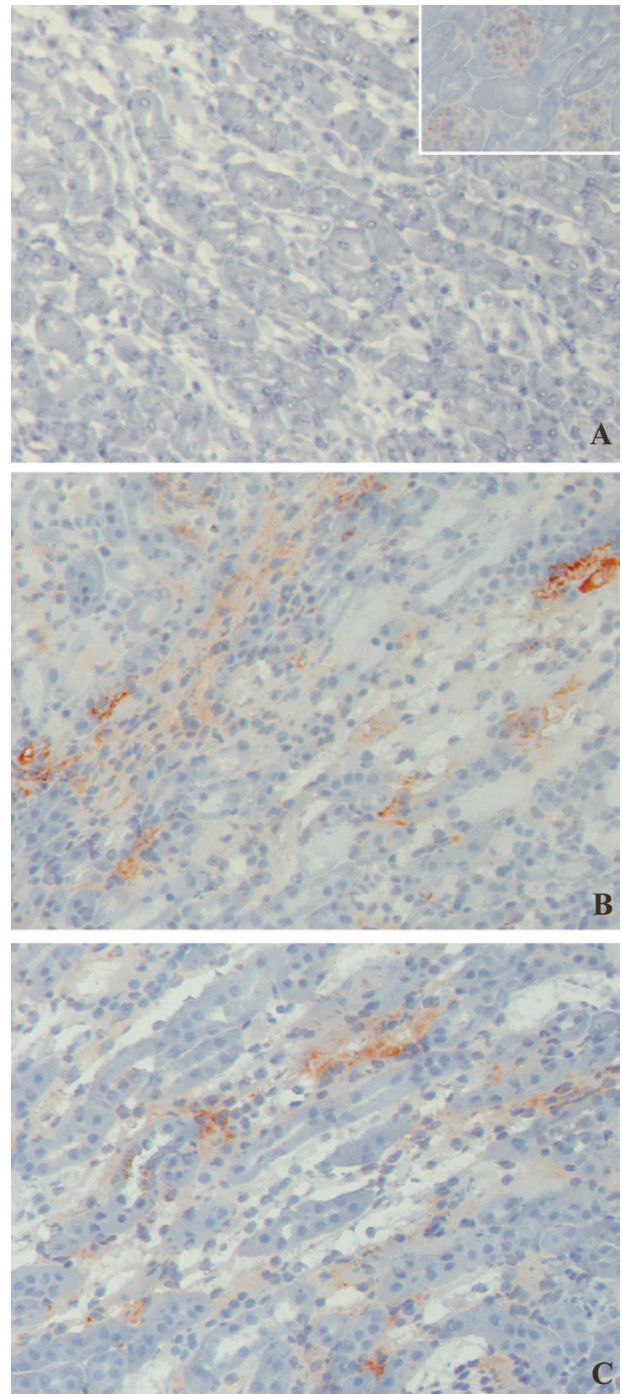

Figure 4. Two hours of reperfusion of ischemically damaged kidneys results in early MBL-C deposition, similar in $Mpo^{-/-}$ and WT mice. MBL-C binding in WT sham-treated (not shown) and KO sham-treated (**A**) animals was only observed in glomeruli. MBL-C deposition was evident in peritubular capillaries, the interstitium, and along the epithelial brush border of damaged tubules in both WT (**B**) and $Mpo^{-/-}$ (**C**) mice subjected to renal I/R. Shown are representative microphotographs of all groups. Original magnifications: ×200; ×600 (**inset**).

circulating MPO (Figure 5), indicating I/R-mediated neutrophil activation. This idea was strengthened by the lack of MPO in sham-treated animals. Immunohistology revealed high levels of MPO in the kidney, mostly comprised to NIMP-R14-positive PMNs and their direct sur-
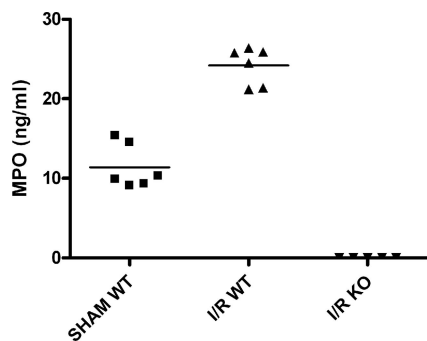
Figure 5. MPO plasma levels, representing total neutrophil activation, are increased 24 hours after reperfusion of ischemically damaged WT kidneys. No MPO was detected in $Mpo^{-/-}$ mice. Statistical significance of WT MPO levels as compared with sham-treated animals was denoted at $P < 0.0001$.

Figure 7. MPO deficiency reduces neutrophil infiltration after renal I/R. Statistical significance as compared with WT animals was denoted at $P = 0.017$.

roundings (Figure 6, A–C). As expected MPO was absent in samples from $Mpo^{-/-}$ mice (Figures 5 and 6F). To quantify the neutrophil infiltration, we counted cells positive for NIMP-R14, a neutrophil marker not influenced by the lack of MPO. Analysis revealed a significant reduction of I/R-induced PMN infiltration of the $Mpo^{-/-}$ kidneys in comparison to the WT (I/R $Mpo^{-/-}$ = 28.2 ± 3.3 PMN/high-power fields versus I/R WT = 40.4 ± 2.3 PMN/high-power fields, $P = 0.017$), indicating an important *in vivo* role for MPO in the extravasation of neutrophils after I/R (Figure 7).

Complement Activation

Studies of the complement system during renal I/R revealed the lectin and alternative as well as the subsequent common complement pathways as key effectors in the induction of I/R-induced organ failure.[4,31] MPO has been described as an activator of complement.[20] *In vitro* experiments revealed complement component C5 activating properties for various proteolytic enzymes that were released on PMN stimulation. Similarly, purified MPO was shown to activate C5 by hypochlorite formation *in vitro*. From this we hypothesized that MPO might regulate complement activation and influence renal function in an *in vivo* renal I/R model. Activation of the complement system was assessed after prolonged 24 hours of reperfusion (Table 1). Immunohistochemistry revealed similar activation of early and late common pathway comple-
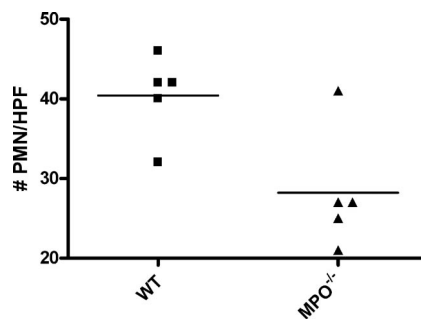
ment proteins in $Mpo^{-/-}$ and WT mice. The deposition of early complement factors MBL-A, MBL-C, and C3 increased in both $Mpo^{-/-}$ and WT mice after 24 hours of reperfusion when compared with the 2-hour reperfusion samples. MBL-C and C3 deposition was abundantly present in peritubular capillaries and interstitium as well as on the epithelial lining of damaged tubules after I/R. Surprisingly, no differences in the quantity of complement proteins MBL-A, MBL-C, and C3 were detected between $Mpo^{-/-}$ and WT groups subjected to I/R (Figure 8). Because C5 convertase-like properties have been described for MPO *in vitro*, the *in vivo* deposition of common pathway complement components was analyzed. Similar to the results observed for early complement proteins, the deposition of common pathway proteins C6 (Figure 9), analyzed by immunohistochemical staining and Western blot, and C9 (Figure 10) were not substantially reduced in MPO-deficient mice after 24 hours of reperfusion, suggesting no considerable role for MPO in the activation of common pathway complement components *in vivo* in response to kidney I/R. C6 and C9 depositions were similarly observed in WT and $Mpo^{-/-}$ mice on the epithelial lining of damaged tubules as well as on tubular cast formations observed after 24 hours of reperfusion.

Discussion

The results provide evidence that a lack of MPO reduces renal function loss after I/R. By showing that MPO-deficient mice are partially protected from I/R-induced renal failure,
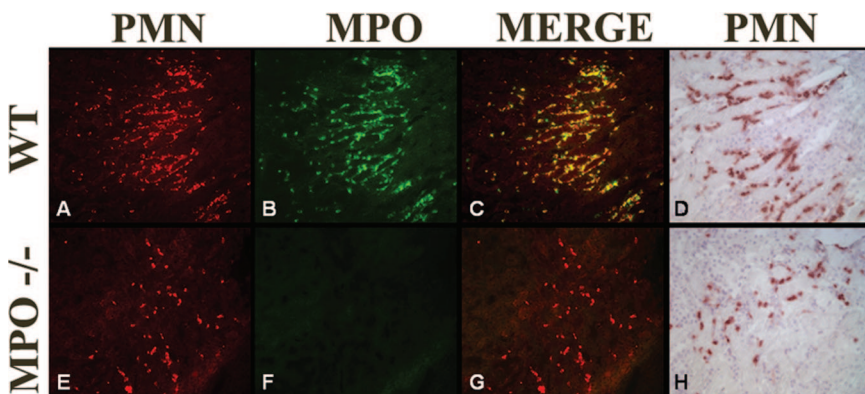

Figure 6. Immunofluorescent staining showing PMN sequestration (red, Alexa 568) and MPO presence (green, Alexa 488) in kidneys of WT (**A–C**) and $Mpo^{-/-}$ (**E–G**) mice subjected to I/R. Overlapping PMN and MPO data depict the fact that MPO is predominantly located around NIMP-R14-positive PMNs. **D** (WT) and **H** ($Mpo^{-/-}$) show PMN infiltration in relation to structural changes. Shown are representative microphotographs of all groups. Original magnifications, ×200.
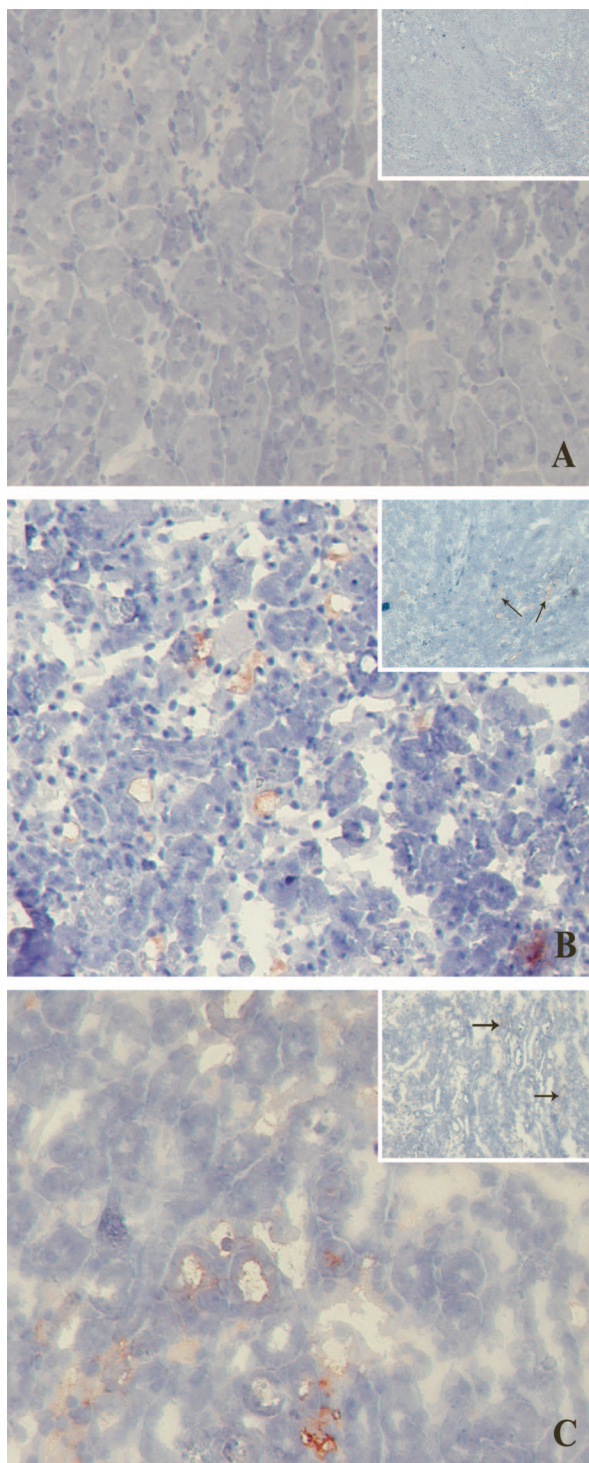
Figure 8. MBL-C and C3 (**insets**) deposition in WT and $Mpo^{-/-}$ mice after 24 hours of reperfusion. MBL-C and C3 deposition was not different in WT (**B**) or $Mpo^{-/-}$ (**C**) mice. **A:** Virtually no MBL-C or C3 staining was detected in sham-treated WT (not shown) and $Mpo^{-/-}$ mice. Corresponding data were obtained for MBL-A deposition (data not shown). Shown are representative microphotographs of all groups. Original magnifications: ×200; ×100 (**insets**).

Figure 9. Common pathway activation as determined by immunohisto-chemical and Western blot analyses of C6 deposition in WT and $Mpo^{-/-}$ mice after 24 hours of reperfusion. C6 deposition in $Mpo^{-/-}$ mice (**C**) was similar as compared with WT (**B**) controls. **A:** No C6 staining was detected between WT sham (not shown) and $Mpo^{-/-}$ sham-treated mice. Shown are representative microphotographs of all groups. Western blot results for C6 (sham in **A**; WT in **B**; KO in **C**) under reducing conditions are shown in the **insets**. Original magnifications, ×100.

the results demonstrate that this enzyme plays an important role in the pathogenesis of I/R-induced renal damage. MPO deficiency reduced PMN infiltration but failed to abrogate complement activation in reperfused murine kidneys.
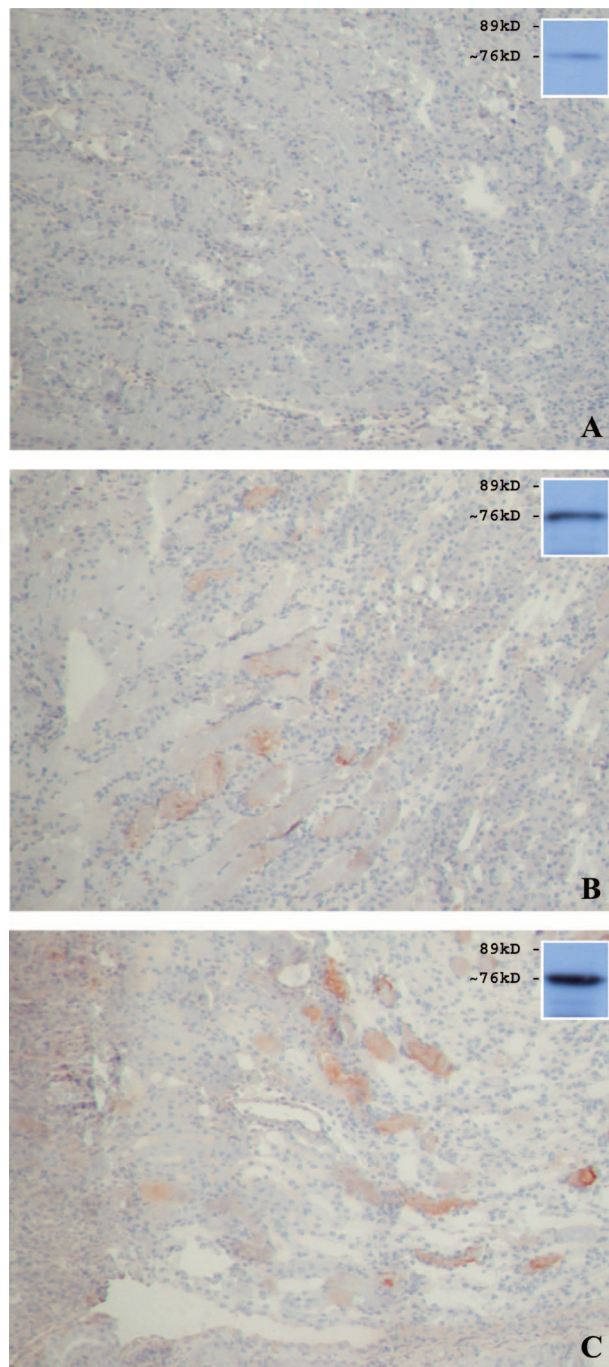
MPO is a neutrophil-derived enzyme with the capacity to catalyze the formation of the proinflammatory oxidant HOCl and chlorinating species out of H_2O_2 and chloride ions.[11] Apart from MPO's contribution to innate immunity, there is *in vitro* evidence that MPO plays a role in apoptosis. Neutrophil-derived proteinase 3 and MPO mediate
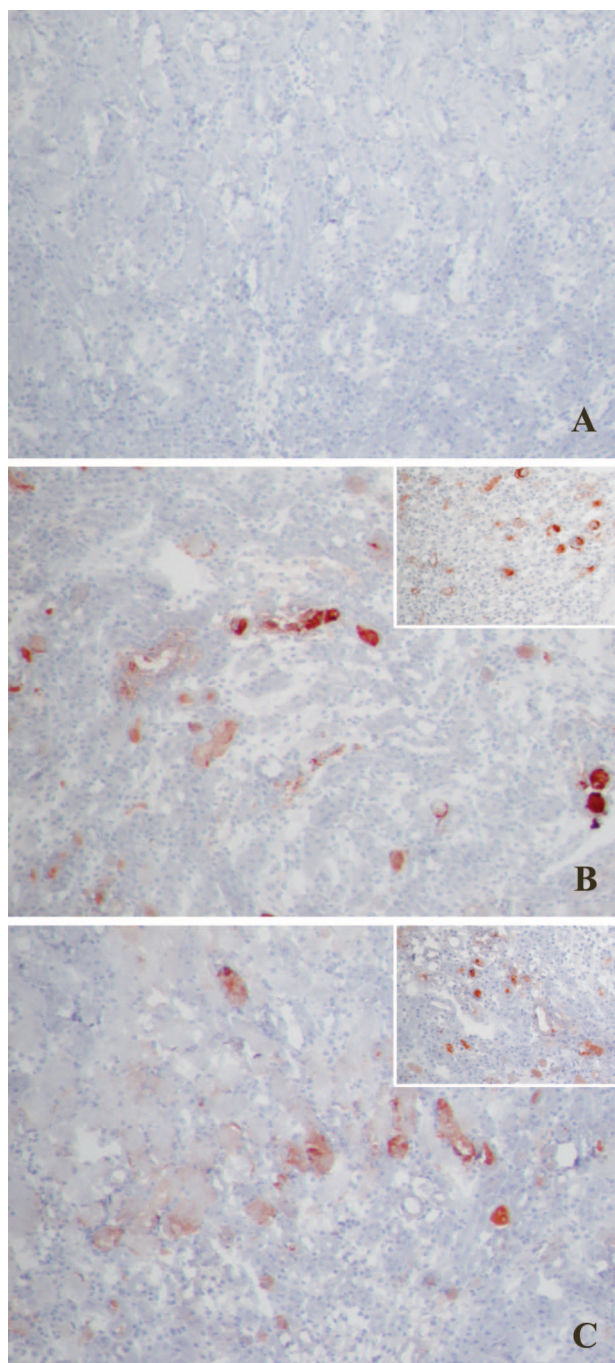
Figure 10. Similar common pathway activation between $Mpo^{-/-}$ (**C**) and WT (**B**) mice after 24 hours of reperfusion was confirmed by C9 immunostaining. WT sham (not shown) and $Mpo^{-/-}$ sham-treated mice (**A**) did not demonstrate any C9 staining. Original magnifications: ×100; ×200 (**insets**).

proapoptotic caspase-3 activation or induce direct HL-60 leukemia cell and endothelial cell apoptosis *in vitro*.[34–36] Throughout the years, a massive body of literature has emerged describing the activation of apoptotic pathways in renal I/R.[37,38] Furthermore, inhibition of apoptosis through administration of the anti-apoptotic agents such as IGF-1 or ZVAD-fmk (a broad caspase inhibitor) has been shown to preserve renal function after I/R.[28] The hypothesis is tested that, in our model, a decrease in

MPO-mediated apoptosis is one of the mechanisms through which $Mpo^{-/-}$ mice are protected from injury caused by renal I/R. Analyzing the overall levels of apoptosis, by detecting specific DNA fragmentation, it is shown that renal ischemia followed by 2 hours of reperfusion induces a marked increase in the level of apoptosis in both KO and WT animals. Moreover, the observed increase in apoptosis in $Mpo^{-/-}$ mice is similar to the rise of apoptosis in WT animals during this early phase of reperfusion. Similarly, studies by Vasilyev and colleagues[19] could not show a significant contribution for MPO or its derived oxidants in the induction of apoptosis and necrosis *in vivo*. MPO rather adversely influenced organ function by the production of cytotoxic aldehydes[19] or the oxidative inactivation of plasminogen activator inhibitor 1 (PAI-1).[39] The present data indeed suggest that MPO has no significant *in vivo* role in the induction of renal cell death throughout the first moments of I/R but rather has a profound effect on organ function during late reperfusion, known as progression phase.

PMNs influence I/R-induced tissue damage in a multitude of organs by capillary plugging,[9] induction of tubular leakage,[40] release of oxygen-free radicals,[41] and lysosomal enzyme activity.[42–45] MPO specifically mediates neutrophil activation by binding to CD11b/CD18 (MAC1) integrins,[32] as well as PMN adhesion via the $\alpha_m\beta_2$ integrin,[33] thereby facilitating PMN extravasation. Inhibiting PMN extravasation abrogates renal I/R injury.[14,46] The reduced levels of BUN, observed in this model in $Mpo^{-/-}$ mice, are accompanied by a decrease in PMN influx during late (24 hours) reperfusion. Our data are in line with the effect of MPO on PMNs and indicate that the absence of MPO prevents PMN activation and adhesion, thereby effectively reducing the amount of neutrophils invading the damaged tissue and preserving organ function.

A major role in the induction and continuation of local inflammation is played by the complement system. Complement proteins contribute to the development of I/R-induced organ injury.[6,31,47] Predominantly the activation of the lectin[30,31] and alternative pathway[48] as well as the formation of the membrane attack complex (MAC)[49,50] and small cationic proteins (C3a, C4a, and C5a), known as anaphylatoxins, have been shown to be involved in I/R-induced tissue injury.[6,26] The deposition of early complement-activating proteins after 2 or 24 hours of reperfusion was similar between $Mpo^{-/-}$ and WT treated mice. Neutrophils produce several proteins, such as properdin and MPO, which have been shown to activate the complement cascade. Myeloperoxidase is reported to directly activate C5, generating a functional common pathway convertase capable of activating C6.[20] We hypothesized that with the absence of MPO adequate means to locally activate common complement components in reperfused kidneys would be reduced. To elucidate common complement pathway activation, we analyzed C6 and C9 deposition in reperfused kidneys of $Mpo^{-/-}$ and WT controls after 24 hours of reperfusion. Similar to the activation of MBL and C3, no reduction in common complement pathway activity was shown in $Mpo^{-/-}$ mice. Our data suggest that complement activa-

tion initiated by the local presence of MPO is not of particular importance during renal I/R and consequently has no significant contribution to I/R-induced renal function loss. It has been described that several important proteins of the common complement cascade, such as C6 and C7,[51] are directly produced by PMNs and released on their activation. This would mean that PMNs provide the necessary elements, ie, C6 and C7, to boost common pathway activation at sites of ongoing inflammation. This could imply a limited presence of these important common pathway components in case of a reduced PMN influx, as was observed in the reperfused kidneys of $Mpo^{-/-}$ mice. However, our data do not support that this quality attributed to the PMN has a detrimental role in our renal I/R model.

In conclusion, a reduced function loss in $Mpo^{-/-}$ mice as compared with WT controls was observed in a well established model of renal I/R. Apoptosis and activation of the early complement lectin and alternative pathway proteins, MBL-A, MBL-C, and C3, occurred similarly in $Mpo^{-/-}$ and WT mice during the first hours of reperfusion. After 24 hours of reperfusion, $Mpo^{-/-}$ mice exhibited preservation of renal function along with a strongly reduced number of neutrophils, present in the damaged renal tissue. The absence of MPO and the decrease in the number of neutrophils, however, did not correlate with a diminished activation of the common complement pathway in $Mpo^{-/-}$ mice. This observation might well illustrate that the contribution of MPO to renal organ damage after I/R is determined more by its influence on neutrophil extravasation and tissue infiltration than by its ability to mediate local complement activation *in vivo*. The results clarify important mechanisms by which PMNs and their derived activation products mediate I/R-induced renal injury on a local level.

Acknowledgments

References

1. Daemen MA, van de Ven MW, Heineman E, Buurman WA: Involvement of endogenous interleukin-10 and tumor necrosis factor-alpha in renal ischemia-reperfusion injury. Transplantation 1999, 67:792–800

2. Daemen MA, van't Veer C, Wolfs TG, Buurman WA: Ischemia/reperfusion-induced IFN-gamma up-regulation: involvement of IL-12 and IL-18. J Immunol 1999, 162:5506–5510

3. Thakur ML, Gottschalk A, Zaret BL: Imaging experimental myocardial infarction with indium-111-labeled autologous leukocytes: effects of infarct age and residual regional myocardial blood flow. Circulation 1979, 60:297–305

4. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y, Stahl GL, Sacks SH: Predominant role for C5b-9 in renal ischemia/reperfusion injury. J Clin Invest 2000, 105:1363–1371

5. Vakeva AP, Agah A, Rollins SA, Matis LA, Li L, Stahl GL: Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. Circulation 1998, 97:2259–2267

6. De Vries B, Matthijsen RA, Wolfs TG, Van Bijnen AA, Heeringa P, Buurman WA: Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. Transplantation 2003, 75:375–382

7. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN: Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am J Physiol 1987, 253:H699–H703

8. Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR: Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 1983, 67:1016–1023

9. Schmid-Schönbein GW: Capillary plugging by granulocytes and the no-reflow phenomenon in the microcirculation. Fed Proc 1987, 46:2397–2401

10. Kilgore KS, Lucchesi BR: Reperfusion injury after myocardial infarction: the role of free radicals and the inflammatory response. Clin Biochem 1993, 26:359–370

11. Klebanoff SJ: Myeloperoxidase: friend and foe. J Leukoc Biol 2005, 77:598–625

12. Nagra RM, Becher B, Tourtellotte WW, Antel JP, Gold D, Paladino T, Smith RA, Nelson JR, Reynolds WF: Immunohistochemical and genetic evidence of myeloperoxidase involvement in multiple sclerosis. J Neuroimmunol 1997, 78:97–107

13. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Munzel T, Simoons ML, Hamm CW: Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation 2003, 108:1440–1445

14. Miura M, Fu X, Zhang QW, Remick DG, Fairchild RL: Neutralization of Gro alpha and macrophage inflammatory protein-2 attenuates renal ischemia/reperfusion injury. Am J Pathol 2001, 159:2137–2145

15. Malle E, Buch T, Grone HJ: Myeloperoxidase in kidney disease. Kidney Int 2003, 64:1956–1967

16. Hazell LJ, Arnold L, Flowers D, Waeg G, Malle E, Stocker R: Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J Clin Invest 1996, 97:1535–1544

17. Podrez EA, Schmitt D, Hoff HF, Hazen SL: Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. J Clin Invest 1999, 103:1547–1560

18. Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, Heinecke JW: The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. Proc Natl Acad Sci USA 2004, 101:13032–13037

19. Vasilyev N, Williams T, Brennan ML, Unzek S, Zhou X, Heinecke JW, Spitz DR, Topol EJ, Hazen SL, Penn MS: Myeloperoxidase-generated oxidants modulate left ventricular remodeling but not infarct size after myocardial infarction. Circulation 2005, 112:2812–2820

20. Vogt W: Complement activation by myeloperoxidase products released from stimulated human polymorphonuclear leukocytes. Immunobiology 1996, 195:334–346

21. Eiserich JP, Baldus S, Brennan ML, Ma W, Zhang C, Tousson A, Castro L, Lusis AJ, Nauseef WM, White CR, Freeman BA: Myeloperoxidase, a leukocyte-derived vascular NO oxidase. Science 2002, 296:2391–2394

22. Aratani Y, Koyama H, Nyui S, Suzuki K, Kura F, Maeda N: Severe impairment in early host defense against Candida albicans in mice deficient in myeloperoxidase. Infect Immun 1999, 67:1828–1836

23. Huugen D, Xiao H, van Esch A, Falk RJ, Peutz-Kootstra CJ, Buurman WA, Tervaert JW, Jennette JC, Heeringa P: Aggravation of antimyeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide: role of tumor necrosis factor-alpha. Am J Pathol 2005, 167:47–58

24. Lopez AF, Strath M, Sanderson CJ: Differentiation antigens on mouse eosinophils and neutrophils identified by monoclonal antibodies. Br J Haematol 1984, 57:489–494

25. Wolfs TG, de Vries B, Walter SJ, Peutz-Kootstra CJ, van Heurn LW, Oosterhof GO, Buurman WA: Apoptotic cell death is initiated during normothermic ischemia in human kidneys. Am J Transplant 2005, 5:68–75

26. de Vries B, Kohl J, Leclercq WK, Wolfs TG, van Bijnen AA, Heeringa P, Buurman WA: Complement factor C5a mediates renal ischemia-reperfusion injury independent from neutrophils. J Immunol 2003, 170:3883–3889

27. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S:

Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 2005, 115:2894–2903

28. Daemen MA, van 't Veer C, Denecker G, Heemskerk VH, Wolfs TG, Clauss M, Vandenabeele P, Buurman WA: Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. J Clin Invest 1999, 104:541–549

29. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP, Solomon SD, Ezekowitz RA, Stahl GL: Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. J Immunol 2005, 175:541–546

30. Møller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K, Shi L, Ezekowitz A, Jensenius JC, Gadjeva M: Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. Scand J Immunol 2005, 61:426–434

31. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA: The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. Am J Pathol 2004, 165:1677–1688

32. Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, Brummer J, Rudolph V, Munzel T, Heitzer T, Meinertz T, Baldus S: Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. Proc Natl Acad Sci USA 2005, 102:431–436

33. Johansson MW, Patarroyo M, Oberg F, Siegbahn A, Nilsson K: Myeloperoxidase mediates cell adhesion via the alpha M beta 2 integrin (Mac-1, CD11b/CD18). J Cell Sci 1997, 110:1133–1139

34. Wagner BA, Buettner GR, Oberley LW, Darby CJ, Burns CP: Myeloperoxidase is involved in H2O2-induced apoptosis of HL-60 human leukemia cells. J Biol Chem 2000, 275:22461–22469

35. Tsurubuchi T, Aratani Y, Maeda N, Koyama H: Retardation of early-onset PMA-induced apoptosis in mouse neutrophils deficient in myeloperoxidase. J Leukoc Biol 2001, 70:52–58

36. Myzak MC, Carr AC: Myeloperoxidase-dependent caspase-3 activation and apoptosis in HL-60 cells: protection by the antioxidants ascorbate and (dihydro)lipoic acid. Redox Rep 2002, 7:47–53

37. Kaushal GP, Basnakian AG, Shah SV: Apoptotic pathways in ischemic acute renal failure. Kidney Int 2004, 66:500–506

38. Dagher PC: Apoptosis in ischemic renal injury: roles of GTP depletion and p53. Kidney Int 2004, 66:506–509

39. Askari AT, Brennan ML, Zhou X, Drinko J, Morehead A, Thomas JD, Topol EJ, Hazen SL, Penn MS: Myeloperoxidase and plasminogen activator inhibitor 1 play a central role in ventricular remodeling after myocardial infarction. J Exp Med 2003, 197:615–624

40. Hellberg PO, Kallskog TO: Neutrophil-mediated post-ischemic tubular leakage in the rat kidney. Kidney Int 1989, 36:555–561

41. Linas SL, Shanley PF, Whittenburg D, Berger E, Repine JE: Neutrophils accentuate ischemia-reperfusion injury in isolated perfused rat kidneys. Am J Physiol 1988, 255:F728–F735

42. Rabb H, O'Meara YM, Maderna P, Coleman P, Brady HR: Leukocytes, cell adhesion molecules and ischemic acute renal failure. Kidney Int 1997, 51:1463–1468

43. Jordan JE, Zhao ZQ, Vinten-Johansen J: The role of neutrophils in myocardial ischemia-reperfusion injury. Cardiovasc Res 1999, 43:860–878

44. Jaeschke H, Farhood A, Smith CW: Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J 1990, 4:3355–3359

45. Klausner JM, Paterson IS, Goldman G, Kobzik L, Rodzen C, Lawrence R, Valeri CR, Shepro D, Hechtman HB: Postischemic renal injury is mediated by neutrophils and leukotrienes. Am J Physiol 1989, 256:F794–F802

46. Kelly KJ, Williams Jr WW, Colvin RB, Meehan SM, Springer TA, Gutierrez-Ramos JC, Bonventre JV: Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. J Clin Invest 1996, 97:1056–1063

47. Weisman HF, Bartow T, Leppo MK, Marsh Jr HC, Carson GR, Concino MF, Boyle MP, Roux KH, Weisfeldt ML, Fearon DT: Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. Science 1990, 249:146–151

48. Lin T, Zhou W, Farrar CA, Hargreaves RE, Sheerin NS, Sacks SH: Deficiency of C4 from donor or recipient mouse fails to prevent renal allograft rejection. Am J Pathol 2006, 168:1241–1248

49. Kilgore KS, Ward PA, Warren JS: Neutrophil adhesion to human endothelial cells is induced by the membrane attack complex: the roles of P-selectin and platelet activating factor. Inflammation 1998, 22:583–598

50. Kilgore KS, Flory CM, Miller BF, Evans VM, Warren JS: The membrane attack complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. Am J Pathol 1996, 149:953–961

51. Høgåsen AK, Wurzner R, Abrahamsen TG, Dierich MP: Human polymorphonuclear leukocytes store large amounts of terminal complement components C7 and C6, which may be released on stimulation. J Immunol 1995, 154:4734–4740