Does temperature matter?

The role of hypothermia in sterile inflammation resulting from renal ischemia/reperfusion

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Abstract

Ischemia/reperfusion (IR) injury represents an emerging risk in the successful outcome of renal transplantation. Although IR is deleterious for the kidney, subsequent sterile inflammatory responses significantly exacerbate tissue injury. Activation of the innate immune system can result in serious complications, such as delayed graft function (DGF). DGF impacts short- and long-term outcome of kidney grafts, and may even lead to rejection. Hypothermia is widely used as a protective method during the transport and storage of organs and tissues. Although hypothermia has strong beneficial effects, it can also trigger cellular injury. Furthermore, hypothermia can activate a sterile inflammatory response by mechanisms different to those in normothermic IR. As sterile inflammation is of great importance in the development of graft injury, this paper will focus on the mechanisms leading to sterile inflammation in both normothermic and hypothermic ischemia/reperfusion.

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Abbreviations used in the paper

Abbreviation	Description
IR	Ischemia/reperfusion
DAMP	Damage-associated molecular pattern
PRR	Pathogen recognition receptor
TLR	Toll-like receptor
NLRP3	NOD-like receptor subtype NLRP3
ROS	Reactive oxygen species
HS	Hypothermic-storage

I. Introduction

To date, the treatment of choice for patients with end stage renal failure is renal transplantation. While numerous variables influence the outcome of the transplantation procedure, ischemia/reperfusion (IR) injury represents an emerging risk (1). IR is a state in which blood supply to an organ is interrupted and then later reinstated (2). Ischemic conditions can be separated into normothermic ischemia, which occurs with kidney vessel clamping, and hypothermic ischemia, which results from cooling during graft preservation. The latter is a problem in organ transplantation from cadaveric donors, as the *ex-vivo* ischemic period has to last several hours (up to 24 hours for the kidney (3)) in order to have time to locate an appropriate recipient and transport the organ (4). The current strategy in cadaveric donor transplantation remains rapid cooling with the use of preservation fluid. Hypothermia considerably slows the injurious reactions to ischemia since metabolism is lowered with a factor 10-11 at 4°C. However, hypothermia itself is also detrimental to tissue, causing changes similar to those observed in normothermic ischemia even during normoxic cooling. In IR, the ischemic insult causes the initial injury. During prolonged ischemia, the switch to anaerobic metabolism and the accumulation of lactate causes a decrease in ATP and intracellular pH (1). Consequently, ATPase-dependent ion transporters become dysfunctional, causing increased intracellular and mitochondrial calcium levels. Calcium overload leads to cell swelling and subsequent cell rupture. The ischemic phase is followed by reperfusion, characterized by reestablishment of blood flow, which further augments tissue damage. Although the precise mechanisms underlying the induction of reperfusion injury are complex and not yet fully understood (5), reintroduction of oxygen, reintroduction of oxygen with subsequent production of reactive oxygen species (ROS) plays a major role in the induction of organ injury during reperfusion.

Sterile inflammation in ischemia/reperfusion

Although IR is deleterious for the kidney, it is thought that the subsequent sterile inflammatory response significantly exacerbates tissue injury. Cell death, resulting from IR injury, can participate in

the development of the inflammatory response (6). Recently, necroptosis, a programmed cell necrosis characterized by a loss of plasma membrane and extracellular release of damage-associated molecular pattern molecules (DAMPs), has been recognized as an important factor in IR injury (7). DAMPs can trigger innate immune responses in the kidney and include the nuclear protein HMGB1, heat shock proteins, several mitochondrial components, and extracellular DNA and ATP (8). These sterile stimuli can activate the immune system by several mechanisms (6). In general, DAMPs can function as ligands for pathogen recognition receptors (PRRs), such as the Toll-like receptors (TLRs), and the nucleotide-binding oligomerization domain (NOD)-like receptor. TLRs in the kidney are mainly expressed on tubular epithelial cells, glomerular endothelial cells, and podocytes (9). In these cells, specifically TLR2 and TLR4 are upregulated following renal IR. In addition to TLR, the NOD-like receptor NLRP3 has been found to induce a sterile inflammatory response after the release of mitochondria, ATP and other cell components from necrotic cells. Furthermore, NLRP3 augments inflammation after IR (10). Signaling of NLRP3 involves a two-step pathway, with the first being PPR- or cytokine-dependent upregulation of NLRP3 and the second being the actual activation of NLRP3 (6). Activation of both NLRP3 and TLR results in the recruitment of transcription factors, such as NF-κB mitogen-activated protein-3 and interferon (IFN) regulator 3. Subsequently, the upregulation of interleukin-1β (IL-1β) and chemokines leads to recruiting and activation of inflammatory cells. Thus, both TLR and NLRP3 are fundamental in the sterile inflammatory response following renal IR.

The role of hypothermic preservation

Activation of the innate immune system can result in delayed graft function (DGF). DGF impacts short- and long-term outcome of kidney grafts, and may even lead to rejection (11). Moreover, graft rejection puts further pressure on the organ availability, as these patients require a retransplantation or go back to dialysis. More recently, it has been observed that inflammation is also associated with progressive fibrosis and chronic humoral rejection, which are both associated with poor allograft survival (12). Hypothermia is widely used as a protective method during the transport and storage of organs and tissues. Although hypothermia has strong beneficial effects, it can also trigger cellular

injury (13). Since hypothermic preservation is marked by prolonged ischemia, kidney preservation involves a combination of ischemia/reperfusion injury and hypothermic injury (14). Hypothermic storage has recently been associated with sterile inflammation (15), which raises the question to what extend hypothermia is protective in kidney preservation. Therefore, in this review of current literature on normothermic and hypothermic ischemia/reperfusion, I aim to shed light on the role of temperature in kidney preservation. As sterile inflammation is of great importance in the development of graft injury, this paper will focus on the mechanisms leading to sterile inflammation in both normothermic and hypothermic ischemia/reperfusion.

II. Normothermic ischemia-reperfusion

The processes of normothermic ischemia are well described within the context of acute kidney injury (AKI). Experimental animal models for AKI consist of renal arterial clamping while body temperature remains unchanged. Subsequently, IR will lead to necrotic and necroptotic cell death (7). As a consequence, DAMPs are released to the extracellular environment where they provoke sterile inflammatory responses that exacerbate tissue injury (6). In this section mechanisms leading to sterile inflammation in normothermic IR will be reviewed.

Extracellular DAMPs

Hypoxia during early ischemia injures cells, causing the release of endogenous inducers of inflammation, including pro-inflammatory cytokines, complement products, and DAMPs. The latter is currently seen as an important factor in the development of sterile inflammation, particularly the ability of DAMPs to induce influx of neutrophils (16). DAMPs released into the extracellular environment, as a consequence of cell death, can be recognized by PPRs like TLR (Figure 1.) (6). Proximal tubular cells constitutively express TLR subtype TLR4. After 4 h of normothermic ischemia, TLR4 is markedly increased on renal endothelium. Furthermore, 24 h after reperfusion the receptor is significantly upregulated in the cortex and outer medulla (17). Increased TLR4 is still detectable in the

endothelium of the medullary thick ascending limb and distal tubules 3–5 days following reperfusion (17).

Ligands for TLR4 include several DAMPs, including HMGB-1, extracellular matrix (ECM) components, and heat-shock proteins (HSPs) (18). Under normal circumstances, HMGB-1 is located in the nucleus, where it functions in nucleosome stabilization and promotion of DNA transcription. When cell death occurs, the release of HMGB-1 into the extracellular environment triggers inflammatory responses mediated through TLR4 (19). ECM includes biglycan, heparan and hyaluronian (18). Other matrix components like intracellular enzymes released as a consequence of cell rupture, have been shown to cleave the proteoglycan fibronectin (FN), resulting in FN containing the alternatively-spliced extra domain A (EDA+-FN). EDA is capable of interacting with TLR4 resulting in increased expression of IL-6, IL-1β and TNF-α while other recombinant fragments or intact fibronectin fail to elevate cytokine levels (20). The role of HSPs in renal IR is still being debated. HSP-70 stimulates TLR4, which induces the production of TNF-α (21). However, it is unclear to what extend HSP-70 activates TLR4, as it has been recognized that endotoxin also contributed significantly to the inflammatory response. Therefore, the role of HSP-70 in sterile inflammation requires further investigation. Mkaddem et al. (22) observed interaction of HSP-90 with TLR4 on renal tubular epithelial cells and this interaction associated with hypoxia-induced apoptosis. Contrary to the detrimental role of HSPs, HSP-70 can limit the pro-inflammatory action of NF-κB signaling in kidney IR in two ways. Firstly, HSP-70 stabilizes IκB, thus inhibiting the nuclear translocation of NF-κB p65. Secondly, HSP-70 marks pro-inflammatory HSP-90 client proteins for degradation (23). The interaction between HSP-70 and HSP-90 needs further investigation, especially since HSP-70 shows anti-inflammatory potential by inhibition of NF-κB, but is also linked to TLR4 upregulation. Taken together, HMGB-1, ECM and HSPs are able to interact with TLR4 upon their release into the extracellular environment. Interaction with TLR4 leads to subsequent activation of downstream signaling pathways, which initiate the transcription of pro-inflammatory genes.

Downstream signaling of activated Toll-like receptors

Binding of DAMPs to TLR4 leads to activation of signaling cascades, which is distinguished in responses dependent and independent of the Myeloid differentiation primary-response protein 88 (MyD88) / TLR-4 signaling pathways (18). The MyD88 dependent pathway is initiated by activation of TLR4. Then, activated myD88 recruits IL-1 receptor-associated kinase (IRAK)-4 and subsequent phosphorylation of IRAK-1 by IRAK-4 recruits TNF-receptor-associated factor 6 (TRAF6). TRAF6, ubiquitin-conjugating enzymes (UEV1A, UBC13) and binding proteins (TAB1, TAB2) interact to activate TGF-β-activated kinase 1 (TAK1). TAK1 activation leads to the activation of the inhibitor of nuclear factor-ϰB kinase (IKK) complex, which releases NF-ϰB from its inhibitor and promotes its translocation into the nucleus. Translocation of NF-ϰB initiates the expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines responsible for neutrophil (IL-8 and MIP-2) and macrophage (MCP-1) accumulation (24). On the other hand, the MyD88-independent pathway downstream of TLR4 involves activation of TRIF and recruitment of IKKε, which possibly forms a complex with TBK1 to activate IFN-regulatory factor 3 (IRF-3) (18). Translocation of IRF-3 initiates expression of IFN- β (24). Wu et al. observed that MyD88-deficient cells significantly blocked the renal proinflammatory cytokine-chemokine response in normothermic IR, confirming the importance of the MyD88 pathway in sterile inflammation (24). However, using a similar IR model, Pulskens et al. (25) observed no change in the degree of inflammation and renal injury in MyD88 and TRIF knockouts. The fact that sterile inflammation continued in these knockouts raises the question whether there are unknown MyD88- and TRIF-independent pathways responsible for the initiation of renal injury. Elucidation of these pathways might provide new targets for therapeutic intervention.

Intracellular DAMPs

The NLRP3 inflammasome, a member of the NLR family that is expressed in myeloid cells, can sense sterile activators including ATP, cholesterol crystals and hyaluronan (26). ATP binds to P2X7 and triggers the formation of apannexin-1 hemichannel, which activates NLRP3. Other sterile particles are sensed after phagocytosis, upon which NLRP3 recruits the adaptor protein Asc (apoptosis-associated

speck-like protein which contains a caspase recruitment domain [CARD] protein) through a pyrin domain (PYD)-PYD interaction. Asc then recruits inflammatory caspases (e.g., caspase 1) to the assembly complex through CARD-CARD interactions. The inflammatory caspases are brought into proximity by the assembly complex, which leads to their activation. Cleavage by caspase 1 then activates IL-1β and IL-18 (27).

Iyer et al. (10) showed a connection between NLRP3 activation and IR injury. They observed that macrophages that had undergone hypoxic injury, were capable of activating caspase-1 in an NLRP3-dependent manner after cells had undergone necrotic cell death (10). In line with this study, knockout of NLRP3 protects cells from renal IR injury, thereby greatly improving cell survival (28). NLRP3 can thus be recognized as an important factor in the development of IR. However, the absence of Asc or caspase 1 does not protect cells from renal injury (28, 29). Even so, blockade of IL-1 and IL-18 has no significant effect on renal IRI (28). Therefore, NLRP3 mediated injury might involve an inflammasome-independent mechanism. NLRP3 has recently been found to play a distinct role in non-professional immune cells, like vascular endothelial cells, independent of the inflammasome (30). Here, mitochondrial-localized NLRP3 potentiates reactive oxygen species (ROS) to augment R-Smad activation. Wang et al. (31) showed that NLRP3 plays a role in promoting TGF-β signaling and R-Smad activation in renal tubular epithelial cells. Unfortunately, no model for IR was used in this study. Instead, they used a model where they induced inflammation by exposure of epithelium to cytokines. However, the absence of NLRP3 impaired TGF-β signaling in renal tubular epithelium. Impaired TGF-β signaling resulted in reduced expression of TGF-β–stimulated genes that are necessary for epithelial–mesenchymal transition (EMT). As EMT contributes to the development or renal tissue fibrosis, NLRP3 is thus an important factor in the development of renal fibrosis after IR (32)

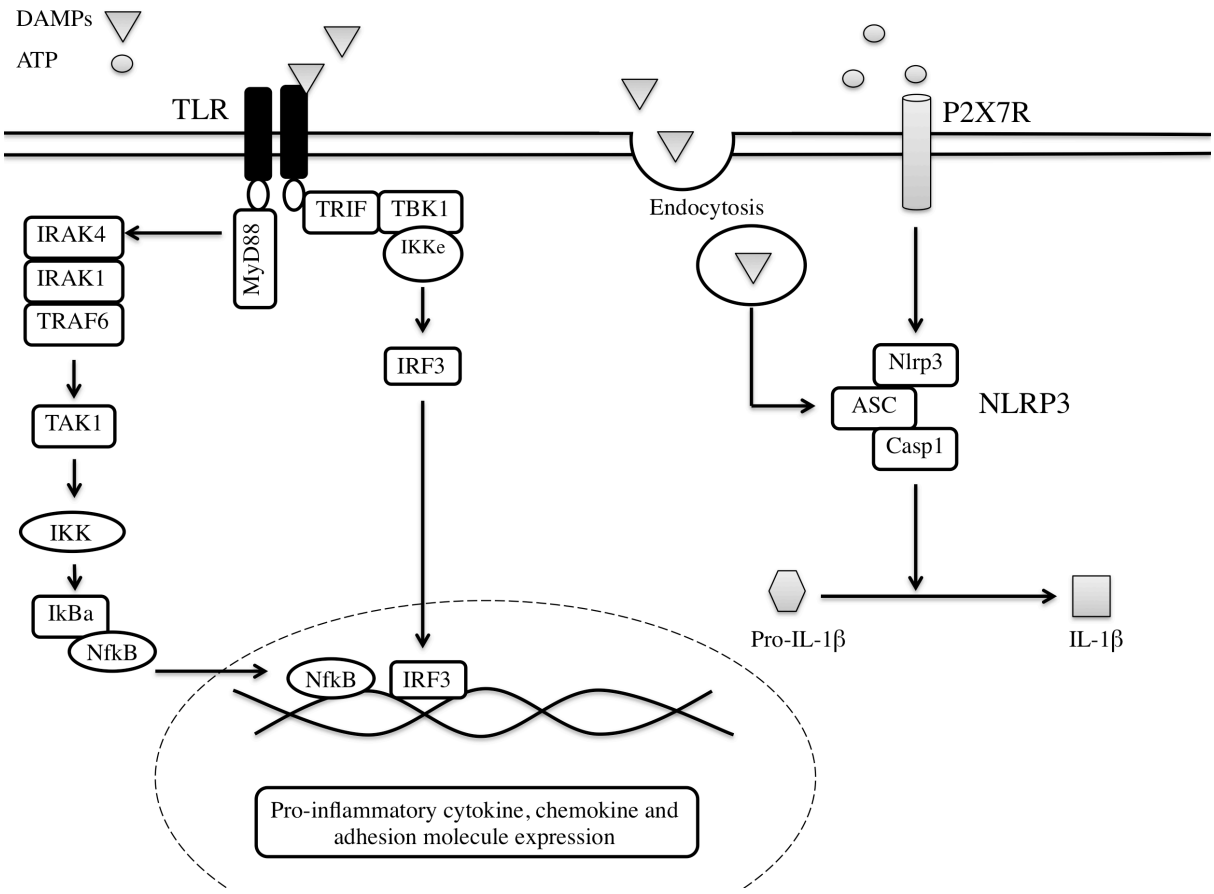

Figure 1. Pathogen Recognition Receptors in sterile inflammation. Ischemia/reperfusion injures cells leading tot predominant necrotic cell death. Release of DAMPs and ATP from necrotic cells activate Toll-like receptors (TLR) and NOD-like receptor NLRP3. Downstream signaling of TLR can be MyD88 dependent or MyD88 independent. MyD88 dependent signaling involves activation of the IRAK complex and translocation of NfkB to the nucleus. MyD88 independent signaling is mediated through TRIF and will lead to nuclear translocation of IRF3. Both NfkB and IRF3 initiate transcription of pro-inflammatory cytokines, chemokines and adhesion molecules. Activation of NLRP3 activates caspase-1, which cleaves pro-IL-1β into active IL-1β.

Endothelial activation and leukocyte infiltration

The endothelium is important in the sterile inflammatory response in kidney IR, since it promotes the accumulation of leukocytes (33). IRI namely leads to increased expression of the adhesion molecule ICAM-1, thereby promoting leukocyte infiltration. Furthermore, release of HMGB-1 after 4h of ischemia increased the expression of proinflammatory adhesion molecules (17). The absence of endothelial TLR4 inhibits the upregulation of adhesion molecules and thereby ameliorates

inflammation and injury. Contrary, exposure to H2O2 leads to upregulation of TLR4 receptor mRNA (17). ROS initiate upregulation of TLR4, where HMGB-1 serves as a ligand for this receptor and activates downstream pathways. Thus, ROS, HMGB-1 and TLR4 are part of a triangle initiating the upregulation of adhesion molecules. Deficiencies of TLR2 or TLR4 in tubular epithelial cells result in defect neutrophil infiltration (34, 35). Downstream signaling of TLR2 induces production of CCR5 chemokine for NK cell chemotaxis. CD137 on the surface of NK binds to CD137L on tubular epithelial cells, stimulating these cells to produce high levels of CXCL1 and CXCL2. Subsequent attraction of neutrophils by CXCL causes further damaging of TECs (36).

Taken together, normothermic IR injury causes cell death, which induces the release of DAMPs. These DAMPs can function as ligands for PRRs, of which TLR4 and NLRP3 play a significant role in IR. Upon activation of TLR4 and NLRP3, downstream signaling leads to the transcription of pro-inflammatory cytokines and chemokines. During the reperfusion phase, the released cytokines and chemokines attract leukocytes to the injured tissue. The accumulation of leukocytes exacerbates tissue injury and completes the sterile inflammatory response.

III. Hypothermia in ischemia/reperfusion

A combination of ischemia/reperfusion and hypothermia/rewarming characterizes kidney transplantation. During hypothermic storage (HS) of transplant organs, oxygen is rapidly depleted by ongoing metabolism. Eventually, hypoxia will lead to cell death caused by mechanisms similar to those described in warm IR (37, 38). It has now been firmly established that, although hypothermic preservation limits ischemic damage, hypothermia perse may also lead to cell damage (14).

Necrosis versus apoptosis in hypothermia

The combination of ischemia and hypothermia-induced damage eventually lead to cell death, of which apoptosis mainly appears in the rewarming phase. Hypothermia-induced apoptosis is triggered by

exposure to low temperatures (4°C) for several hours and develops a significant part of the final cell injury during rewarming of the cells to 37°C (39). HS per se does not result in apoptosis, but is primarily of necrotic nature. However, rewarming is associated with significant apoptosis in the presence of ongoing necrosis, speculatively due to the activation of the apoptotic enzymic process of sublethally injured cells. The addition of antioxidants in the storage solution of kidneys confers protection against both HS and rewarming-induced necrosis and apoptosis (13). Upon HS, the pool of the cytosolic labile (chelatable) iron ions increases in several cell types, such as endothelial cells, hepatocytes, and renal tubular cells. In the reperfusion phase, these iron ions may trigger an apoptotic response also involving the formation of reactive oxygen species. In both examples, an increase in the reduced state of the cellular redox systems increases the reduction of labile iron ions during the ischemic period (40). Predominant apoptosis in the rewarming phase may provoke sterile inflammation in a less 'aggressive' way, compared to necrosis in normothermic ischemia. As apoptosis is a silent form of cell death, in which the release of DAMPs is also attenuated, activation of PPRs is possibly decreased. On the contrary, redox imbalance may stimulate sterile inflammatory pathways independent from DAMP release. Thus, release of DAMPs is less prominent in hypothermic IR compared to normothermic IR, while ROS might play an important role in hypothermic IR mediated sterile inflammation.

Toll-like receptor expression

As described earlier, TLRs are one of the principle innate immune receptors that have been implicated to play an important role in sterile inflammation (41). TLR4 can initiate a proinflammatory response in the early phase of renal IR injury, contributing to renal injury and dysfunction (25, 35). Both epithelium-associated and leukocyte-associated TLR4 contribute to the IR-induced effects *in vivo*. A study by Krüger et al. (42) provides strong evidence that TLR4 expression is increased in deceased donor kidneys, compared with living donor kidneys. Since deceased donor graft outcome is worse, compared to living donor grafts, the increased TLR4 expression might be detrimental to the kidney (43). As cooling is a common procedure in the preservation of deceased donor grafts, increased

expression of TLR4 might be associated with HS. The expression of TLR4 is likely controlled by activation of NF-κB and MAPK signal transduction pathways (44). Subsequently activated transcription factors bind to the promoter of the TLR4 gene, initiating transcription. Here, the activity of the transduction signaling determines the extent of the transcriptional upregulation. ROS are an important mediator in hypothermic injury and are known to activate NF-κB (15). Hence, ROS might function as inducers of TLR4 in HS, by activation of NF-κB.

Endothelium activation by ROS

Exposure to cold *in vitro* facilitates cellular infiltration, through increased expression of adhesion molecules (15). Similar findings in non-hypoxic conditions by means of *in vivo* assessment of chilled tissue, results in inflammation as well, evidenced by ROS production, ICAM-1 upregulation and recruitment of neutrophils. ROS has been implied in hypothermic injury (45). The main cause of ROS production under hypothermic conditions is depletion of SH-reduction equivalents, leading to redox imbalance (46). Generation of ROS, as a result from exposure to cold and subsequent rewarming, in human umbilical vein endothelial cells (HUVECs) is associated with nuclear translocation of NF-κB. Transcriptional activation by NF-κB, together with increased TNF-a, induces upregulation of cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 (15). As NF-κB functions as a transcription factor for many other proinflammatory genes, it is likely that genes such as IL1, IL-8 and COX-2 are upregulated as well (47).

Mitochondrial ROS

Mitochondria seem to be a prime target of hypothermia-induced injury. Furthermore, oxidative stress has been shown to play a detrimental role in hypothermic preservation and mitochondria are a potential source of reactive oxygen species (ROS) during hypothermic storage (48). Human tubular cells subjected to HS show a marked increase in free radicals (49). These free radicals are likely of mitochondrial origin, as there is an induction of SOD2 (Mitochondrial-SOD) (48). Other studies have implicated the presence of ROS in HS induced damage by the use of oxidant scavengers (49). Thus,

evidence exists that mitochondrial-derived ROS play a part in hypothermic IR. A recent study on mitochondrial-derived oxidants revealed several radicals as key signaling molecules of HS mediated injury, including mitochondrial superoxide ($O_2^{\bullet-}$), nitric oxide ($^{\bullet}NO$) and their reaction products (50). Generation of these molecules is linked to mitochondrial membrane depolarization, which further increases ($O_2^{\bullet-}$) levels and leads to peroxynitrite ($ONOO^-$) formation.

Mitochondrial ROS can have direct inflammatory effects in ischemic cells (51). This mechanism involves NLRP3 inflammasome activation, leading to subsequent caspase-1 cleavage of pro- IL-1β into IL-1β (Figure 2.). Mitochondrial ROS can activate NLRP3 through several mechanisms, such as sustaining MAPK activity, increasing the stability and accumulation of HIF-1 and NF-ϰB activation. NLRP3 can also directly sense mitochondrial ROS, via the trx and thioredoxin interacting protein (TXNIP). This complex dissociates upon sensing of ROS, allowing TXNIP to bind and activate the NLRP3 inflammasome (51). While ROS in general have a deleterious effect on cells subjected to HS, the potential to induce acute inflammatory responses aggravates tissue injury. Interestingly, Ali et al. (52) hypothesize ROS production to be a physiological, rather than a pathological response to hypothermia. It is possible that, to a certain degree, mitochondria are capable of autonomous thermoregulation, in which ROS function as a signal molecule. For instance, superoxide formation stimulates mitochondrial uncoupling protein (UCP) activation, leading to the mitochondrial generation of heat instead of ATP (53). Alternatively, UCP activation reduces ROS, thereby completing a negative feedback loop (54). Therefore, activation of UCP represents a potential target in reducing ROS.

Exposure to hypothermia in rat and human renal proximal tubular cells causes mitochondrial permeability transition (mPT), which is marked by an increase in the permeability of the mitochondria (55, 56). Initiation of mPT leads to the formation of the mitochondrial permeability transition pore (mPTP), causing release of ROS and mitochondrial swelling, which, in turn, triggers key apoptotic events and sets the stage for apoptosis during rewarming (56). The Bcl-2 family of proteins, located at the junction between inner and outer mitochondrial membranes, regulate the formation of mPTPs.

Usually the Bcl-2 protein counteracts the activity of pro-apoptotic pore-forming Bax protein. During the cold, the ratio between Bax and Bcl-2 is shifted towards Bax, promoting the formation of mPTP (56). The mPTP also allows for leakage of cytochrome c and other proapoptotic proteins such as Apaf-1, which in turn leads to the formation of apoptosome complexes composed of cytochrome c, Apaf-l, ATP, and procaspase-9 (57). During the rewarming phase, the complex recruits capsases-1, -2, -3 and -4 through its recruitment domain (CARD), initiating the caspase cascade.

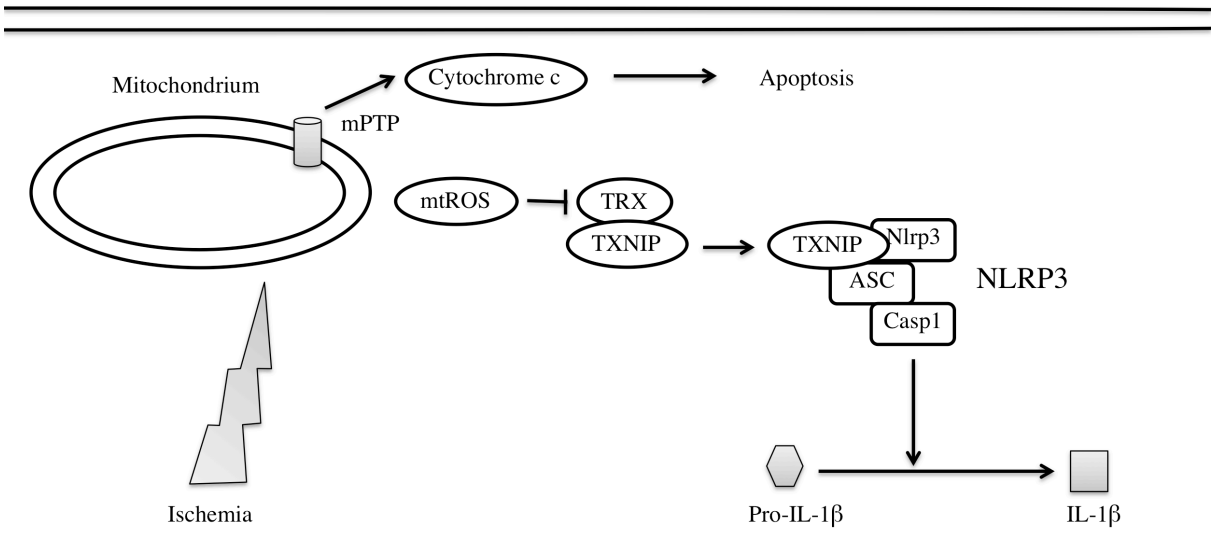

Figure 2. Mitochondrial ROS can have direct inflammatory effects by activating NLRP3. Ischemia/hypoxia induces mitochondrial dysfunction and generation of mitochondrial ROS. Formation of mPTP leads to leakage of more ROS and cytochrome c. The latter initiates apoptosis, leading to cell death. Mitochondrial ROS interacts with the TRX/TXNIP complex, leading to dissociation of TXNIP. TXNIP subsequently activates the NLRP3 complex, which leads to cleavage of pro-IL-1β into active IL-1β.

Mitochondrial-derived DAMPs

While caspase cascade marks apoptotic cell death, the principal mode of cell death in IR is probably a combination of necroptosis (regulated necrosis) during ischemia (7), and apoptosis during reperfusion (13). Cell death in HS per se is characterized by necrotic cell death while rewarming is associated with significant apoptosis, caused by the activation of apoptotic enzymes in injured cells (13). Next, cell death leads to the release of mitochondrial-derived DAMPs (mito-DAMPs), which activate innate immune systems and contribute to sterile inflammation (58). Release of mito-DAMPs is most prominent during necrosis (59) and to a lesser extent apoptosis (58). Since hypothermic ischemia is characterized by long HS times and thus long periods of necrosis, release of mito-DAMPs plays an important role in hypothermic sterile inflammation.

DAMPs derived from mitochondria include mitochondrial DNA, N-formyl peptides, ATP and cytochrome c (described earlier) (58). ATP released into the extracellular environment activates the NLRP3 inflammasome in macrophages, leading to cleavage of active IL-1β. Detection of this cytokine by endothelial cells causes upregulation of adhesion molecules on their surface and production of chemokines (27). Subsequently, neutrophils are recruited to the injured tissue, where formyl peptide receptor signals guide the neutrophils to the site of necrosis. Furthermore, mRNA expression of TGF-β1 increase gradually as hypothermic ischemic time becomes longer. If renal injury occurs, TGF-β1 can accelerate the progression of inflammation at the early stage of injury. TGF-β1 is released by macrophages and stimulates the transformation of renal tubular epithelial cells into myofibroblasts. The myofibroblasts and the other mesenchymal cells produce a large amount of stromatin protein and collagen during renal fibrosis (60).

Mitochondrial dysfunction and significant generation of ROS thus characterize hypothermic IR, where ROS function as pro-inflammatory signal molecules. Furthermore, ROS might induce increased expression of TLR4. During hypothermic storage long ischemia times allows for significant necrotic cell death to occur. Necrotic cell death and mitochondrial dysfunction promote the release of DAMPs and mito-DAMPs, which can activate PRRs and provoke sterile inflammatory responses.

IV. Future perspectives

While short-term effects of sterile inflammation in normothermic and hypothermic IR are now better understood, knowledge of long-term effects remains incomplete. The sterile inflammatory response sets the stage for progressive fibrosis and chronic humoral rejection (12). Research should thus focus on the prevention of acute inflammation, as this is the root of the problem. Many therapeutic strategies to prevent inflammatory reactions have proved to mitigate IR injury and have focused on blocking of transcription factors, cytokines, chemokines, adhesion molecules, and other pro-inflammatory compounds (61). Furthermore, new preservation strategies like normothermic machine perfusion are effective, especially in marginal or deceased donor kidney (62). Further, supplying ATP during hypothermic preservation, such as during machine perfusion, improves preservation but only allows a minor increase in preservation times (63). In this section, new potential therapies are described that interfere with pathways involved in sterile inflammation.

Protective function by Nrf2 activation

Pharmaceutical activation of the Nrf2 pathway has been shown to protect against acute renal IR damage in mice (64, 65). The cell normally attenuates oxidative stress situations via activation of the Nrf2 pathway, by transactivation of many antioxidant proteins, including heme oxygenase-1, catalase, glutathione peroxidase, superoxide dismutase, and thioredoxin. These proteins directly or indirectly scavenge free radicals and decrease the dose-dependent toxicity of ROS (66). Nrf2 mediated protection is thus based on increased scavenging of ROS, which are described earlier as detrimental to renal tissue in the context of sterile inflammation. Shokeir et al. (67) found that ischemic pre-conditioning activates the Nrf2 pathway and thereby protects the kidney from IR damage. Pre-conditioning involves short cycles of IR, prior to a longer ischemic insult. The renoprotective mechanisms of ischemic pre-conditioning besides Nrf2 activation include reduction in inflammatory cytokines (TNF-α, IL1β and ICAM-1) and inhibition of apoptosis (inhibition of caspase-3) (67). These findings suggest Nrf2 to be a mechanism by which ROS mediated sterile inflammation can be

attenuated. Here, Nrf2 activation can be achieved either by renal pre-condition or pharmaceutical intervention.

Xenon mediated HIF-1α activation

Xenon treatment attenuates kidney injury in warm (68) as well as hypothermic ischemia (69). Precondition with the noble gas results in upregulation of HIF-1α, via mTOR activation leading to increased downstream signaling of the mammalian target of rapamycin (mTOR) pathway (68). Zhao et al. (69) demonstrated that upregulation of HIF-1α stabilized the structural integrity of both the nucleus and cytoskeleton of tubular cells in response to oxidative and inflammatory. Nuclear stabilization preserves HMGB-1 within the nuclei (70) and thereby prevents activation of TLR4. Subsequently, decreased activation of TLR4 prevents the release of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β. However, in a rat model for lung IR injury, HIF-1α upregulation increases TLR4 expression (71). TLR4 signaling, on the other hand, results in HIF-1α upregulation in the MyD88-dependent way. Upregulation of TLR4 by HIF-1α has potential inflammatory effects and contradicts the protective function of HIF-1α as described by Zhao et al. (71). Thus, while Xenon shows to be a promising therapy in the prevention of IR injury, further investigation is required in order to understand the mechanisms by which HIF-1α regulates sterile inflammation.

V. Conclusion

Kidney transplantation is marked by the occurrence of ischemia/reperfusion, which results in a sterile inflammatory reaction of the graft tissue. Sterile inflammation can be induced by sterile particles released by dying cells, with the mode of cell death determining the release of these sterile particles (6). Three modes of cell death can be distinguished. First, Apoptosis involves a programmed signaling cascade of caspases that leads to the formation of apoptotic bodies. As phagocytes can effectively clear these bodies, apoptotic cell death is a self-contained event with little or no release of endogenous molecules. Secondly, Necrotic cell death involves cellular and organelle swelling and eventually

rupture of the plasma membrane. Lastly, regulated necrosis (necroptosis) causes the release of DAMPs, despite of it's regulated nature (7). Normothermic IR injury is marked by the release of DAMPs as a result of necrotic and necroptotic cell death. The released DAMPs subsequently activate PRRs and provoke a sterile inflammatory response. Contrary to the normothermic IR, hypothermic IR is characterized by apoptosis during the rewarming phase and it is therefore possible that hypothermia prevents large-scale release of DAMPs. However, ROS play an important role in hypothermic injury by directly activating PPRs and leading to mitochondrial dysfunction. Mitochondrial dysfunction causes the release of mitochondrial DAMPs and subsequent activation of PPRs. Despite the protection from acute necrosis due to a reduced metabolism in the cold, hypothermia can activate a sterile inflammatory response by mechanisms different to those in normothermic IR. Therefore, it can be concluded that the hypothermic insult during HS induces sterile inflammation, independent from mechanisms of normothermic IR. Thus, *temperature matters* in the development of renal IR induced sterile inflammation.

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