Pattern recognition receptors, sensing renal danger
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The kidney senses mitochondrial-derived DAMPs to induce sterile inflammatory responses

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Abstract

Systemic inflammation can cause severe collateral damage including the development of acute kidney injury. Inflammation is induced by different pattern recognition receptors (PRRs) of the innate immune system that sense damage-associated molecular patterns (DAMPs) that are released upon tissue injury. Mitochondria are a source of DAMPs and are thought to form a molecular link between tissue injury and inflammation in trauma patients. The role of the kidney herein remained however unstudied. Hence, in this study we investigated the role of renal tissue in sensing mitochondrial derived DAMPs to elicit inflammatory responses that may lead to renal injury and the impact of mitochondrial DNA on systemic inflammation and acute kidney injury (AKI).

Primary renal tubular epithelial cells (TECs) were stimulated with necrotic supernatant or purified mitochondrial ligands, after which the inflammatory response was determined. Circulating mitochondrial DNA (mtDNA) was determined in plasma of mice subjected to ischemic AKI. Renal function and injury were assayed in mice that received an injection with purified mtDNA. The impact of systemic inflammatory response syndrome (SIRS) and AKI on the levels of plasma and urinary mtDNA was determined in patients on the Intensive Care Unit (ICU) and associated to inflammatory mediators.

Our data reveal that renal TECs express several PRRs involved in sensing mitochondrial DAMPs and partly respond to necrotic cells in an mtDNA-dependent pathway. Mice that were subjected to ischemic AKI displayed elevated plasma mtDNA levels. Systemic administration of purified mtDNA provoked renal inflammation but did not affect renal function. Critically ill patients with SIRS demonstrated elevated levels of plasma and urinary mtDNA and proinflammatory cytokines compared to control ICU-patients. The presence of AKI did however not further statistically affect the levels of circulating or urinary mtDNA in SIRS patients.

Our data demonstrate that the kidney recognizes and responds to mitochondrial-derived ligands to initiate inflammation. This study further shows that experimental AKI is associated with high circulating mtDNA and that circulating mtDNA levels are associated with severity and outcome of systemic inflammation in critically ill patients. Together, these data suggests that mitochondrial DNA may form a link between tissue injury and inflammation.
Introduction

The onset of systemic inflammatory response syndrome (SIRS) significantly contributes to morbidity and mortality in critically ill patients on the intensive care unit (ICU) \(^1\). During SIRS, multiple inflammatory pathways are activated that damage cells and may result in clinical symptoms similar to sepsis. A common complication is the development of acute kidney injury (AKI) that affects more than 35% of critically ill patients \(^2-5\), the severity of which associated with an incremental increase in mortality \(^6\). AKI by itself is also characterized by massive renal inflammation that in turn influences kidney function and morphology \(^7-9\). Currently, it is however unclear which intrinsic factors trigger the onset of SIRS and lead to the development of AKI \(^8\) or distant organ injury \(^10\).

Sterile cellular injury results in the release of several endogenous danger molecules that are collectively referred to as ‘damage-associated molecular patterns’ (DAMPs) \(^11, 12\). These DAMPs are sensed and activate different families of pattern recognition receptors (PRRs). These include Toll-like receptors (TLRs), Nod-like receptors (NLRs) and the candidate formyl-peptide receptors (FPRs) \(^13-15\), that upon activation provoke local production of cytokines and chemokines and the recruitment of immune cells. We and others have demonstrated that necrotic cells can drive sterile inflammation, in part through mitochondria that are released by damaged cells \(^16-18\). Mitochondrial components closely resemble bacterial molecular patterns among which formylated peptides and the unmethylated CpG-enriched mitochondrial DNA (mtDNA). As such, mitochondria have the ability to trigger the (innate) immune system as a result of their suspected evolutionary origin as bacterial endosymbionts \(^19-25\). Indeed, recent studies of Zhang \textit{et al.} showed that circulating mitochondrial DAMPs link cellular injury to inflammation in trauma patients that explains why both trauma-induced SIRS and sepsis elicit a similar inflammatory response \(^26, 27\).

The role of circulating mitochondrial components in eliciting renal injury, a frequent complication of SIRS in critically ill patients, is unstudied. Hence, in this study we aimed to elucidate the role of the kidney in sensing mitochondrial DAMPs following sterile injury and their subsequent ability to elicit inflammatory responses that contribute to systemic or local inflammation and kidney injury.
Material & Methods

Induction of sterile tissue injury
Wild type C57Bl/6N male mice (Charles River Laboratories) were subjected to sublethal bilateral renal ischemia/reperfusion (I/R) injury as described extensively before and sacrificed after 24 hours \(^{28,29}\). Sham-operated mice underwent the same procedure, without clamping of the renal pedicles. Upon sacrifice, kidneys were isolated and snap-frozen into liquid nitrogen or formalin-fixed. Blood was collected by heart puncture in heparin-coated tubes. All blood samples were immediately processed on ice and spun down twice for 20 minutes at 1550g to obtain platelet-free plasma fractions \(^{30,31}\). Free circulating DNA was subsequently extracted using QIAamp DNA blood mini kit (Qiagen) according to the manufactures’ protocol. DNA was eluted by the supplied buffer and stored at -20°C until use. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Quantitative RT-PCR
Real-time quantitative PCR was performed on a Roche Lightcycler with SYBR green PCR master mix. SYBR green dye intensity was analyzed with linear regression analysis. The following murine primer sets were used: 12S (12S ribosomal RNA; for: 5’-CTAGCCACACCCCCACGGGA-3’, rev: 5’-CGTATGACCGCGGTGGCTGG-3’), ND1 (NADH dehydrogenase subunit 1; for: 5’-CAAACCGGGCCCCCTCGAC-3’, rev: 5’-CGAATGGCGCCGCTGCTAT-3’), COX1 (cytochrome C oxidase subunit 1; for: 5’-CCAGTGCTAGCCGGAGCA-3’, rev: 5’-TTGGGTCCCCCTCCTCAGCG-3’), B2M (Beta2-Microglobulin; for: 5’-CCCTCAGAAACCCTCTCAAAT-3’, rev: 5’-CGTTCCTTCAGGATTGT-3’), Cyclophilin G (Ppg; for: 5’-AAGGGAATGGAAGAGGAGA-3’, rev: 5’-CCCTCTGTTGGCCGATGTA-3’), FPR1 (for: 5’-TGTCAGAAGCAGTCCAGAAGACG-3’, rev: 5’-TCATGAGGTTCACTGAGACT-3’), FPR2 (=FPRL1; for: 5’-ATGACCCACATCACARCA-3’, rev: 5’-TTGGGTCCCCCTGCTT-3’), TLR9 (for: 5’-GAATCCTCCATCTCCCAACA-3’, rev: 5’-GGTGTGCTGGATGTCTTCTT-3’), NLRP3 (for: CCACGTCTCAGAGCACTTT-3’, rev: 5’-GGTGTGCTGGATGTCTTCTT-3’), HPRT (for: 5’-TCCTCCTCAGAGCACTTT-3’, rev: 5’-CCTGGTGTTCTGCTAATC-3’).

To detect and amplify human mtDNA, the following primers were used: COXIII (for: 5’-ATGACCCACCTACATGC-3’, rev: 5’-实际序列−3’), NADH1 (for: 5’-ATACCATGGCTAGGCCGGAG-3’, rev: 5’-实际序列−3’), NADH2 (for: 5’-TCCTCACAGACAAACTAGCCG-3’, rev: 5’-实际序列−3’).
Immunohistochemistry
Formalin-fixed, paraffin-embedded renal tissue of sham-operated mice was cut into 5μm sections. Slides were deparaffinised, endogenous peroxidase activity was blocked and antigen retrieval was subsequently performed by boiling tissue sections for 10 minutes in 1mM sodium citrate buffer (pH 6.0). Slides were exposed to rabbit anti-mouse FPR1 antibodies (Abcam) in blocking diluent (SkyTek Laboratories) overnight, followed by poly-rabbit-HRP (ImmunoLogic) incubation. Slides were developed using 1% H₂O₂ and DAB (Sigma-Aldrich) in 0.05M Tris-HCl (pH 7.9).

Western blotting
Snap-frozen renal tissue was lysed into cold RIPA buffer supplemented with 4mM Na₃VO₄, 0.5M NaF and protease inhibitors (Sigma). Total renal lysates (25μg) were denatured using 2-mercaptoethanol and heated 5 minutes at 100°C before loaded onto 12% SDS-PAGE gel. Proteins were transferred to Immobilon-P membranes (Millipore) using a semi-dry Trans-Blot system (Biorad). After blocking with 3% BSA/Tris-buffered saline (TBS)-Tween for 2 hours, membranes were incubated with the primary rabbit anti-mouse FPR1 antibody (1:500 dilution) at 4°C overnight. After washing, membranes were incubated with appropriate HRP-conjugated secondary antibodies and developed using ECL reagent (GE Healthcare).

In vitro stimulation assays
Primary renal TECs (cells bearing mtDNA) were harvested and cultured as described before and grown to confluence in HK2 culture medium, supplemented with 10% fetal calf serum, 100IU/ml penicillin, 100μg/ml streptomycin, 2mM L-Glutamine (all Invitrogen), 1% ITS and 1% S1 hormone mixture (Sigma). mtDNA-depleted cells (murine ρ0 L929 fibroblasts) were cultured in DMEM supplemented with 10% FCS, 100IU/ml penicillin, 100μg/ml streptomycin, 2mM L-Glutamine, 1mM pyruvate (all Invitrogen) and 50μg/ml Uridine (Sigma). As a consequence of mtDNA-depletion, these cells lack formylated peptides.

In the first in vitro stimulation assay, a predetermined amount of ‘donor’ cells (either cells bearing mtDNA (primary TECs) or mtDNA-depleted cells (ρ0 cells)) was subjected to three cycles of freeze-thaw into liquid nitrogen and subsequently centrifuged for 5 minutes at 1500rpm to obtain the supernatant of necrotic cells. Cultured ‘receiver’ primary TECs were subsequently stimulated with necrotic supernatant derived from indicated ratios of donor cells in HK2 culture medium. After 24 hours, supernatant was harvested for subsequent cytokine/chemokine release measurement by ELISA.

In the second in vitro stimulation assay, primary renal TECs were stimulated with different highly purified ligands, including CpG-DNA oligonucleotides (Hycult Biotech), the prototype synthetic formylated peptide N-formyl-Met-Leu-Phe (fMLF; GenWay...
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Biotech Inc.) and N-formylated peptides corresponding to the N-terminus of the mtDNA-encoded proteins NADH dehydrogenase subunit 2 (NADH2; fMNPITLA) and cytochrome C oxidase subunit 1 (COX I; fMFINRW) that were purchased from Genscript (Genscript USA Inc.). Peptides were >90% pure as determined by mass spectrometry and HPLC and dissolved in DMSO. After 6 hours of stimulation, supernatant was harvested for further investigation as described above.

Isolation of mtDNA and nDNA:
Donor wild type male C57Bl/6N mice (Charles River laboratories) were sacrificed under general anaesthesia and whole liver tissue was isolated. Mitochondrial fractions were extracted using cold Isolation Buffer (50mM Sucrose, 200mM Mannitol, 5mM KH$_2$PO$_4$, 5mM MOPS, 1mM EGTA and 0.1% BSA (all Sigma), pH 7.15) as described before 37, 38. Massively cultured mtDNA-depleted cells (murine ρ0 L929 fibroblasts) were used to isolate nuclear DNA (nDNA). The obtained mitochondrial fraction and mtDNA-depleted cells were subsequently processed using the QIAamp DNA mini kit (Qiagen) according to the manufacturers’ instructions in order to obtain either purified mtDNA or nDNA and eluted in the appropriate elution buffer. DNA concentrations were determined by spectrophotometer after which samples were stored at -20°C until used for in vivo injection.

In vivo injection of mtDNA or nDNA
Wild type C57Bl/6N male mice (Charles River) were anesthetized using isoflurane inhalation and received a single dose of mtDNA (60μg or 100μg), nDNA (60μg) or saline in 200μl total volume intravenously (n=2/group). To preferentially distribute the systemic injected DNA towards renal tissue, the left kidney was exposed after which the kidney was pressed once for ~2 seconds with a plunger of a syringe as described before 39, 40. The abdomen was closed and mice received a subcutaneous injection of 50μg/kg buprenorphin (Temgesic; Schering-Plough) for analgetic purposes. Mice were subsequently monitored and sacrificed after 24 hours by heart puncture after which plasma and organs were isolated for further investigations. Plasma levels of urea, creatinine, ASAT and ALAT and LDH were determined by routinely used clinical diagnostic assays at the hospital facilities. Kidneys were homogenized into Greenberger Lysis buffer as described before 29.

ELISA
Cytokines and chemokines (KC, MCP1, IL1β, TNFα) were measured in cell culture supernatants and kidney homogenates using specific ELISA (R&D systems) according to the manufacturers’ protocol. The detection limits were 15.6 pg/ml (KC), 6.3 pg/ml (MCP1), 31.3 pg/ml (IL1β) and 31.3 pg/ml (TNFα).
Patient inclusion and sample preparation

We included 61 patients that were admitted to the mixed medical-surgical ICU of the Academic Medical Centre in Amsterdam, The Netherlands. Patients were diagnosed and classified for onset of Systemic Inflammatory Response Syndrome (SIRS) and the development of acute kidney injury (AKI). SIRS was defined when patients met at least two of the following criteria (according to the SIRS consensus definition by the American College of Chest Physicians (ACCP) and society of Critical Care Medicine (SCCM)): fever or hypothermia, tachycardia, tachypnea or abnormal white blood cell count. The development of AKI was diagnosed and classified according to the Acute Kidney Injury Network (AKIN) criteria: either an abrupt (within 48hrs) absolute increase in serum creatinine concentrations of ≥0.3mg/ml from baseline, a percentage increase in the serum creatinine concentrations of ≥50% or oliguria of less than 0.6ml/kg per hour for more than 6hrs. Patients that had positive cultures of otherwise sterile sites or with a strong clinical suspicion of sepsis despite negative cultures, were excluded. The control group included ICU patients after elective major surgery without a diagnosis of either SIRS or AKI.

Blood (n=61) and urine (n=56; due to practical inability to obtain urinary samples) samples were collected within 24 hours after admission to the ICU. Arterial blood was isolated into heparin-coated tubes and urine was collected directly from the urinary bag and both were immediately processed. In order to obtain platelet-free plasma and sediment-free urine, all samples were spun down twice for 20 minutes at 1550g at room temperature and subsequently stored at -80°C until usage. Free circulating DNA was isolated from 400μl plasma or urine using the QIAamp DNA Blood Midi Kit (Qiagen) according the manufacturers’ protocol and eluted into the appropriate elution buffer, as also described before. The level of free circulating mtDNA was determined by RT-PCR. As all analyses were done on waste material, the Institutional Review Board of the University of Amsterdam, Amsterdam, the Netherlands consented to a waiver for obtaining informed consent.

Cytometric Beads Array:

The levels of inflammatory cytokines and chemokines (Interleukin (IL)-8, IL-1β, IL-6, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α) in human plasma and urinary samples were determined using the ‘Human Inflammatory Cytokines’ Cytometric Beads Array (CBA; BD Biosciences) according the manufacturers’ protocol. Measurements were performed using a FACS Calibur (BD). Undetectable samples were set to zero for statistical analysis; detection levels were 2.5pg/ml for all measured cytokines.
Statistics
Differences between experimental groups were determined using Mann-Whitney or ANOVA test. Statistical analysis on human data was performed using the Kruskal-Wallis with Dunn post-hoc testing. Correlations were performed using Spearman’s tests. All data are presented as mean±SEM (standard error of the mean). A p-value of p<0.05 was considered as statistically significant.

Results

Renal epithelial cells express PRRs to detect mitochondrial DAMPs
To determine whether renal tissue is able to sense mitochondrial-derived DAMPs, we first examined the presence of PRRs involved in sensing mitochondrial DAMPs on renal cells. We observed mRNA expression of FPR1, FPR2, TLR9, AIM2 and NLRP3 in total kidney specimens of control mice and mice with ischemic AKI. Moreover, primary wild type TECs with/without LPS priming clearly displayed expression of all receptors (figure 1a). Next, we looked into more detail to the capacity to detect formylated peptides by renal tissue. Interestingly, FPR1 protein was present in total renal lysates of both control and injured kidneys (figure 1b). More specifically, FPR1 expression was confined to specific renal tubular segments in the cortico-medulary region, but not to glomeruli (figure 1c). These data suggest that renal TECs might be able to sense mitochondrial-derived DAMPs.

Figure 1: The murine kidney expresses PRRs that can detect mitochondrial DAMPs.
Gel analysis of the mRNA expression of receptors FPR1, FPR2 and TLR9, AIM2, NLRP3 as sensors for respectively formyl peptides and DNA. All receptors were expressed in total renal specimen of either control or injured kidneys. In addition, primary renal TECs with/without LPS activation demonstrated comparable mRNA expression of all receptors. As a positive control, macrophages (MØ) were used (A). FPR1 protein was expressed in total renal lysates of sham-operated and renal I/R-injured mice (B), whereas immunohistochemical staining using an anti-FPR1 primary antibody displayed a specific tubular expression pattern of FPR1 in the cortico-medullary region of the kidney (C).
Necrotic cells trigger a renal proinflammatory response, partially dependent on mitochondrial-derived DAMPs

In order to investigate whether renal cells can respond to mitochondrial DAMPs, primary renal TECs were stimulated with necrotic supernatant derived from mtDNA-depleted cells or cells bearing mtDNA. Necrotic supernatant of mtDNA-depleted cells contained significant less mtDNA compared to supernatant of cells bearing mtDNA (data not shown). A clear proinflammatory response in a ratio-dependent manner was observed following stimulation with necrotic sup of cells bearing mtDNA, as reflected by profound KC production and a tendency towards higher MCP1 levels (figure 2a-b). Interestingly, KC release was significantly lower upon stimulation with necrotic sup of mtDNA-depleted cells, while MCP1 shows a tendency to lower levels, suggesting that the mitochondrial part of necrotic cells is, at least partially, responsible for the proinflammatory effect observed.

Figure 2: Immunostimulatory effect of necrotic supernatant on renal TECs partially depends on mitochondrial-derived DAMPs.

(A; B): Primary renal TECs were stimulated for 24 hours with necrotic sup derived from different ratios (6:1; 3:1 or 1:1) of either cells bearing mtDNA or mtDNA-depleted cells (n=4/group), after which KC (A) and MCP1 (B) levels were measured in the supernatant. (C; D): Primary renal TECs were stimulated for 6 hours with highly purified hexapeptides mimicking mtDNA-encoded peptides fCOX1 and fND2 (10 or 100μM), formyl peptide fMLF (1, 10, 100μM), CpG DNA oligonucleotides (1, 10, 25, 50μg) or LPS (10ng/ml) (n=4/group) after which KC (C) and MCP1 (D) levels were measured in the supernatant. Data are presented as mean±SEM. *: indicates significantly different compared to control samples, #: indicates significantly different compared to (6:1) cells bearing mtDNA.
In addition, the immunopotency of highly purified ligands was determined. After 6 hours of stimulation, CpG-DNA oligonucleotides, present in mtDNA, induced a strong proinflammatory response in a dose-dependent manner, whereas no effect could be observed upon stimulation with synthetic mitochondrial formyl peptides (figure 2c-d). Taken together, these data indicate that renal TECs can sense endogenous danger signals released by necrotic cells, in particular the mtDNA fraction and subsequently induce a proinflammatory response. Moreover, these data indicate that the immunostimulatory potential of individual highly purified mitochondrial DAMPs is minor, except for CpG-DNA motifs.

**Sterile tissue injury causes elevated plasma mtDNA levels in vivo**

In order to investigate whether sterile renal trauma in vivo causes release of mtDNA into the circulation, the presence of free circulating mtDNA was determined in plasma of mice subjected to ischemic AKI. Interestingly, a clear increase was observed in the amount of mitochondrial DAMPs in plasma of I/R-injured mice compared to non-injured (sham) mice, as reflected by enhanced levels of mtDNA-encoded genes 12S, COX1 and ND1 (figure 3). Significant tissue trauma was reflected by elevated plasma creatinine levels compared to sham-mice (5.6±0.4 vs. 135.2±22.2 μmol/l; p=0.004). To exclude a potential confounder effect of cellular contaminants during the isolation procedure of plasma samples, nuclear DNA-encoded genes B2M and Cyclophilin-G were determined. Both genes were undetectable in all measured plasma samples (data not shown).

![Figure 3: Ischemic AKI causes elevation of circulating mitochondrial DNA.](image)

The level of free circulating mtDNA is enhanced in plasma of mice subjected to renal ischemic AKI (n=7) compared to sham-operated mice (n=6), as reflected by plasma levels of the mtDNA-encoded genes 12S (A), COX1 (B) and ND1 (C). Data are mean±SEM. *: p<0.05

**Systemic administration of purified mtDNA induces local inflammation, but not renal dysfunction**

We next determined whether systemic administration of purified mtDNA results in the development of kidney injury. Systemic administration of high doses of mtDNA induced a local renal proinflammatory response, as reflected by the concentrations
of KC, MCP1, IL1β and TNFα in kidney homogenates that was even further enhanced when the mtDNA was preferentially distributed towards the kidney (figure 4c-f). Administration of mtDNA did however not result in renal dysfunction, as reflected by plasma urea and creatinine levels (figure 4a-b), nor differences in plasma ASAT, ALAT or LDH levels (data not shown).

Figure 4: Systemic administration of purified mtDNA does not affect renal function but enhances local renal inflammation.
Systemic injection of nuclear DNA (60μg) and mtDNA (60μg or 100μg) did not induce renal dysfunction in wild type mice (n=2/group; n=1 for nDNA), as reflected by similar plasma urea (A) and creatinine (B) levels compared to controls after 24 hours. High doses of mtDNA administration induced renal inflammation, as reflected by elevated levels of KC (C), MCP1 (D), IL1β (E) and TNFα (F) in kidney homogenates. The additional use of the renal pressure-method (+P) in order to preferentially distribute injected mtDNA towards the kidney did not affect renal function (A-B), but further enhances renal inflammation (C-F). Data are mean±SEM.

Table 1: Patient characteristics

<table>
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<th>ICU Controls</th>
<th>SIRS – AKI</th>
<th>SIRS + AKI</th>
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<tr>
<td>Plasma samples N</td>
<td>25</td>
<td>22</td>
<td>14</td>
<td>61</td>
</tr>
<tr>
<td>Urinary samples N</td>
<td>24</td>
<td>20</td>
<td>12</td>
<td>56</td>
</tr>
<tr>
<td>Male/Female (%)</td>
<td>72 (28)</td>
<td>59 (41)</td>
<td>50 (50)</td>
<td>62 (38)</td>
</tr>
<tr>
<td>Mean age</td>
<td>61±3.2</td>
<td>60±3.6 *</td>
<td>60±4.7</td>
<td>57±2.2</td>
</tr>
<tr>
<td>Length ICU stay (days)</td>
<td>3.5±1.4</td>
<td>6.7±1.3 *</td>
<td>6.7±2.3 *</td>
<td>5.4±0.9</td>
</tr>
<tr>
<td>28 day mortality (%)</td>
<td>0.0</td>
<td>27.3 *</td>
<td>35.7 *</td>
<td>18.0</td>
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<tr>
<td>90 day mortality (%)</td>
<td>4.0</td>
<td>31.8 *</td>
<td>50.0 *</td>
<td>24.6</td>
</tr>
<tr>
<td>Mean plasma creatinine (μmol/l)</td>
<td>68.4±3.4</td>
<td>61.7±3.1</td>
<td>197±18.6 *#</td>
<td>95.7±8.5</td>
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<tr>
<td>Mean urinary creatinine (μmol/l)</td>
<td>10.5±1.1</td>
<td>6.3±0.8 *</td>
<td>7.8±1.5</td>
<td>8.5±0.7</td>
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<td>Oliguria (%)</td>
<td>0</td>
<td>0</td>
<td>50 *#</td>
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<td>Hemofiltration (%)</td>
<td>0</td>
<td>0</td>
<td>21.4 *#</td>
<td>4.9</td>
</tr>
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</table>

Data are presented as mean±SEM or percentages. *: indicates significantly differences compared to ICU Controls; #: indicates significantly differences compared to SIRS-AKI group.
Mitochondria behind sterile inflammation

SIRS development in critically ill patients associates with increased levels of plasma and urinary mtDNA, independently of the development of AKI

In order to investigate whether acute kidney injury influences the levels of circulating mtDNA in patients with SIRS, mtDNA levels were determined in plasma and urine of ICU-patients with SIRS and/or AKI as well as control ICU-patients without a SIRS/AKI diagnosis. Patient characteristics are displayed in table 1. Of the patients with AKI, 21% were on continuous renal replacement therapy. The clinical diagnosis of SIRS corroborated with increased plasma levels of proinflammatory cytokines. SIRS patients displayed enhanced plasma IL8, IL6 (tendency) and IL10 levels compared to ICU-control patients (figure 5a-c). In addition, urinary IL8 was significantly elevated in SIRS+AKI patients compared to ICU-control patients and SIRS-AKI patients, whereas IL6 did not differ (figure 5d-e). Plasma and urinary levels of TNFα, IL1β, IL12p70 and urinary IL10 all remained below detection level (data not shown). Plasma mtDNA levels were increased in SIRS patients when compared to ICU-control patients, as reflected by the mtDNA-encoded genes COXIII, NADH1 and NADH2 (figure 6a-c). The additional development of AKI did not result in a further increase. Similar tendencies of increased mtDNA levels were observed in urine of SIRS patients with/without AKI compared to ICU-control patients, as reflected by the levels of urinary COXIII, NADH1 and NADH2 (figure 6d-f; P>0.05).
Interestingly, correlations were observed between levels of circulating mtDNA and plasma leukocyte counts (as an important criterion of SIRS), between circulating mtDNA and levels of plasma IL8 and between circulating mtDNA and the length of ICU-stay (as measure of clinical outcome) (table 2).

Figure 6: SIRS results in elevated levels of plasma mtDNA and a tendency toward higher urinary mtDNA levels, whereas AKI does not further affect mtDNA levels.

Plasma mtDNA levels are significantly elevated in SIRS patients without AKI (n=22) compared to ICU-control patients (n=25), as reflected by the mtDNA-encoded genes COXIII (A), NADH1 (B) and NADH2 (C). SIRS patients characterized with AKI (n=14) did not display significantly altered mtDNA expression as compared to ICU-control patients. Moreover, a tendency towards higher levels of urinary mtDNA was observed in SIRS+AKI (n=12) and SIRS-AKI (n=20) patients compared to ICU-control patients (n=24), as reflected by the levels of mtDNA-encoded genes COXIII (D), NADH1 (E) and NADH2 (F). Data are mean±SEM. *: p<0.05 vs controls.

Table 2: Correlations between patient characteristics:

<table>
<thead>
<tr>
<th></th>
<th>Plasma COXIII levels</th>
<th>Plasma NADH1 levels</th>
<th>Plasma NADH2 levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma leukocyte count</td>
<td>0.005 (0.35) *</td>
<td>0.0025 (0.38) *</td>
<td>0.0020 (0.39) *</td>
</tr>
<tr>
<td>Plasma IL8 levels</td>
<td>0.066 (0.24)</td>
<td>0.077 (0.23)</td>
<td>0.04 (0.27) *</td>
</tr>
<tr>
<td>Plasma IL6 levels</td>
<td>0.97 (-0.004)</td>
<td>0.98 (0.002)</td>
<td>0.93 (-0.011)</td>
</tr>
<tr>
<td>Plasma IL10 levels</td>
<td>0.11 (0.22)</td>
<td>0.059 (0.26)</td>
<td>0.06 (0.25)</td>
</tr>
<tr>
<td>ICU length of stay</td>
<td>0.013 (0.314) *</td>
<td>0.0023 (0.38) *</td>
<td>0.018 (0.30) *</td>
</tr>
<tr>
<td>Plasma creatinine levels</td>
<td>0.79 (0.035)</td>
<td>0.98 (-0.003)</td>
<td>0.46 (0.096)</td>
</tr>
</tbody>
</table>

Data presented are p-values and (spearman r-value) between measured parameters of all patients. *: indicates significant correlations between parameters.
Discussion

In patients with SIRS, multiple inflammatory pathways are activated that damages tissues and cells and may result in clinical symptoms similar to sepsis. A common complication that affects more than 35% of these critically ill patients is the development of acute kidney injury (AKI) \(^2-6\). The primary mechanism through which these inflammatory pathways leading to SIRS and/or AKI are initiated is however not completely understood. We and others have already shown the contribution of several PRRs in sterile renal injury models, such as for TLR2 \(^28, 47, 48\), TLR4 \(^29, 32, 48, 49\) and NLRP3 \(^16, 18\). Moreover, we and others demonstrated that the mitochondrial fraction of necrotic cells in particular was able to trigger a proinflammatory response in macrophages and monocytes \(^16, 17\). Mitochondria are an endogenous source of DAMPs with immunopotency \(^50-52\). Recent studies of Zhang \(et\ al\). showed that circulating mitochondrial DAMPs link cellular injury to inflammation in trauma patients \(^26, 27\). It however remained undefined whether mitochondrial DAMPs may elicit renal inflammation. In the current project we therefore investigated the role of renal tissue in inducing inflammation upon exposure to mitochondrial structures and investigated the impact of AKI on the levels of circulating mtDNA.

We first assessed the ability of renal TECs to sense mitochondrial-derived DAMPs by determining the presence of formylated peptide receptors FPR1 and FPR2 \(^53\), and DNA-sensors TLR9 and inflammasome (AIM2 and NLRP3) \(^54-60\). Interestingly, this study is the first to show renal expression of FPR1, FPR2 and AIM2. We also confirm renal NLRP3 expression \(^61\) and show the presence of renal TLR9, although expression of TLR9 in the kidney remains controversial \(^62-64\). In order to determine whether renal cells actively respond to mitochondrial danger ligands we subsequently performed a stimulation assay with necrotic supernatant that was derived from cells bearing or lacking both mtDNA and formyl peptides \(^33-36\). Interestingly, renal TECs display a proinflammatory response upon stimulation with necrotic supernatant, at least partially dependent on the presence of mtDNA and/or formylated peptides. Together, these data suggest that renal cells indeed can sense and respond to mtDNA. These results were in line with the study of Crouser \(et\ al\). demonstrating that the mitochondrial part of necrotic cells in particular was immunostimulatory \(^17\).

To determine the immunopotency of highly purified mitochondrial DAMPs on renal TECs, we next stimulated cells with different doses of formylated hexapeptides corresponding to the N-terminus of mtDNA-encoded formyl-peptides which are potent chemoattractants \(^24, 65\), the prototype formylated peptide fMLF or CpG-DNA oligonucleotides. This revealed that only CpG-DNA oligonucleotides demonstrated a clear dose-dependent proinflammatory effect on renal TECs. Since necrotic sup containing many endogenous PRR ligands induced a proinflammatory response
in renal TECs, and purified formylated peptides not, we propose at least two distinct stimuli are required for this response. This phenomenon was also shown by studies of Crouser et al. and Zhang et al. demonstrating that combinations of mitochondrial-derived DAMPs synergistically induce proinflammatory effects 17, 26. Besides, the reason for the reduced KC and MCP1 production observed upon high doses of fMLF stimulation (100μM) on renal TECs remains unclear, but might reflect desensitization of the FPR receptors 66.

In order to investigate whether circulating mtDNA could form a link between renal injury and inflammation, we first determined whether ischemic AKI results in elevation of systemic mtDNA levels. Indeed, we observed enhanced levels of plasma mtDNA in mice subjected to ischemic AKI compared to sham-operated mice. This is in line with the study of Zhang et al. that showed elevated plasma mtDNA levels in patients after trauma or haemorrhagic shock 27. To investigate whether systemic administration of purified mtDNA could induce renal inflammation and/or injury, mice received an intravenous injection of different doses of mtDNA. In order to preferentially distribute the injected mtDNA towards the kidney, the renal-pressure method 39, 40 was additionally used. Mice did not display signs of organ failure, since plasma levels of urea, creatinine, ASAT, ALAT and LDH were not altered. Interestingly however, cytokine/chemokine levels in kidney homogenates were elevated upon injection of high doses of mtDNA or upon preferential distribution of the mtDNA towards the kidney, indicating that high levels of systemic or local mtDNA might result in secondary kidney injury. Even though, these data might also indicate that purified mtDNA alone is insufficient to induce renal dysfunction, suggesting that a combination of additional mitochondrial-derived DAMPs might have more immunopotency, as we also showed for renal TECs. In line, the study of Zhang et al. demonstrated that systemic injection of a mixture of mitochondrial-derived DAMPs (MTD; including mtDNA and formyl peptides) induced early rat lung and liver injury 26. Finally, we investigated whether development of AKI affects the circulating and/or local levels of mtDNA in patients diagnosed with SIRS and whether this was related to SIRS-parameters, AKI and clinical outcome. Our results indicate that patients defined with SIRS display higher circulating mtDNA levels compared to control ICU-patients and also have elevated plasma inflammatory cytokine levels. Circulating mtDNA levels correlated positively with plasma leukocyte counts and with levels of circulating proinflammatory IL8. Moreover, circulating mtDNA showed a significant positive correlation with clinical outcome, as reflected by the length of stay on the ICU. These data suggest that high circulating mtDNA associates with the severity of SIRS and the resulting inflammatory responses. These data agree with the study of Zhang et al. 26 and indicate that mtDNA might indeed form a link between systemic inflammation and tissue injury. In line, a study demonstrated elevated plasma
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mtDNA levels in HIV patients, revealing a possible role for mtDNA in inflammation or as biomarker for virus-induced damage. Besides, the elevated levels of anti-inflammatory cytokine IL10 observed in SIRS patients might reflect the onset of a compensatory anti-inflammatory response syndrome (CARS) in patients suffering severe inflammation. Our data indicate that additional development of AKI does not further influence circulating mtDNA levels in SIRS patients and no correlation was observed between circulating mtDNA and plasma creatinine levels. These data suggest that high circulating mtDNA due to systemic inflammation does not lead to secondary acute kidney injury per se.

Furthermore, we observed that urinary mtDNA levels were detectable and elevated in SIRS patients and further increased in patients with AKI compared to control ICU-patients, although not statistically significant. In addition, urinary IL8 concentrations were markedly enhanced in SIRS+AKI patients. These data indicate that systemic inflammation with/without additional AKI leads to enhanced levels of urinary mtDNA levels, although the inclusion of additional patients is necessary in order to improve statistical power and draw further conclusions.

In conclusion, our data demonstrate that the kidney recognizes mitochondrial-derived ligands and subsequently induces proinflammatory responses. This study further shows that experimental AKI is associated with high circulating mitochondrial DNA and that circulating mtDNA levels are associated with severity and outcome of systemic inflammation in critically ill patients. Together, this suggests that mitochondrial DNA may form a link between tissue injury and inflammation.

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References

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