Selective mitochondrial antioxidant MitoTempo reduces renal dysfunction and systemic interleukin-1beta without impairing macrophage oxidative burst in experimental sepsis

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Abstract

**Background:** Increased production of mitochondrial reactive oxygen species (mROS) in sepsis is associated with organ failure. However, immune cell non-mitochondrial ROS is required for pathogen clearance, while mROS induces NLRP3 inflammasome activation. We determined the impact of a mitochondria-targeted antioxidant (MitoTEMPO) on renal proximal tubular epithelial cells mitochondrial dysfunction and peritoneal immune cell NLRP3-mediated IL-1-beta release and ROS burst *ex vivo*, and organ dysfunction in a rat model of sepsis.

**Methods:** The effects of MitoTEMPO were assessed *ex vivo* using ATP and lipopolysaccharide-stimulated rat CD11b positive peritoneal immune cells and fresh rat kidney slices exposed to serum from septic rats. We assessed mROS production and phagocytotic capacity using flow cytometry, mitochondrial functionality using multiphoton imaging and respirometry, and NLRP3 inflammasome activation in cell culture. The effect of MitoTEMPO on organ dysfunction was evaluated in a rat model of faecal peritonitis.

**Results:** MitoTEMPO decreased septic serum-induced mROS (*p*<0.001) and maintained normal NADH redox state (*p*=0.02) and mitochondrial membrane potential (*p*<0.001) in renal proximal tubular epithelial cells *ex vivo*. In lipopolysaccharide-stimulated peritoneal immune cells, MitoTEMPO abrogated the rise in mROS (*p*=0.006) and IL-1-beta (*p*=0.03) without affecting non-mitochondrial oxygen consumption, or the phagocytotic-induced respiratory burst (*p*>0.05). *In vivo*, compared to untreated septic animals, MitoTEMPO reduced systemic IL-1β (*p*=0.01), reduced renal oxidative stress as determined by urine isoprostane levels (*p*=0.04), and ameliorated renal dysfunction as determined by serum urea (*p*<0.001) and creatinine (*p*=0.05).

**Conclusions:** Reduction of mROS by a mitochondria-targeted antioxidant reduced IL-1-beta, and protected mitochondrial, cellular and organ functionality following septic insults.

**Keywords:** Reactive oxygen species; Sepsis; Acute kidney injury; Animal model; NLRP3 inflammasome; macrophage, mitochondria
Introduction

Sepsis is associated with an increase in reactive oxygen species (ROS) generation and oxidative stress, contributing to organ failure. While excessive production of mitochondrial ROS (mROS) may be toxic, they play an important signalling role in health and disease. ROS are primarily generated during oxidative phosphorylation in mitochondria, and by NADPH oxidase principally from immune cells. mROS are considered essential for immune cell functionality including NLRP3 inflammasome activation and phagocytic killing of pathogenic bacteria by macrophages, while NADPH oxidase-generated ROS are necessary for pathogen clearance and regulation of inflammation.

The ability to selectively reduce pathological levels of mROS while maintaining adequate levels required for innate immunity and other important roles is a therapeutic challenge. MitoTEMPO, an antioxidant that contains piperidine nitroxide TEMPOL conjugated with a positively charged triphenylphosphonium cation. This charge facilitates several hundred-fold accumulation within the mitochondrial matrix driven by the membrane potential, as the mitochondria is a compartment with a negatively charged membrane.

We previously reported that renal proximal tubular epithelial cell (PTEC) mitochondrial dysfunction with oxidative stress occurs ex vivo on exposure to serum from septic animals. Here, we investigated whether selectively scavenging mROS with MitoTEMPO would limit renal PTEC mROS generation, NLRP3-mediated inflammation and mitochondrial and renal dysfunction. We also evaluated the effect of MitoTEMPO on mROS-induced peritoneal macrophage NLRP3 inflammasome activation and non-mitochondrial phagocytic ROS to investigate whether innate immune function would be affected by MitoTEMPO in sepsis.
Methods and Materials

Methods are described in full in the Supplementary data. Male Wistar rats (Charles River, Margate, UK) weighing 325-400g were used throughout. All experiments were performed under a Home Office Project License (PPL 70/7029) and local UCL Ethics Committee approval. All experiments were performed in accordance with relevant guidelines and regulations.

A previously developed ex vivo imaging technique using mitochondrial dyes was utilized to probe temporal changes in mitochondrial function in thin slices of kidney tissue, visualized using dual photon beam confocal microscopy \(^{10,11}\). Rat peritoneal immune cells were isolated and cultured were identified using CD11b antibodies. The effect LPS and MitoTEMPO on mROS and IL-1\(\beta\) production was assessed using flow cytometry and ELISA respectively.

The Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of peritoneal cells.

A fluid-resuscitated rat model of sepsis was used to assess the effect of MitoTEMPO in vivo\(^{12}\). Based on previous data, a 24-hour timepoint was chosen to capture the peak of renal injury, as measured by serum creatinine \(^{12}\). Animals were randomized to receive either MitoTEMPO (Sigma) or placebo. The effects of MitoTEMPO in previous experimental studies of intra-abdominal sepsis are conflicting \(^{13,14}\); as the daily dose given varied 200-fold. As the higher dose (10 mg kg\(^{-1}\) per day) was beneficial, we selected a total dose of 10 mg kg\(^{-1}\) infused over 18 hours.
Results

*MitoTEMPO decreased septic serum-induced mROS, maintained NADH redox state, and preserved mitochondrial membrane potential in renal proximal tubular epithelia ex vivo*

We first assessed the effect of serum from sham-operated and septic animals on renal PTEC mitochondrial function

**i) Effect of serum from sham-operated animals on fresh kidney slices**

There were no significant changes in mitochondrial membrane potential (tetramethylrhodamine methyl ester, TMRM; Fig 1a(i)), nicotinamide adenine dinucleotide (NADH) redox state (Fig 1a(ii)), reactive oxygen species production (dihydroethidium, DHE; Fig 1a(iii)), and cell viability (calcein-AM fluorescence) in renal PTEC cells within the kidney slices over the 90 min incubation period in both physiologic saline solution (PSS) and serum from non-septic (sham-operated) animals.

**ii) Effect of serum from septic animals on fresh kidney slices**

Compared to incubation of fresh kidney slices for 90 min with serum from healthy rats, incubation of PTEC with serum from septic animals resulted in a significant decrease in mitochondrial membrane potential (Fig 1a (i); p<0.001), oxidation of the NADH pool (Fig 1a(ii); p<0.001), and an increase in the rate of ROS generation (Fig 1a(iii); p<0.001). Cell viability did not differ between PSS, sham serum and septic serum groups (p=0.794). Representative multiphoton confocal images of kidney slices are shown in Fig 1c, demonstrating fluorescence from the proximal tubular cells with each dye/native fluorescent marker before and after exposure to septic serum.

**iii) Effect of MitoTEMPO and genipin on septic serum-exposed fresh kidney slices**

To evaluate the mechanism of septic serum induced PTEC mitochondrial dysfunction, we evaluated the effects of MitoTEMPO and genipin. Compared to kidney slices incubated in septic serum alone, co-incubation with MitoTEMPO (added at time zero) prevented changes in PTEC mitochondrial membrane potential (Fig 1b(i); p<0.001), NADH redox state (Fig 1b(ii); p=0.024), and rate of ROS production (Fig 1b(iii); p<0.001). Addition of MitoTEMPO at 50 min following exposure to septic serum also rescued mitochondrial membrane potential (Fig 1b(i); p<0.001)
and NADH redox state (Fig 1 b(ii); p=0.023) and blunted the increase in ROS production (Fig 1 b(iii); p<0.001).

The addition of genipin, an inhibitor of mitochondrial uncoupling protein (UCP)-2, reduced the fall in mitochondrial membrane potential (p=0.002) and NADH reduction (p=0.003) induced by septic serum (Fig 1 b(i-ii)); however, the rate of ROS production increased even further compared to kidney slices incubated in septic serum alone (p=0.005; Fig 1 b(iii)).

**iv) Verification of targeted effects of MitoTEMPO on mROS using the mitochondria-specific superoxide indicator, MitoSOX Red**

Incubation of fresh kidney slices in septic serum resulted in a significant increase in MitoSOX Red fluorescence at 90 min compared to kidney slices incubated in sham serum (Fig 1 a(iv); p<0.001). Co-incubation of kidney slices in septic serum with MitoTEMPO was associated with significantly lower MitoSOX Red fluorescence (p<0.001 compared to untreated; Fig 1 b(iv)). Addition of the NADPH oxidase inhibitor, apocyanin to kidney slices exposed to septic serum did not affect MitoSOX Red fluorescence (p=0.558; Fig 1 b(iv)).

**MitoTEMPO abrogated LPS/ATP-induced IL-1β release in peritoneal immune cells ex-vivo**

The effect of MitoTEMPO and NLRP3 inhibitor MCC950 on peritoneal immune cell mROS and LPS/ATP- induced IL-1β release ex vivo was assessed.

**i) Isolation and culture of rat peritoneal immune cells**

CD11b-stained cells obtained by peritoneal lavage with a typical macrophage structure were visualized using confocal microscopy (Fig 2a). Between 50-55% of peritoneal cells were CD11b positive with >98% viability on flow cytometry. MitoSOX Red co-localized with MitoTracker Green (Fig 2b).

**ii) Establishing an effective concentration for MitoTEMPO**

MitoSOX Red fluorescence was assessed in CD11b positive peritoneal immune cells using flow cytometry (Fig 2c). Treatment of cells with the mitochondrial Complex III inhibitor, antimycin A resulted in a significant increase in MitoSOX Red fluorescence in CD11b positive peritoneal immune cells; this was prevented by co-incubation with MitoTEMPO at doses
between 5µM and 150µM (Fig 2d). A MitoTEMPO dose of 100µM was therefore used for subsequent *ex vivo* experiments.

**iii) mROS induces NLRP3 inflammasome activation in CD11b positive (peritoneal) immune cells *ex vivo***

Addition of LPS and ATP increased MitoSOX Red fluorescence (*p*<0.001) and IL-1β release (*p*<0.001) compared to unstimulated peritoneal immune cells, with minimal cell death (Fig 2 e (i-iii)). Co-incubation with the NLRP3 inhibitor, MCC950 reduced IL-1β release (*p*=0.020) but MitoSOX Red fluorescence was unaffected (*p*=0.597) compared to LPS/ATP treated cells. By contrast, co-incubation with MitoTEMPO significantly decreased MitoSOX Red fluorescence (*p*=0.006) and IL-1β release compared to LPS/ATP treated cells (*p*=0.030). No measurable IL-1β levels were achieved across any of the groups (LPS alone, antimycin (at doses between 5-100 µM), or LPS followed by antimycin.

**MitoTEMPO did not alter the increase in non-mitochondrial oxygen consumption and did not prevent metabolic reprogramming of peritoneal cells towards glycolysis following LPS exposure *ex vivo***

The effect of MitoTEMPO on peritoneal immune cell mitochondrial and non-mitochondrial oxygen consumption on exposure to LPS *ex vivo* was assessed using respirometry.

**i) ATP-induced mitochondrial depolarization**

Although extracellular ATP was used as a co-stimulus for NLRP3 activation, ATP was not added during respirometry studies due to its known rapid and reversible depolarizing effect on mitochondrial membrane potential 15 (Supplementary Figure 1).

**ii) Respirometry with LPS and MitoTEMPO**

Mitochondrial and non-mitochondrial oxygen consumption of rat peritoneal cells were assessed using respirometry following 6-hour exposure of the cells to 200 ng ml⁻¹ LPS (Fig 3). Compared to untreated cells, incubation with LPS resulted in a significant increase in non-mitochondrial oxygen consumption (*p*=0.014; Fig 3 a(i)). This was associated with a shift in energy metabolism from oxidative phosphorylation towards glycolysis as evidenced by an increase in basal extracellular acidification rate (ECAR) (*p*=0.045; Fig 3 a(ii)), and a decrease in ATP-coupled respiration (*p*=0.046; Fig 3 a(iii)). Compared to untreated cells, maximal respiration
was significantly reduced by LPS exposure, whereas basal respiration (p=0.396; Fig 3 a(v)) and mitochondrial proton leak (p=0.819; Fig 3 a(vi)) remained unaltered. Co-incubation of LPS-exposed cells with MitoTEMPO did not reverse any of the changes seen in the respirometry parameters (Fig 3 a-b).

**MitoTEMPO did not affect phagocytosis or the phagocytosis-induced respiratory burst in peritoneal cells ex vivo**

The effect of MitoTEMPO on peritoneal immune cell phagocytosis and total ROS release ex vivo was assessed. Incubation of CD11b positive peritoneal immune cells with opsonized E. coli resulted in a significant increase in phagocytosis (pHRhodo fluorescence) compared to untreated cells (p=0.007; Fig 3 c); this was unaltered by MitoTEMPO co-incubation (p=0.435; Fig 3 c). Dichlorofluorescein diacetate (DCFDA) fluorescence, a marker of total cellular ROS production, was significantly increased following phagocytosis of opsonized E. coli (p<0.001; Fig 3 d); this too was unchanged by MitoTEMPO co-incubation (p=0.329; Fig 3 d).

**24-hour fluid-resuscitated rat faecal peritonitis model**

Eight of 13 (62%) septic animals survived in our 24-hour model. Compared to naïve animals, septic rats had higher core temperature (p=0.025), serum lactate (p=0.031), and IL-1β (p=0.013) at 24 hours (Fig 4 b-d). Serum urea (p=0.055), creatinine (p=0.001) and urine isoprostanes, a marker of renal ROS production (p<0.01), were elevated compared to sham-operated animals (Fig 5 a-c). Likewise, renal homogenate IL-1β measured by ELISA (p=0.007) and the active 17 kDa fragment measured by Western blot (p=0.040) were also significantly elevated (Fig 5 d-f).

**MitoTEMPO reduced systemic IL-1β and abrogated renal oxidative stress and dysfunction in a 24-hour fluid-resuscitated rat faecal peritonitis model**

The effect of mitoTEMPO on systemic inflammation and renal function in vivo was assessed using a rat model of faecal peritonitis. Survival at 24 hours was similar between placebo-treated (8/13) and MitoTEMPO treated rats (8/12). Compared to placebo-treated septic animals (Fig 4 b-d), MitoTEMPO treated septic animals had similar core body temperature (p=0.999) and arterial lactate (p=0.999) at 24 hours, although serum IL-1β was significantly lower (p=0.025).
Renal dysfunction was abrogated in MitoTEMPO treated animals compared to placebo-treated animals, as evidenced by a lower serum urea ($p<0.01$; Fig 5 a) and creatinine ($p=0.052$). This was associated with a lower urine isoprostane level ($p=0.038$; Fig 5 c). However, treatment with MitoTEMPO did not affect renal homogenate IL-1β values either on ELISA ($p=0.083$; Fig 5 d) nor on Western blot of the active 17kDa fragment ($p=0.912$) compared to placebo-treated septic animals (Fig 5 e-f).
Discussion

Mitochondrial antioxidants have been shown to ameliorate organ dysfunction and inflammation in animal models of sepsis\textsuperscript{16,17}. However, clinical trials evaluating non-specific antioxidants have failed to demonstrate benefit in sepsis, reflecting the complexity of the underlying pathology and the difficulty of targeting a mediator as ubiquitous and pluripotent as reactive oxygen species \textsuperscript{1}. A shotgun approach to ROS scavenging may also not be beneficial given the importance of ROS in pathogen clearance and immune signalling, among other roles. Here we demonstrate a contribution of mROS towards the development of sepsis-induced kidney injury, and amelioration through selective scavenging.

Scavenging mROS with MitoTEMPO offered mitochondrial protection. The ROS generated in mitochondria is likely to be superoxide (O$_2^-$) as mitochondrial dysfunction in sepsis is largely attributed to complexes I and III\textsuperscript{18}. This was explored further through manipulation of uncoupling. Uncoupling proteins (UCP) enable leak of protons across the inner mitochondrial membrane. Within the kidney, UCP2 expression is upregulated by an increase in superoxide, thus providing a negative feedback mechanism to decrease mROS production through uncoupling\textsuperscript{19}. A reno-protective role of UCP2 has been demonstrated \textit{in vivo}\textsuperscript{20,21}. Genipin inhibits UCP2-mediated proton leak in isolated mitochondria\textsuperscript{22}. We found that genipin protected the mitochondrial membrane potential from depolarisation on exposure to septic serum but caused a concomitant increase in ROS production which could be potentially deleterious. These results are consistent with an increase in UCP2 activity in our model, which mitigates the increase in mROS production at the expense of mitochondrial membrane potential. Although genipin alone gives only indirect indications about UCP-2 involvement, we have previously shown that the level of renal UCP-2 protein is increased in the rat model of sepsis\textsuperscript{9}.

The inflammasomes are part of the innate immune system that regulate activation of IL-1β to induce inflammation\textsuperscript{23}. IL-1β is a key pyrogen and induces haemodynamic shock in experimental models; with tissue damage and metabolic derangements similar to those associated with sepsis in humans\textsuperscript{24}. 
MitoTEMPO was associated with a reduction in mROS and IL-1β in peritoneal immune cells ex vivo. mROS stimulate NLRP3 activation and IL-1β release in monocytes and macrophages. This is achieved by a process of reverse electron transport -ROS generation, in which macrophages reorganize their respiratory complexes, decreasing the activity of complex I and increasing activity of complex II. Treatment of macrophages with mitoTEMPO inhibited the effect of succinate on LPS-induced pro-IL-1β confirming that ROS was critical for IL-1β production. Additionally, mROS-dependent disulphide linkage of nuclear factor κB essential modulator (NEMO) leads to release of proinflammatory cytokines including IL-1β in macrophages. In patients with intra-abdominal sepsis, impaired monocyte NLRP3 activation was associated with mitochondrial dysfunction and a greater mortality risk.

mROS are important for phagocytic killing of pathogenic bacteria by macrophages. Stimulation of toll-like receptors in cultured macrophage-like cell lines increased mROS production and enhanced bactericidal capacity. Conversely, depletion of mROS by catalase overexpression impaired clearance of intracellular organisms. LPS induces switching of the tricarboxylic-acid cycle towards succinate generation via glutamine-dependent anaplerosis. This may facilitate increased succinate oxidation to fumarate via complex II of the mitochondrial electron transport chain to inhibit bacterial growth. Our findings are consistent with the metabolic reprogramming of classically stimulated macrophages from oxidative phosphorylation to glycolysis. NADPH-oxidase dependent ROS play an important part in pathogen clearance via the respiratory burst. This increase in LPS-induced non-mitochondrial oxygen consumption and phagocytosis-induced ROS do not appear to be affected by MitoTEMPO.

Excessive mitochondrial superoxide results in opening of the mitochondrial permeability transition pore (MPTP) with subsequent collapse of the mitochondrial membrane potential, impaired oxidative phosphorylation and, eventually, cell death. Scavenging of mROS with MitoTEMPO may have prevented MPTP activation and preserved mitochondrial membrane potential, thereby maintaining oxidative phosphorylation. Alternatively, a decrease in
mitochondrial membrane potential could be caused by mROS-induced upregulation of uncoupling proteins, a process that could also be reversed by selective mROS scavenging. Components of the NLRP3 inflammasome are expressed in renal epithelial cells and play a key role in many inflammatory renal diseases. Renal NLRP3 activity, as reflected by the cleaved fragment of IL-1β, was not influenced by MitoTEMPO treatment, suggesting that the renoprotective effect of MitoTEMPO primarily arises from its ability to scavenge mROS rather than by limiting renal inflammasome activation.

We were unable to demonstrate IL-1β release even with incremental doses of antimycin A and concurrent LPS priming as described by others. The optimal timing and/or dose conditions for rat peritoneal cell stimulation may not have been established, or differences in cell types requiring different stimuli. Although all flow cytometric analyses focused on CD11b positive cells, cell culture experiments included all peritoneal cells. Therefore, we are not able to conclude that the modulation of IL-1β was specific to peritoneal macrophages. While we intended to measure mROS levels in isolated CD11b positive peritoneal immune cells from our in vivo studies, we could not isolate a sufficient yield for flow cytometry due to significant intraperitoneal soiling. We isolated peritoneal immune cells from healthy rats and performed ex-vivo stimulation. The proportion of cells from peritoneal lavage would thus be similar to that seen in healthy rats, consisting of mononuclear phagocytes (approximately 70%), lymphocytes (15%) and granulocytes (15%) respectively. Whilst majority of CD11b positive peritoneal cells identify macrophages, additional cell surface markers may identify macrophages and their subsets with greater specificity.

Renal tubular cell death is not a major feature of sepsis although hydric mitochondria and increased autophagosomes are reported in critically ill septic patients. Similarly, in our rat model, significant histological changes are not evident as previously published which is likely related to the duration and intensity of the septic insult. As such renal histological injury was thus not analyzed in MitoTEMPO treated animals. The analysis of renal macrophages will provide further information in renal inflammation and dysfunction; however, it was beyond the scope of this study. The lack of mortality benefit of MitoTEMPO in sepsis may relate to
undesirable off-target effects, or that the attributable effect of mROS on mortality may not be significant. MitoTEMPO reduced IL-1β levels but had no effect on core temperature, despite IL-1β being a key regulator of fever.\textsuperscript{38}

Covalent attachment of lipophilic cations such as triphenylphosphonium (TPP\textsuperscript{+}) are rapidly taken up \textit{in vivo} by mitochondria, driven by its negative membrane potential in the matrix. Extensive accumulation of lipophilic cations within isolated mitochondria disrupts membrane integrity, respiration and ATP synthesis, which may limit clinical use.\textsuperscript{39}

A major strength of our model is the description of multiple features of sepsis similar to the human condition, reflecting its clinical relevance. This is of particular importance when investigating modulation of ROS in sepsis, as ROS are generated from different sources (mitochondrial and non-mitochondrial) and cells (non-immune and immune) with both functional and pathological roles.

In summary, we demonstrate the physiological role of mROS in immune cell signalling but a harmful effect from excessive mROS on organ dysfunction in sepsis. While mitoTEMPO cannot be used in patients, the ability to selectively scavenge mROS in this experimental sepsis model to ameliorate organ dysfunction without any obvious detrimental effects opens the possibility for MitoTEMPO, or similar agents, to be developed as a future therapeutic strategy.
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Declaration of interest

None

Author contributions

N.A and MS contributed to study concept and design; NA, SJP, CG, VP, GS, and TS contributed to ex vivo experiments; NA and RT contributed to in vivo experiments; NA and SJP contributed to data analysis; NA drafted the manuscript; MD and MS reviewed the manuscript; and all authors reviewed and approved the final version of the manuscript.
References

Figure Legends

Figure 1. MitoTEMPO ameliorates mitochondrial ROS, maintains NADH redox state, and preserves mitochondrial membrane potential in ex vivo renal proximal tubular epithelia exposed to septic serum

a. In proximal tubular epithelial cells, markers of mitochondrial functionality (membrane potential (TMRM), NADH redox state (NADH autofluorescence) and ROS production (dihydroethidium, MitoSOX Red)) showed no differences following 90 min perfusion with serum from sham-operated animals (sham serum) compared to perfusion with physiological saline solution (PSS) (a-i-iv). By contrast, perfusion with serum from septic animals significantly decreased membrane potential (a-i) and increased both NADH oxidation (a-ii) and ROS production (a-iii-iv), without affecting cell viability.

b. Co-incubation with MitoTEMPO attenuated these effects, either when given at Time 0 (baseline) or at the experiment midpoint (50 min) (b-i-iii). Co-incubation with the UCP2 inhibitor, genipin prevented the fall in membrane potential and NADH oxidation seen with septic serum alone, but resulted in an increase in ROS production (b-i-iii). mROS production was similar between kidney slices in septic serum co-incubated with the NADPH-oxidase inhibitor, apocyanin compared with septic serum alone (b-iv)

Scatter plots represent individual biological replicates and bar represents mean of 4-6 replicates. ANOVA was used to determine statistical significance between groups (*p<0.05, ** p<0.01, ***p<0.001).

c. Representative multiphoton confocal images of kidney slices at 0 and 90 min

Scale bars represent 100 µm.

PSS= physiologic saline solution, TMRM= tetramethylrhodamine methyl ester.
Figure 2. MitoTEMPO abrogated LPS-ATP induced mitochondrial ROS and IL-1β release in CD11b positive (peritoneal) immune cells

a. Representative images of cells following CD11b positive (peritoneal) immune cell isolation. Most isolated cells are CD11b-positive and present a typical macrophage phenotype. The round cell presents a different phenotype and is CD11b-negative (white arrow). Scale bar = 10 µm.

b. Representative images of CD11b positive (peritoneal) immune cells exposed to LPS-ATP with or without MitoTEMPO. The MitoSOX Red signal co-localizes with the mitochondria, identified by MitoTracker Green FM. Scale bar = 10 µm.

c. Gating of CD11b positive (peritoneal) cells on flow cytometry.

d. Increased MitoSOX Red fluorescence following 120 min exposure to 50 microM antimycin A, and reduced MitoSOX Red fluorescence with increasing doses (in MmicroM) of MitoTEMPO.

e. Addition of LPS and ATP: (e-i) significantly increased MitoSOX Red fluorescence which was reduced on addition of MitoTEMPO (100microMol) but not the NLRP3 inhibitor MCC950, (e-ii) significantly increased IL-1beta release which was reduced by both MitoTEMPO and MCC950 yet (e-iii) CD11b positive (peritoneal) immune cell death was minimal.

Scatter plots represent individual biological replicates and bar represents mean of 4-6 independent replicates. ANOVA was used to determine statistical significance between groups (*p<0.05, ** p<0.01, ***p<0.001).
Figure 3. MitoTEMPO does not affect respirometry following LPS stimulation, phagocytosis or phagocytosis-induced ROS burst in peritoneal cells \textit{ex vivo}

3a. Respirometry of peritoneal cells. Incubation of peritoneal cells with LPS (a-i) significantly increased non-mitochondrial oxygen consumption and shifted metabolism from oxidative phosphorylation towards glycolysis as evidenced by an increase in (a-ii) basal ECAR and (a-iii) a decrease in ATP-coupled respiration. (a-iv) Maximal respiration was significantly reduced with LPS stimulation, whereas (a-v) basal respiration and (a-vi) proton leak remained unaltered. 3a-b. Co-incubation of LPS with MitoTEMPO did not alter any respirometry parameters.

3c. Incubation of \textit{CD11b positive (peritoneal) immune cells} with opsonized \textit{E. coli} lead to phagocytosis (measured by pHRhodo fluorescence) and this was unaltered with MitoTEMPO co-incubation. 3d. Total cellular ROS (measured by DCFDA) were significantly increased following phagocytosis of opsonized \textit{E. coli}, and this was not prevented by co-incubation with MitoTEMPO. Scatter plots represent individual biological replicates and bar represents median of 4-6 independent replicates. Kruskal-Wallis test was used to determine statistical significance between groups (**p<0.05, *** p<0.01, ****p<0.001). ECAR = Extracellular Acidification Rate, OCR = oxygen consumption rate, MT = MitoTEMPO.
Figure 4. MitoTEMPO reduces systemic IL-1β in a rat model of sepsis

(a). Experimental protocol timeline. Compared to naïve rats, septic rats had a (b), lower oxygen consumption (VO_2), (c) higher serum IL-1beta (d) higher arterial lactate (d) higher core temperature. MitoTEMPO treated septic animals demonstrated quicker recovery of whole body VO_2 following treatment (p<0.05), had a similar core temperature and arterial lactate, although serum IL-1beta was significantly reduced.

Scatter plots represent individual biological replicates and bar represents median of 4 (naïve) to 8 (septic) independent replicates. Kruskal-Wallis test was used to determine statistical significance between groups (*p<0.05, ** p<0.01, ***p<0.001).
Figure 5. MitoTEMPO abrogates renal oxidative stress and dysfunction in a rat model of sepsis

Serum urea (a) was elevated in septic animals compared to naïve animals albeit not statistically significant (p=0.055). Serum creatinine (b) and urine isoprostanes (c) were significantly elevated in septic animals as were renal homogenate IL-1beta on ELISA (f) and the active IL-1beta 17kDa fragment on Western blot (e). Renal dysfunction was attenuated in MitoTEMPO treated animals as evidenced by reductions in serum urea and creatinine (a-b). This was associated with a significantly lower urine isoprostane level (c). Treatment with MitoTEMPO did not, however, affect renal homogenate IL-1beta values (e-f).

Scatter plots represent individual biological replicates and bar represents median of 4 (naïve) to 8 (septic) independent replicates. Kruskal-Wallis test was used to determine statistical significance between groups (*p<0.05, ** p<0.01, ***p<0.001).