Primary Research Article

DJ-1/PARK7 impairs bacterial clearance in sepsis
Hajera Amatullah1,2, Yuexin Shan1, Brittany L. Beauchamp3, Patricia L. Gali1, Sahil Gupta1,4, Tatiana Maron-Gutierrez5,6, Edwin R. Speck1, Alison E. Fox-Robichaud7, Jennifer L.Y. Tsang1,8, Shirley H.J. Mei9, Tak W. Mak10, Patricia R.M. Rocco6, John W. Semple1, Haibo Zhang1, Pingzhao Hu11, John C. Marshall1, Duncan J. Stewart9, Mary-Ellen Harper3, Patricia C. Liaw8, W. Conrad Liles12, Claudia C. dos Santos1,4* On behalf of the Canadian Critical Care Translational Biology Group (CCCTBG).

1) The Keenan Research Centre for Biomedical Science of the Li Ka Shing Knowledge Institute of St. Michael's Hospital, Toronto, ON, CA.
2) Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, ON, CA.
3) Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, CA.
4) Institute of Medical Sciences, University of Toronto, Toronto, ON, CA.
5) Laboratory of Immunopharmacology, Oswaldo Cruz Institute, FIOCRUZ, RJ, Brazil.
6) Laboratory of Pulmonary Investigation, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, RJ, Brazil.
7) Thrombosis and Atherosclerosis Research Institute, Department of Medicine, McMaster University. Hamilton, ON, CA
8) Department of Medicine, McMaster University, Hamilton (Niagara Campus), ON, CA.
9) Ottawa Hospital Research Institute and the University of Ottawa, Ottawa, ON, CA.
10) Department of Medical Biophysics and Immunology, The Campbell Family Institute for Breast Cancer Research at Princess Margaret Hospital, Ontario Cancer Institute, University Health Network, Toronto, ON, CA.
11) Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MA, CA.
12) Department of Medicine, University of Washington, Seattle, WA, USA.

*Correspondence and reprint requests to:
C. C. dos Santos, MSc, MD.
Clinician-Scientist, Associate Professor of Medicine,
Interdepartmental Division of Critical Care,
St. Michael's Hospital/University of Toronto, 30 Bond Street, Room 4-008, Toronto, ON, M5B 1WB, CA.
Tel: (+1416)-864-6060 (3198)
E-mail: dossantosC@smh.ca

Running Title: Role of DJ-1 in bacterial sepsis

Descriptor Number: 4.12, 13.4

Word Count: (text excluding abstract, figure legends, references): 3,454

Figure + Table Count: 8 Figures + 9 Supplementary; 1 Table

Reference Count: 75

Other Information: This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Contributions: Conception and design: CCDS, WCL and JCM; Analysis and interpretation: HA, PLG, YS, SG, TMG, ERS, AFR, JLYT, SHJM, TWM, PRMR, JWS, PZ, PCL; Drafting the manuscript for important intellectual content: HA, JCM, WCL, DJS, MEH, PCL, HZ, CCDS.
At a Glance Commentary:

Scientific knowledge on the subject: Cellular redox status plays a complex and dynamic role in host innate immune regulation in sepsis. DJ-1 (PARK7) is a well-established antioxidant with a protective role in the nervous system. Mutations in PARK7 result in autosomal recessive familial Parkinson’s disease. There is emerging evidence that DJ-1 can also modulate immune signaling pathways; however, the role of DJ-1 in sepsis remains largely uninvestigated.

What this study adds to the field: DJ-1 expression increases following CLP-induced sepsis. Targeted deletion of DJ-1 in mice resulted in increased ROS and pro-inflammatory markers and surprisingly improved survival in polymicrobial sepsis. These findings are similar to those observed for other deletion studies involving negative immune regulators. Our novel findings reveal that DJ-1 expression supports bacterial clearance in sepsis and a robust innate immune response is vital for resolution of infection and subsequent host survival. Our findings are in line with the most recent Sepsis-3 definition, which emphasizes the importance of a dysregulated host response (rather than hyper- or hypo-inflammatory) to infection as a critical determinant of outcomes.

Abstract

Rationale & Objectives: Effective and rapid bacterial clearance is a fundamental determinant of outcome(s) in sepsis. DJ-1 is a well-established reactive oxygen species (ROS) scavenger. As cellular ROS status is pivotal to inflammation and bacterial killing, we determined the role of DJ-1 in bacterial sepsis.
**Methods:** We used cell and murine models with gain and loss of function experiments, plasma and cells from septic patients.

**Measurements & Results:** Stimulation of bone marrow derived macrophages (BMM) with endotoxin resulted in increased DJ-1 mRNA and protein expression. Cellular and mitochondrial ROS increased in DJ-1 deficient (-/-) BMMs compared to wild type (WT). In a clinically relevant model of polymicrobial sepsis (cecal ligation and puncture, CLP), DJ-1/-/- mice had improved survival and bacterial clearance. DJ-1/-/- macrophages exhibited enhanced phagocytosis and bactericidal activity *in vitro*, and adoptive transfer of DJ-1/-/- bone marrow derived mononuclear cells rescued WT mice from CLP-induced mortality. In stimulated BMMs, DJ-1 inhibited ROS production by binding to p47^{phox}, a critical component of the NADPH oxidase complex, disrupting the complex and facilitating Nox2 (gp91^{phox}) ubiquitination and degradation. Knocking down DJ-1 (siRNA) in THP-1 (human monocytic cell line) and polymorphonuclear cells (PMNs) from septic patients enhanced bacterial killing and respiratory burst. DJ-1 protein levels were elevated in plasma from septic patients. Higher levels of circulating DJ-1 were associated with increased organ failure and death.

**Conclusions:** These novel findings reveal DJ-1 impairs optimal ROS production for bacterial killing with important implications for host survival in sepsis.

**Keywords:** DJ-1/PARK7, ROS, sepsis, CLP, bacterial clearance, NADPH oxidase

**Introduction**

Sepsis with accompanying organ dysfunction remains one of the leading causes of morbidity and mortality in intensive care units (ICUs) (1). Despite recent advances, no specific therapies are available to reduce the burden of illness (2, 3). Traditional approaches including resuscitation, antibiotics, source control and supportive care have improved outcomes (4, 5)
while strategies to blunt the inflammatory response have failed to improve survival (6, 7). Alternative strategies that enhance host defenses have recently gained attention (8). Our group has used systemic administration of bone marrow derived-mesenchymal stem/stromal cells (MSCs) to reduce inflammation, organ dysfunction and mortality, while enhancing bacterial clearance in experimental models of polymicrobial sepsis (9-13). Network analysis of global transcriptional responses modulated by mesenchymal stem cell (MSC) administration in sepsis identified the KEGG-Parkinson’s Disease pathway as markedly altered in septic tissues (14). Here we pursue one of the statistically significant gene products modulated in this pathway, Parkinson disease (autosomal recessive, early onset) 7 [PARK7], also known as DJ-1.

Originally identified as an oncogene (15), DJ-1 functions as a ubiquitous cytoprotective protein with diverse functions including transcriptional and mitochondrial regulation (16-22). Its main role, however, is providing protection from oxidative stress (15, 16). Loss of functional protein results in autosomal recessive familial Parkinson’s disease (23, 24). In the case of sporadic disease, overwhelming and/or persistent oxidative stress results in loss of DJ-1 function, accumulation of reactive oxygen species (ROS) and eventual neuronal death (25-27). A recent report has questioned the role of DJ-1 as an antioxidant suggesting that rather than reducing ROS, DJ-1 is required for increased ROS production in sepsis (28).

Cellular redox status plays a complex and dynamic role in host innate immune regulation and survival in sepsis. While excessive ROS can contribute to cell and tissue injury, free oxygen radicals as well as their oxidized substrates are key signaling molecules involved in pathogen recognition and clearance (29-32). Although little is known about the role of DJ-1 outside the nervous system, DJ-1 deficient Caenorhabditis elegans develop marked p38 mitogen-activated protein kinase activation and enhanced pattern recognition receptor expression (33). Moreover,
increased respiratory burst occurs in DJ-1 deficient *Litopenaeus vannamei* following bacterial challenge (34). These data suggest a conserved and protective role for DJ-1 to minimize inflammation (ROS) during acute infections. Here we address the role of DJ-1 in bacterial sepsis. Some of the results of these studies have been previously reported as abstracts (35-37).

**Materials & Methods**

**Plasma and Polymorphonuclear (PMNs) Cells from Sepsis Patients:** The Ethics Committee at McMaster University and St. Michael’s Hospital approved all study protocols. Study criteria and patient selection have been published (38). Written informed consent was obtained from all enrolled patients or substitute decision makers and from consenting adult healthy volunteers. Neutrophil isolation is described in supplement (39).

**Animals:** Protocols were approved by the Animal Care Committee at St. Michael’s Hospital. Wildtype (WT) C57Bl/6J (Jackson Laboratories) and DJ-1 deficient mice (targeted deletion of DJ-1, DJ-1/-/- (40)) on a C57Bl/6J background (20 backcrosses).

**Cecal Ligation and Puncture (CLP) Model:** Male mice (25-30 g) were randomized to CLP or sham surgery (9, 41, 42) detailed in supplementary material).

**Plasma/Serum DJ-1 Levels:** DJ-1 levels in human plasma and mouse serum were determined by ELISA (Cusabio Biotech Co., Ltd. China), according to manufacturer’s instructions.

**Serum Biochemistry Analyses and measurement of levels of Inflammatory Mediators:** VetScan Comprehensive Diagnostic Profile (VetScan Test Panels, University Health Network, Ontario) was used to perform serum biochemistry analysis. Inflammatory mediators were measured using Procarta Cytometric Bead Array (Affymetrix Panomics, Santa Clara, CA), according to manufacturer’s instructions (see supplementary file).
Isolation of Bone Marrow-Derived Macrophages: BMMs were isolated as described (43).

ROS measurements: Cellular and mitochondrial ROS in BMMs were assessed by CM-H2DCFDA and MitoSOX (supplementary). DCF fluorescence was measured in lung and spleens lysates using the OxiSelect ROS/RNS assay kit (Cell BioLabs), according to manufacturer’s instructions. Values were normalized to protein input.

Respiratory Burst in PMNs: Burst measurement in septic PMNs described in supplement (44).

Assessment of Cellular Bioenergetics, Phagocytosis and Bacterial killing: Detailed in supplemental file.

Loss- and Gain-of-Function: BMMs or THP-1 cells were transfected with mouse or human siRNA against DJ-1 (DJ-1 siRNA, loss-of-function), or a control scrambled siRNA (ctrl siRNA, Ambion). Alternatively, BMMs were infected (50 multiplicity of infection, MOI) overnight with recombinant adenovirus overexpressing DJ-1 (Ad-DJ-1, gain-of-function) or a control adenovirus (Ad-Ctrl), detailed in supplement.

Statistical Analyses: Mice were randomized (random number generator) to treatment groups, investigators blinded to genotype and evaluators blinded to group assignment. Survival studies were analyzed using Log Rank (Mantel-Cox) tests. Based on sample size calculation eight animals per group would allow us to detect a significant difference in 7-day mortality with 95% confidence. Unless otherwise stated, data are presented as mean +/- standard error of mean (SEM). Differences between groups were determined using Mann-Whitney, Student’s t-test, one-way ANOVA, or two-way ANOVA followed by Bonferroni post-hoc test to account for both “genotype” and “treatment”.

Results

Page 7
DJ-1 reduces ROS production and inflammation in BMMs following endotoxin challenge

Bone marrow derived-macrophages (BMMs) were isolated from wildtype (WT) and DJ-1 deficient (DJ-1/-/-) mice. DJ-1 mRNA and protein expression were upregulated in WT BMMs in response to LPS (1µg/ml) (Fig 1A & B). Levels of cellular ROS and mitochondrial ROS were comparable between genotypes at baseline. Following stimulation, both cellular and mitochondrial ROS increased in DJ-1/-/- BMMs compared to WTs (Fig 1C & D). Pro-inflammatory mediator levels were also higher in DJ-1/-/- BMM (Fig 1F). In addition, we have previously shown DJ-1 deficiency results in Nrf2 (Nuclear Factor, Erythroid 2-Like 2, NFE2L2) degradation (16, 43). Consistent with a decrease in Nrf2 transcriptional activity, glutathione, heme oxygenase 1 (Hmox-1), glutathione peroxidase 1 (Gpx-1) and manganese superoxide dismutase (MnSOD) expression was not increased in DJ-1/-/- BMMs (Fig 1E and Supplementary Fig 1A). DJ-1 deficiency in BMMs had no effect on cellular viability (Supplementary Fig 1B).

DJ-1 deficiency increases ROS production and inflammation following CLP

We randomized WT and DJ-1/-/- mice to a fluid resuscitated model of CLP-induced polymicrobial sepsis versus sham surgery. Elevated circulating DJ-1 levels were present in WT septic mice 24 hrs post-CLP (Fig 2A). ROS levels increased in lung lysates were higher in DJ-1/-/- mice (Fig 2B). In the absence of DJ-1, circulating and pulmonary levels of pro-inflammatory mediators IL-1β, IL-6, TNF, MIP-1b and MIP-2 increased at 24 and 48 hrs (except for TNF and MIP-1b in lungs) (Fig 2C). Increased pulmonary mediator levels were associated with enhanced cellular recruitment into the alveolar space by 48 hrs (Supplementary Fig 2C). CLP resulted in a 2-3 fold increase in BALF total protein and IgM levels in both DJ-1/-/- and WT mice at 24 hrs (Supplementary Fig 2C), and this was sustained in DJ-1/-/- mice at 48 hrs.

DJ-1 deficient mice had improved survival and organ function in response to CLP
Despite evidence of increased ROS production and inflammation, DJ-1 deficiency significantly attenuated 7-day mortality in fluid resuscitated, antibiotic treated CLP mice (81.82 vs. 18.18%, p=0.0005, Fig 23E). While WT mice became lethargic, stopped grooming, and showed moderate distress after CLP, DJ-1/-/- mice showed no, or only mild distress. Body weight and temperature were not significantly different between genotypes (supplementary Fig 2A). Resistance to CLP-induced mortality was observed in DJ-1/-/- mice even in the absence of antibiotics (60 vs. 88.8% survival, p=0.006, Fig 23D) and irrespective of perforation size (supplementary Fig 2B).

Assessment of serum biochemical markers of organ dysfunction determined lactate levels, although increased in both CLP groups at 24 hrs post CLP, were significantly lower in DJ-1/-/- mice (Fig 2F). Albumin levels were decreased, while alanine amino transferase (ALT) was increased equally in both genotypes. Total bilirubin, blood urea nitrogen (BUN), amylase and glucose levels were lower in the DJ-1/-/- mice (Fig 2F).

**DJ-1 impairs bacterial clearance and M1 polarization of professional phagocytes**

A potential explanation for improved survival is enhanced source control. DJ-1/-/- mice had significantly lower bacterial counts in blood, lung and spleen (12 and 24 hours after CLP, Fig 3A). Because M1 macrophages may enhance bacterial clearance, we isolated peritoneal cells (PC) from WT and DJ-1/-/- mice 12 hours after CLP. In addition to enhanced expression of proinflammatory cytokines, absence of DJ-1 resulted in an increase in the proportion of CD80 (cell surface marker for M1 phenotype) and inducible nitric oxide synthase (iNOS) mRNA expression. In parallel, CD206 (marker for M2 phenotype, Fig 3B) positive cells, and YM1 expression was significantly decreased, and a trend towards lower Arginase 1 (Arg1) mRNA expression (Fig 3C). iNOS protein was increased in WT and DJ-1/-/- BMMs (Fig 3D & E), while
Arg1 protein levels were decreased. iNOS expression was increased in DJ-1/- lungs and spleens 24 hours following CLP surgery (supplementary Fig 3A & B).

**DJ-1 impairs bacterial killing**

Improved bacterial clearance *in vivo* was associated with enhanced phagocytosis by DJ-1/- PC *in vitro*. We incubated WT and DJ-1/- PCs isolated 12 hours post-CLP (Fig 4A and supplementary Fig 4A) or following thioglycollate stimulation (supplementary Fig 4B) with fluorescent *E. coli* bioparticles. Phagocytosis of *E. coli* bioparticles also increased in DJ-1/- compared to WT BMMs and this effect was sustained over time (Fig 4B). Compared to WT BMMs, increased pHrodo (pH sensitive dye) labeled *E. coli* and *S. aureus* was also observed in DJ-1/- BMMs at baseline (non-stimulated, NS) and following 1h pre-stimulation with LPS (Fig 4C), consistent with acidification of the phagolysosomal compartment. Inhibition of ROS generation with Bayb117082 (an inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha [IκBα]) or mitoTEMPO (a mitochondria-targeted superoxide dismutase mimetic), attenuated the enhanced phagocytic phenotype in DJ-1/- BMMs (supplementary Fig 5A and 5B).

To determine bactericidal activity, we infected WT and DJ-1/- BMMs with *E. coli* or *S. aureus* for 30 or 60 min and treated with gentamicin. Bacteria that are not engulfed (remain extracellular) are killed by the antibiotic. After cell lysis, the resulting bacterial colonies represent intracellular viable bacteria that phagocytes have failed to kill. DJ-1/- BMMs had significantly decreased number of viable bacterial colonies 30 and 60 minutes post infection compared to WT BMMs (Fig 4D).

**Adoptive transfer of DJ-1 deficient bone marrow derived mononuclear cells (BMCs) improved survival in WT mice following CLP surgery**
To demonstrate that DJ-1 deficient phagocytes are more effective in eliminating bacteria early in the course of sepsis, conferring a beneficial survival effect, we isolated bone marrow derived-mononuclear cells (BMCs) from WT and DJ-1-/- mice and administered to WT mice 6 hours after the induction of CLP (Fig 5A). Treatment with DJ-1-/- BMCs significantly improved 7-day survival following CLP surgery compared with WT BMCs and saline controls (Fig 5B).

**DJ-1 reduces mitochondrial ROS production**

It is unknown whether loss of DJ-1 affects mitochondrial respiration in professional phagocytes but ineffective mitochondrial respiration generates ROS. Accordingly, we measured mitochondrial respiration and uncoupling in WT and DJ-1-/- BMMs. DJ-1 partially localized to mitochondria in unstimulated and LPS-stimulated BMMs (Fig 5C). Basal oxygen consumption rate (OCR), a measure of mitochondrial respiration, was not significantly different between DJ-1-/- and WT BMMs. Exposure to TNF however (6 hrs), unmasked a significant decrease in OCR in DJ-1-/- BMMs (Fig 5D & E). No difference was noted after 24 hrs treatment (data not shown). Although there was a modest increase in proton leak in DJ-1-/- BMMs at baseline, there was no significant difference following treatment (Fig 5F). Furthermore, addition of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), which uncouples proton pumping from ATP synthesis, maximized OCR in WTs while DJ-1-/- BMMs remained unresponsive (Fig 5F & G). Therefore, while 6h of TNF treatment had profound effects on cellular metabolic pathways (decreased basal and maximal respiration) in DJ-1-/- cells compared to WT cells, ROS emission increased normally in DJ-1-/- cells in response to a longer exposure to TNF (24h) suggesting another mechanism may explain increased ROS following pro-inflammatory stimulation.

**DJ-1 reduces NADPH oxidase-dependent ROS generation**
ROS generation by the NADPH oxidase complex is critical in host defense. The NADPH oxidase is a multi-subunit complex consisting of two membrane proteins, p22phox and the catalytic subunit, Nox2 (gp91phox), and the cytosolic proteins, p47phox, p67phox, p40phox, and Rac-1. We first assessed whether DJ-1 deficiency resulted in regulation of the subunits of the NOX complex. Expression of Nox2 (gp91phox) mRNA (Fig 6A) and Nox2 and p47phox protein were increased in DJb1b/b vs. WT BMMs at baseline and in response to LPS (Fig 6B). In parallel, knockdown of DJ-1 expression (supplementary Fig 6A) in WT BMMs resulted in increased Nox2 and p47phox protein expression following LPS stimulation (Fig 6B). Conversely, overexpression of DJ-1 (supplementary Fig 6A) attenuated Nox2 and p47phox expression (Fig 6C). NADPH oxidase activity (supplementary Fig 7A) was also increased in DJ-1 -/- BMMs. Treatment with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, normalized ROS burst in WT and DJ-1-/- BMMs (supplementary Fig 7B). Phosphorylation of p47phox mediates interaction of the various NADPH complex and Nox2 activation (45). Phosphorylation of p47phox is enhanced in DJ-1-/- BMMs following stimulation with LPS or live E. coli bacteria (EB) (Fig 6D). This was replicated in WT BMMs treated with DJ-1 siRNA (supplementary Fig 7C).

**DJ-1 binds to p47phox disrupting NADPH oxidase complex and promoting Nox2 degradation**

We investigated if DJ-1 inhibits ROS production by binding to components of the NADPH oxidase. P47phox subunit coimmunoprecipitated with DJ-1 at baseline and following LPS stimulation in both BMM and RAW cells (Fig 6E) indicating DJ-1 binds to p47phox. We did not observe binding of DJ-1 with the Nox2 or p67phox subunits. We postulated binding of DJ-1 to P47phox could result in decreased ROS production because of loss of complex stability. After inhibition of de novo protein synthesis with cycloheximide, Nox 2 protein levels decreased over
time in WT while remaining stable in DJ-1 BMMs exposed to *E. coli* bacteria (Fig 6F and supplementary Fig 7D). Absence of DJ-1 reduced Nox2 ubiquitination following *E. coli* treatment (Fig 6G and supplementary Fig 8E). The role of Dj-1 in complex disassembly was further supported by the evidence that co-treatment with the proteasome inhibitor, MG132, partially prevented decreased Nox2 protein expression in DJ-1/-/- BMMs (Fig 6H).

**Absence of DJ-1 also protects from *Pseudomonas aeruginosa* peritonitis**

To address whether absence of DJ-1 was protective in other bacterial infection models, we administered *Pseudomonas aeruginosa* intraperitoneally to wild-type and DJ-1/-/- mice. In line with our previous results, DJ-1/-/- mice were resistant to *Pseudomonas* -induced mortality compared with wild-type mice (Fig 7A).

**DJ-1 expression modulates bacterial killing and respiratory burst in human phagocytes**

We further investigated the role of DJ-1 in human cells. DJ-1 protein expression was increased in THP-1 (human monocytic) cells exposed to LPS and in polymorphonuclear neutrophils (PMNs) from septic patients (Fig 7B, D &E). Knockdown of DJ-1 in phorbol 12-myristate 13-acetate (PMA)-activated THP-1 cells (supplementary Fig 6B) resulted in increased *E. coli* or *S. aureus* bacterial killing (Fig 7C). Similarly, DJ-1 knockdown in PMNs from septic patients (n=5) modestly increased baseline respiratory burst in septic PMNs (Fig 7F). This increased following administration of PMA, an activator of respiratory burst (Fig 7F). Conversely, 1 hour pre-treatment with diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, abolished the enhanced respiratory burst in DJ-1/-/- BMMs (Fig 7F). Silencing DJ-1 in PMNs (septic or healthy) did not significantly alter cell death (apoptosis, supplementary Fig 6C).

**Circulating DJ-1 levels are increased and correlate with markers of sepsis severity and organ dysfunction in patients**
To determine if increased DJ-1 levels were also associated with poor outcomes in humans, we further identified elevated circulating levels of DJ-1 in septic patients (N=60) compared with healthy controls (N=12, Fig 8A). Higher DJ-1 protein levels were associated with increased mortality (Fig 8B), documented bacteremia (Fig 8C) and higher multi-organ dysfunction (MODS) scores on day of study enrollment (Fig 8D). Specific components of the MODS score also showed significant correlations with DJ-1 levels (Supplementary Fig 9). Collectively, these findings indicate DJ-1 is a potent anti-oxidant that plays a critical role in human sepsis and clinical outcomes.

**Discussion**

Our findings reveal a role for DJ-1 in the innate immune response to bacterial sepsis and pathogen clearance. Septic patients who die or develop significant organ dysfunction have increased circulating DJ-1. Absence of DJ-1 in vitro and in vivo resulted in increased ROS and inflammatory mediator expression. Despite an increased pro-inflammatory and pro-oxidant state, DJ-1 deficiency confers striking resistance to polymicrobial sepsis in a resuscitated and antibiotic treated clinically relevant animal model of sepsis. Even in the absence of antibiotics, absence of DJ-1 protects against polymicrobial sepsis-induced mortality by inducing ROS-mediated effective and rapid bacterial clearance. Moreover, adoptive transfer of DJ-1 deficient BMCs can confer resistance to bacterial sepsis to WT mice. Here we demonstrate that in innate immune cells, DJ-1 expression increases following an inflammatory (LPS) or bacterial stimulus. Available DJ-1 then binds to p47rophox disrupting NADPH oxidase complex assembly and/or contributing to Nox2 degradation thereby decreasing ROS production. Phagocytes that lack DJ-1 are consequently more efficient at engulfing and killing bacteria.
Regulating ROS balance is vital for mounting an appropriate immune response without excessive oxidative damage to resident host tissues. In macrophages and neutrophils, bacterial phagocytosis results in NADPH oxidase 2 complex assembly at the phagosomal membrane. Membrane (gp91<sub>phox</sub>/Nox2 and p22<sub>phox</sub>) as well as cytosolic (p47<sub>phox</sub>, p67<sub>phox</sub>, p40<sub>phox</sub>, and Rac1) subunits come together leading to an activated complex capable of generating ROS (46). Generation of ROS (specifically superoxide) constitutes a fundamental pathway for pathogen clearance (31, 46-48). In this study, we demonstrate that DJ-1 expression impairs host defense against bacterial infection by substantially limiting ROS production by the NADPH oxidase complex. DJ-1 deficient BMMs had enhanced expression as well as activity of the NOX complex. DJ-1 has been previously shown to regulate Nox4 in renal proximal tubule cells (49), further reinforcing the critical role of DJ-1 in regulating redox status in cells. Moreover, here absence of DJ-1 also results in decreased expression of Nrf2-dependent genes. This is in keeping with our previous data showing DJ-1 protects Nrf2 from Keap-1 mediated degradation (43).

In addition to the NADPH oxidase, the mitochondrial electron transport chain is an alternative contributor of ROS. Mitochondrial ROS are recognized as critical in innate immune activation and facilitation of antibacterial activity (50, 51). ROS emission per unit O2 consumed is highest when OXPHOS flux is lowest, since membrane potential is high. Our observations of impaired cellular bioenergetics in DJ-1<sup>-/-</sup> BMMs are consistent with previous findings (52, 53). In our study, treatment with Bay-11 7082 (specific inhibitor of inflammasome activation in macrophages) and mitoTEMPO (to reduce mtROS) appear to reverse the enhanced phagocytosis observed in the DJ-1<sup>-/-</sup> BMMs. Possible crosstalk between mitochondrial ROS and NADPH oxidant generation may contribute to a positive ROS feedback loop in DJ-1<sup>-/-</sup> mice (26, 54).
Most studies to date have focused on the role of DJ-1 as a ROS scavenger (55, 56). DJ-1, however, has a variety of other functions including protein chaperone, protease, RNA binding and regulator of autophagy (17, 18, 20, 52, 57-62). It is unclear whether multiple or specific functions of DJ-1 are also involved. In addition to its intracellular functions, DJ-1 is secreted into extracellular regions (63, 64) but its extracellular role remains enigmatic. Further studies will elucidate the role of extracellular DJ-1 in sepsis.

The role of DJ-1 in the host immune response has only been recently recognized. DJ-1-/- mice exhibit augmented passive cutaneous anaphylactic reactions and antigen-stimulated mast cell degranulation (65) suggesting a role for DJ-1 in adaptive immune response modulation. In addition, CD3+ T cell migration is increased in DJ-1-/- mice. DJ-1-/- Th1 and Th17 CD3+ T cell subsets had increased production of IFN-γ and IL-17 (66). Lack of DJ-1 leads to enhanced ROS production, higher Sgk1 (serine/threonine-protein kinase Sgk1 or serum glucocorticoid-regulated kinase 1) expression, and development of regulatory T cells (Tregs, (67). DJ-1 deficiency modifies the CD4+/CD8+ T cell ratio (67). These data are in keeping with our findings that absence of DJ-1 promotes early M1 polarization. We speculate that the enhanced bacterial killing phenotype conferred by DJ-1 deficiency may be one explanation for the conservation of this mutation through evolution.

Our results differ from those of a recent report that DJ-1 binding to p47phox is required for NADPH oxidase-dependent ROS production (28). However, those findings are surprising given the well-established antioxidant role of DJ-1 highlighting increased ROS and pro-inflammatory markers in various DJ-1 deficient models of inflammation (25, 40, 49, 58, 68). DJ-1 deficient astrocytes, for instance, have increased ROS, IL-6 and iNOS following LPS stimulation (69). Likewise, bone marrow derived-mast cells and mice lacking DJ-1 have higher ROS and TNF
levels following DNP-specific IgE stimulation (65). These findings as well as the consistency of our *in vivo* and *in vitro* data establish DJ-1 as a negative regulator of ROS production with implications for host immunity (70, 71). Here we show that binding of DJ-1 to p47phox acts to inhibit p47phox phosphorylation, preventing subsequent activation of the complex and ROS production. Phosphorylation of p47phox is a key event in the assembly and translocation of the cytosolic components as well as the activation of the NOX complex (45, 72). Furthermore, in the absence of DJ-1, Nox2 ubiquitination was reduced following exposure to LPS or *E. coli* bacteria, suggesting that DJ-1 regulates the stability of the NADPH oxidase protein. Treating cells with the proteasome inhibitor MG132 prevented Nox2 degradation in DJ-1 competent BMMs. A schematic diagram of our proposed mechanism is presented in Figure 8E.

Importantly, human monocytes and PMNs from septic patients can be made more efficient in bacterial phagocytosis and killing by transfection with an anti-DJ-1 siRNA. Based on our findings, DJ-1 contributes to a critical cell protection negative feedback mechanism to prevent excessive oxidative stress and inflammation. Reduced ROS production, however, significantly affects bacterial killing and clearance resulting in early mortality from bacterial sepsis. Interestingly, deletion of other negative regulators of ROS and innate immune signaling such as NRROS, KLF, ATF3 also results in improved outcomes in acute bacterial infection models (70, 73, 74). Here we also highlight how targeting endogenous mechanisms to maximize early clearance of bacteria may be beneficial for host survival. Collectively, our results challenge the paradigm that morbidity/mortality are determined by ‘degree’ or ‘severity’ of inflammation alone. These findings demonstrate dissociation between sepsis outcomes (organ injury and death) and inflammation. They also emphasize that therapeutic strategies designed to
simply decrease ROS and inflammation in early sepsis may be ultimately detrimental. This is in accordance with the current Sepsis-3 definition, which emphasizes the need to screen and identify underlying infection, as well as to distinguish infection-related organ dysfunction from that of non-infectious insults such as trauma and burns (75).

In summary, our data show that loss of DJ-1 enhances ROS, inflammatory responses, and bacterial clearance, resulting in improved survival from CLP-induced sepsis. DJ-1 binds to the NADPH oxidase complex affecting its stability and its ROS generating capacity, thereby impairing optimal ROS production for bacterial clearance. Furthermore, these observations provide novel insights into the host response during systemic bacterial infection that challenge the conventional paradigm that outcomes in sepsis are primarily determined by the severity of the host inflammatory response.
Acknowledgments: We thank Dr. David Park (University of Ottawa) for the DJ-1 adenovirus vector, Julie Khang (University of Toronto) for her help with the multiplex cytokine assays.

Competing Interests: The authors do not have any potential conflicts of interest. Parts of this work have been previously presented in abstract form (35-37).

Funding Sources: This work was supported by the Canadian Institutes of Health Research (Grant # MOP-130331 to CCDS). HA is the recipient of the Ontario Graduate Scholarship and St. Michael’s Hospital Li Ka Shing Knowledge Institute Graduate Scholarship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abbreviations
MODS – multiple organ dysfunction syndrome; CLP – cecal ligation and perforation; LPS – lipopolysaccharide; TNF - tumour necrosis factor; NOX/NADPH oxidase – Nicotinamide adenine dinucleotide phosphate oxidase; BMMs – Bone marrow derived-macrophages; PCs – Peritoneal cells; ROS – reactive oxygen species; MSCs – mesenchymal stem/stromal cells; PMNs – Polymorphonuclear cells (neutrophils); KEGG - Kyoto Encyclopedia of Genes and Genomes.


Figure Legends

**Fig. 1: DJ-1 reduces inflammation and ROS production in BMMs following endotoxin challenge.** A) Real-time PCR (RT-PCR) results for changes in the expression of DJ-1 messenger RNA (mRNA) in BMMs exposed to saline or LPS (1µg/mL) over 24 hrs. Bar graphs represent fold change of DJ-1 expression over the saline control at each time point normalized to glyceraldehyde dehydrogenase (GAPDH) expression. B) Representative western blot and quantification (n=5) showing increased DJ-1 protein expression in BMMs from WT compared to DJ-1/- mice following 24 hrs LPS stimulation (1µg/ml) normalized to GAPDH protein expression. Bar graphs represent means ± SEM (*p≤0.05, t-test). C) Intracellular and D) mitochondrial ROS in WT and DJ-1/- BMMs following saline and increasing doses of LPS (0.1 - 1µg/mL). E) Relative total glutathione content in WT and DJ-1/- BMMs following 24 hrs exposure to saline or LPS (1µg/mL). Bar graphs represent means ± SEM (*p≤0.05, ***p≤0.001, two-way ANOVA). F) Levels of inflammatory mediators in LPS-treated BMMs cell lysate from WT vs. DJ-1/- mice. BMMs were treated with or without LPS (1µg/mL) for 24 hrs and inflammatory mediator response was determined using multiplex ELISA. Mediators profiled: interleukin-1β (IL-1β), interleukin-6 (IL-6); interleukin-12 (IL-12 p40), macrophage inflammatory protein 1α (MIP-1α), KC (CXCL1) and monocyte chemoattractant protein-1 (MCP-1/CCL2). Bar graphs represent means ± SEM (n=3); (* p≤0.05, **p≤0.01, two-way ANOVA).

**Fig. 2 DJ-1 deficiency increases inflammation, but improves survival and organ function following CLP:** A) Serum levels of DJ-1 protein (pg/mL) in mice 24 hours following sham or CLP surgery. Data are presented as means ± SEM (n=8-10 per group, ***p≤0.001, student’s t-test). B) Levels of ROS in lung homogenates from WT and DJ-1/- mice 24 hours post CLP,
presented as dihydrochlorofluorescein (DCF) levels per microgram of protein. Data are presented as means ± SEM (n=6-8 per group, *p≤0.05, **p≤0.01, two way ANOVA). C) Levels of inflammatory mediators in lung homogenates and serum from WT or DJ-1-/- mice 24 and 48 hrs post-CLP, presented as fold change over sham controls. Mediators profiled: interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNF), macrophage inflammatory protein 1 beta (MIP-1β/CCL4), and macrophage inflammatory protein 2 (MIP-2/CXCL2). Data are presented as means ± SEM (n=6-8 per group, *p≤0.05, **p≤0.01, DJ-1-/- compared to WT, two-way ANOVA). D) Percent survival of WT and DJ-1-/- mice at 48 hours with fluid resuscitation and buprenorphine (***p≤0.001, Log-rank/Mantel-Cox test). E) Percent survival of wildtype (WT) and DJ-1-/- mice at 7 days (fluid resuscitation, buprenorphine and imipenem-cilastatin administration commencing at 6 hours post CLP and every 24 hours after that) following CLP-induced sepsis compared with sham controls. F) VetScan comprehensive diagnostic profile markers of organ dysfunction measured in serum of WT and DJ-1-/- mice 48 hrs after sham (n=5 per group) and CLP (n = 8 – 9 per group). Markers profiled: albumin (g/L), total globulin (g/L), alanine aminotransferase, ALT (U/L), total bilirubin (µmol/L), blood urea nitrogen, BUN (mmol/L), amylase (U/L), and glucose (mmol/L). Serum lactate levels (µM) was measured at 24 hours following sham or CLP surgery (n= 8 - 10 per group, *p≤0.05, **p≤0.01). Data are presented as means ± SEM (*p≤0.05, **p≤0.01, two-way ANOVA).

**Fig 3: DJ-1 impairs bacterial clearance and M1 polarization of professional phagocytes: A)** Bacterial load, represented as colony forming units per mL (cfu/mL), in blood, lungs, and spleen 12 and 24 hrs after CLP surgery. Box and whisker plots, the median is indicated with a horizontal line in the interior of the box, and the maximum and minimum are at the ends of the
whiskers (n = 5 – 8 per group, *p≤0.05 and **p≤0.01, two-way ANOVA). **B** Flow cytometric analysis and bar graph of activation surface markers on M1 and M2 macrophage subsets. F4/80 positive WT (black) and DJ-1-/- (grey) peritoneal cells were assessed for CD80 and CD206 receptor expression. **C** Real-time PCR (RT-PCR) results for changes in the expression of mRNA for inducible nitric oxide synthase (iNOS), Arginase 1 (Arg1), and Ym1 normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in WT and DJ-1-/- BMMs 24 hours after LPS. Data are presented as means ± SEM (n=3 per group, *p≤0.05, **p≤0.01, two-way ANOVA). **D** Representative western blot showing iNOS and Arg1 protein expression in WT and DJ-1-/- BMMs following 8 and 24 hrs of LPS treatment. Gels are normalized to β-actin expression. **E** Densitometry analysis showing quantification of iNOS protein expression at 8 and 24 hours. Data are presented as means ± SEM (n=3 per group, *p≤0.05, **p≤0.01, two-way ANOVA).

**Fig. 4: Effect of DJ-1 deficiency on bacterial clearance and phagocytosis.** **A** Representative images of WT and DJ-1-/- peritoneal cells isolated following 12 hours of CLP surgery and incubated with Alexa Fluor 488 conjugated *E. coli* (K-12 strain) BioParticles (Life Technologies) for 30 mins (Captured at 100x). Bottom panel - Quantified phagocytosis of Alexa Fluor 488 conjugated *E. coli* in WT and DJ-1-/- Peritoneal Cells, represented as means ± SEM of fluorescence intensity per cell from 10 representative images. **B** Representative fluorescence images of WT and DJ-1-/- BMMs with green pHrodo *E. coli* bioparticles and blue (DAPI) nuclear staining over a course of 30 minutes. **C** Measurement of phagocytic function of WT and DJ-1-/- BMMs using *E. coli* or *S. aureus* pHrodo Bioparticles with no stimulation (NS) and 1 hour pre-stimulation with LPS (1µg/mL). Assays were conducted in 96 well plates in triplicate.
and repeated twice. Data were normalized to baseline values at time 0 and presented as % fluorescence intensity. The fluorescence intensity was read using SpectraMax plate reader. Results are presented as means ± SEM (*p≤0.05). D) Measurement of bacterial killing activity in WT and DJ-1-/- BMMs by gentamicin protection assay at 30, 60 or 90 mins following *E. coli* or *S. aureus* infection. Data are presented as means ± SEM (n=5-6 per group, *p≤0.05, two-way ANOVA).

**Fig. 5: Adoptive transfer of Bone Marrow Derived Mononuclear Cells (BMCs) and role of DJ-1 in mitochondrial respiration**

A) Schematic of adoptive transfer of DJ-1 deficient bone marrow derived mononuclear cells (BMCs) in WT mice exposed to sham or CLP surgery. B) Percent survival of wild-type (WT) mice at 7 days (fluid resuscitation, buprenorphine and imipenem-cilastatin administration) after CLP-induced sepsis with administration of WT and DJ-1-/- BMCs six hours after sham or CLP surgery (*p≤0.05, Student’s t-test comparing CLP + WT BMCs vs CLP = DJ-1 -/- BMCs). C) Representative fluorescent images of DJ-1 expression (red), mitochondria (green), merged (yellow), and with nuclear (blue) immunostaining at baseline and following 24 hrs of LPS (1µg/mL) treatment (Captured on Olympus Upright Microscope at magnification of 60x).

Assessment of cellular energetics and oxidative stress: D) Representative Oxygen Consumption Rate (OCR) curve in WT and DJ-1-/- BMMs (5 x 10⁴) following 6 hrs saline or TNF treatment (10ng/mL) using Seahorse Bioanalyzer with sequential treatment of oligomycin (1µg/ml), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone or FCCP (1µM), and antimycin A (1µM). Data presented as means ±SEM, *p≤0.05 ANOVA compared to WT saline, #p≤0.05 compared to WT TNF, ANOVA. E) Basal Respiration Rate, F) ATP production and proton leak,
and G) Maximal Respiration calculated from three independent experiments. Data presented as bar graphs ± SEM (*p≤0.05, **p≤0.01, two-way ANOVA).

**Fig. 6: DJ-1 negatively regulates the NOX2 oxidase complex activity**

A) Real-time PCR (RT-PCR) results for changes in the expression of messenger RNA for Nox2 normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in BMMs exposed to saline or LPS (1µg/mL) over 24 hrs. Data are presented as fold change over WT saline control with means ± SEM (n= 3 - 6 per group, *p≤0.05, p≤0.01 two-way ANOVA). B) Left panel - Representative western blot showing increased p47\(^{phox}\) and Nox2/gp91\(^{phox}\) protein expression in DJ-1/-/ BMMs compared with WT BMMs following 24 hrs of saline or LPS treatment. Right panel - DJ-1 silencing in WT BMMs using short interfering RNA against DJ-1 (DJ-1 siRNA) increased expression of p47\(^{phox}\) and Nox2 protein expression 24 hrs after LPS administration compared with silencing with control scrambled RNA (ctrl siRNA). C) Representative western blots showing: Left panel – decreased expression of p47\(^{phox}\) and Nox2 (gp91\(^{phox}\)) in WT BMMs overexpressing adenovirus DJ-1 vector (Ad-DJ-1) compared with control adenovirus vector (Ad-Ctrl) following 24 hrs of saline or LPS treatment. Right panel – Increased expression of p47\(^{phox}\) and Nox2 in DJ-1/-/ BMMs is mitigated following reconstitution of DJ-1 levels with DJ-1 adenovirus vector (Ad DJ-1) compared with control adenovirus vector (Ad-Ctrl). Gels are normalized to GAPDH expression. D) Representative western blot showing anti-p47\(^{phox}\) immunoprecipitated from WT and DJ-1/-/ BMMs and probed with anti-phosphoserine antibody in 24 hours LPS treated (left panel) and 4 hours *E. coli* bacteria treated (right panel) samples. E) Representative western blot showing anti-DJ-1 and negative control (NC) IgG immunoprecipitated (IP) from WT BMMs or RAW 264.7 cells and probed with anti-p47\(^{phox}\),
Representative western blot expression of Nox2 and p47phox protein over time (0 – 4 hrs) in *E. coli* bacteria (EB) treated samples following pre-treatment with cyclohexamide (CHX, 10µg/mL). Right panel showing anti-ubiquitin protein ligase E3 component n-recong in 7 (UBR7) immunoprecipitated from WT and DJ-1/-/- BMMs and probed with anti-Nox2 antibody in samples treated with *E. coli* bacteria with and without CHX at 4 hours. G) Representative western blot showing UBR7 immunoprecipitated from WT and DJ-1/-/- BMM lysates treated with *E. coli* bacteria (EB) for 4 hrs and probed with anti-Nox2 antibody or reciprocal blot showing Nox2 immunoprecipitated from cell samples and probed with UBR7. I) Representative western blot showing Nox2 protein expression at 4 hours with *E. coli* bacteria (EB) treated samples with and without pretreatment of cycloheximide (CHX, 10µg/mL) and proteasomal inhibitor, MG132 (10µM).

**Fig. 7: DJ-1 expression modulates bacterial killing and respiratory burst in human phagocytes**

A) Percent survival of WT and DJ-1/-/- mice at 7 days with fluid resuscitation and buprenorphine (**p≤0.01, ***p≤0.001, Log-rank/Mantel-Cox test) following intraperitoneal injection of *Pseudomonas aeruginosa* infection. B) Representative western blot showing increased DJ-1 and oxDJ-1 protein expression in THP-1 cells with increasing concentration of LPS (0.1 – 10 µg/mL) and TNF (1 – 50ng/mL) stimulation normalized to β-actin protein expression. C) Measurement of bacterial killing activity in PMA-activated THP-1 cells with control siRNA or DJ-1 siRNA by gentamicin protection assay at 60 mins following *E. coli* or *S. aureus* infection. Data are presented as means ± SEM (n=5 per group, *p≤0.05, ANOVA). D & E) Representative western blot and quantification showing increased DJ-1 protein expression in polymorphonuclear cells (PMNs) collected from healthy donors or septic patients normalized to β-actin protein expression. F) Respiratory burst in septic PMNs at 24 hours with transfected with
control or DJ-1 siRNA at baseline (no treatment), or after treatment with phorbol 12-myristate 13-acetate (PMA) treatment, or 1 hour pre-treatment with diphenyleneiodonium (DPI). Data are presented means ± SEM (n=5 per group *p≤0.05 compared to control baseline, #p≤0.05 compared to DJ-1 baseline, $p≤0.05 compared to PMA treated only groups for respective genotypes).

**Fig. 8: DJ-1 protein expression in septic patients:** A) Plasma DJ-1 protein level (log ng/mL) was increased in septic patients (n=60) vs. non-septic controls (n=12); B) in patients with severe sepsis who died (n=32) compared to those alive at 28 days (n=28); C) in patients without (n=34) and with documented bacteremia (n=23). Data are presented as means ± SEM (**p≤0.01, ***p≤0.001, Mann-Whitney test). D) Correlation analysis of plasma DJ-1 levels with enrollment multi organ dysfunction score, MODS, E) Schematic of DJ-1’s role in regulating NADPH oxidase and bacteria clearance. In innate immune cells, following an inflammatory (LPS) or bacterial stimulus, DJ-1 expression in increased. Available DJ-1 can directly scavenge superoxide ions and reduce ROS. Alternatively, DJ-1 may bind to p47^{phox}, thereby disrupting NADPH oxidase complex assembly, or ubiquitinates Nox2 subsequently leading to decreased ROS production.
Figure 1

A

DJ-1/GAPDH
Fold Change

B

BMMs

WT

DJ-1-/-

C

LPS

C

LPS

GAPDH

D

DJ-1/GAPDH
Fold Change

C

Intracellular ROS
Fold Change

D

Mitochondrial ROS
Fold Change

E

Relative Glutathione

F

IL1β IL6 MIP1α RANTES MIP2 MCP1 KC IL12(p40)

(pg/ml)

Sal 0.1 1.0 Sal 0.1 1.0 LPS

Sal 0.1 1.0 LPS

WT DJ-1-/- Saline WT LPS DJ-1-/- LPS
Figure 3

A

**12 HOURS**

- Blood (x 10^3 cfu/mL)
- Lungs (x 10^3 cfu/mL)
- Spleen (x 10^3 cfu/mL)

**24 HOURS**

- Blood (x 10^4 cfu/mL)
- Lungs (x 10^4 cfu/mL)
- Spleen (x 10^4 cfu/mL)

B

**PERITONEAL CELLS**

- CD80
- CD206

C

- iNOS/GAPDH mRNA
- Arg1/GAPDH mRNA
- Ym1/GAPDH mRNA

D

- BMMs
  - WT
  - DJ-1−/−
  - C
  - LPS

E

- iNOS/β-actin Fold Change
Figure 5

A. Bone Marrow Derived Mononuclear Cells

DJ-1-/- → WT

6 hrs → 7 days → Survival

B. Percent survival

- Sham + saline (n=2)
- Sham + WT BMC (n=3)
- Sham + DJ-1-/- BMC (n=3)
- CLP + saline (n=9)
- CLP + WT BMC (n=13)
- CLP + DJ-1-/- BMC (n=13)

C. DJ-1 Mito Track Merge Merge

Control LPS

D. Oligomycin FCCP Antimycin A

OCR (pMoles/min/mg protein)

WT Sal DJ-1-/- Sal WT TNF DJ-1-/- TNF

E. Basal OCR (pMoles/min/mg protein)

Saline TNF

F. Respiration (pMoles/min/mg protein)

WT DJ-1-/- Saline TNF

G. Maximal Respiration (pMoles/min/mg protein)

Saline TNF
Figure 7

A

P. aeruginosa i.p. Percent survival

![Graph showing percent survival over hours]

WT (n=10) vs DJ-1-/- (n=10)

B

THP-1

<table>
<thead>
<tr>
<th></th>
<th>LPS</th>
<th>TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>

DJ-1
oxDJ-1
β-Actin

C

PMA activated THP-1 cells

E. coli (CFU/mL)
S. aureus (CFU/mL)

![Graph showing bacterial counts]

D

PMNs

Healthy donors vs Septic patients

DJ-1
β-Actin

E

Septic PMNs

DJ-1/β-actin Fold Change

![Graph showing fold change in DJ-1/β-actin]

F

Respiratory Burst Fold Change

![Graph showing respiratory burst fold change]
**Figure 8**

A. Log DJ-1 (ng/mL) for Healthy (n=12) vs. Septic (n=60)

B. Log DJ-1 (ng/mL) for Alive (n=32) vs. Dead (n=28)

C. Log DJ-1 (ng/mL) for Non-bacteremic (n=34) vs. Bacteremic (n=23)

D. Scatter plot showing correlation between MODS and DJ-1 (ng/mL) with correlation coefficient r = 0.3428, p = 0.0073**

E. Diagram illustrating the transition from RESTING STATE to ACTIVE STATE and then to CELLULAR FEEDBACK, with microbial, inflammatory stimuli leading to a redox response.
**Supplementary & Detailed Methods**

**Isolation of septic polymorphonuclear cells (PMNs):** A total of 40mL of whole blood (healthy volunteers) was collected by venipuncture into sodium citrate tubes or 10 mL of whole blood was collected through an indwelling arterial line (septic patients) at day 1 of ICU admission. Whole blood was centrifuged at 1500 g at 4°C for 10 minutes. Neutrophils were isolated by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient as previously described (36). Cell populations were determined to be >95% neutrophils. Viability as assessed by trypan blue exclusion routinely exceeded 95%. Polymorphonuclear neutrophils (PMNs) were resuspended in polypropylene tubes (1 x 10⁶ cells/mL) in Dulbecco’s modified Eagle’s medium with low glucose (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin solution.

**Cecal Ligation and Puncture Model:** Briefly, the chest and abdomen were shaved and prepared with 70% ethanol and betadine. The cecum was exteriorized and ligated 1 cm from the apex with 3–0 silk suture, penetrated through-and-through with an 18-gauge needle and then the abdominal incision was closed. In control sham surgeries, the cecum was exteriorized and manipulated but not ligated or punctured. All animals received subcutaneous (s/c) fluid resuscitation with 50ml/kg saline and 0.2mg/kg Buprenorphine (Buprenex) twice daily. Animals were placed in a temperature-controlled incubator and monitored every 6 hours for the first 48 hours; monitoring was de-escalated to every 8 hrs until the end of the experiments. Welfare-related assessment scores included gross appearance, motor activity and reflexes. For 7 day survival studies exclusively, mice received intraperitoneal (IP) 25mg/kg of Imipenem-Cilastatin (Ranbaxy, Pharmaceuticals Canada Inc. ON) once daily. Body weight, temperature and glucose levels were monitored in these animals for 7 days.
**Tissue Collection:** Mice were sacrificed 12, 24 or 48 hour after sham or CLP and tissues harvested for analysis. Whole blood was collected by cardiac puncture, centrifuged, and serum separated for biochemical and mediator analysis. The right lung was formalin-fixed for histology and the left lung was snap-frozen and stored at -80°C for protein and RNA analyses.

**Bronchoalveolar Lavage Fluid (BALF):** Bronchoalveolar lavage was performed on a subset of animals. The trachea was cannulated with an 18-gauge steel cannula (BD Biosciences, Canada) and instilled with three 0.5mL aliquots of 1x PBS. The collected volume was spun down to collect cells for counting and cytometry, and supernatant was stored at -80°C for total protein and IgM analysis.

**Isolation of bone marrow derived macrophages (BMMs):** Briefly, adult WT or DJ-1-/- mice (25-30g weeks) were sacrificed, then the femurs and tibiae were removed, and cleaned of all connective tissue in Dulbecco’s PBS without calcium and magnesium. The marrow cavity was flushed with PBS and the pellet was suspended and filtered. After washing, cells were cultured in DMEM/F12 GlutaMax medium with Macrophage Colony Stimulating Factor (M-CSF). The medium was changed every 48 h. Cells were used on day 7.

**ROS Measurements:** BMMs plated in 24-well plates were stimulated with 1µg/mL LPS for 12h. Culture medium was removed, cells were washed with PBS, incubated with MitoSOX (to measure the mROS superoxide, 5µM) or CM-H2DCFDA (to measure total cellular H2O2, 10µM) (Invitrogen) in serum-free DMEM/F12 (Invitrogen) for 20 min at 37°C (protected from light). Following incubation, cells were washed with PBS (37°C) three times. Subsequently, 0.5ml warm buffer was added (DMEM/F12 (1X), liquid, containing 15 mM HEPES buffer and L-glutamine, without phenol red) and plates were read at Ex/Em 510/580nm for MitoSOX and 492-495/517-527nm for CM-H2DCFDA by fluorescence microplate reader (Spectra Max M5 ®).
**Respiratory Burst Measurement:** Briefly, intracellular ROS production was measured as the conversion of dihydrorhodamine (DHR, Molecular Probes/Invitrogen) 123 to rhodamine in the presence of H₂O₂. PMNs were incubated for 5 minutes with DHR123 (1µM) at 37°C and 5% CO₂ and then stimulated with phorbol myristate acetate (PMA, BioShop) (500nM) for 20 mins. A group of PMNs were also pretreated with diphenyleneiodenium (DPI, Sigma-Aldrich) (10µM) for 1 hour prior to the DHR123 addition. Following stimulation, PMNs were placed on ice and fluorescence was quantified on the FL3 channel of the FACSCanto flow cytometer and mean fluorescence intensity was analyzed with FACSDiva Software.

**Real-time PCR:** Total RNA was extracted using Trizol (Ambion, Life Technologies) according to manufacturer’s instructions. First Strand cDNA was synthesized with 2µg of RNA samples using the Superscript First strand synthesis system for RT-PCR (Invitrogen, Life technologies). Real-time PCR (qPCR) was performed with the ABI 7900HT Real Time PCR system (Applied Biosystems, Foster City, CA). Primers were generated and purchased from PrimerQuest program (IDT DNA Technologies).

**Immunocytochemistry:** Cells were fixed with 2% paraformaldehyde and permeabilized with 0.25% Triton X-100. Slides were blocked with 1% BSA (with 0.3M glycine) and incubated overnight with primary antibody (DJ-1). The following day, slides were stained with appropriate fluorescent secondary antibodies and counterstained with 0.1µg/mL Hoescht before obtaining pictures on an Olympus microscope.

**Flow Cytometry:** Peritoneal lavage fluid was collected from mice 12 hours after CLP surgery. Peritoneal cells were washed, re-suspended in Pharmingen stain buffer, and incubated with primary antibodies or the matching control isotypes for 30 min at 4°C. The cells were washed, subsequently acquired and analysed by flow cytometer (Fortessa X-20).
Cell Viability Assessment in BMMs: Cell viability was performed by staining the cells with calcein AM (Molecular Probe, Eugene, OR). Calcein AM hydrolyzes to calcein and retains only in live cells, thus serving as an indicator for cell viability. To measure cell viability, BMMs were plated in 24-well plates and cultured at 37°C, 5% CO2 for 48 hrs. After treated with LPS (1µg/mL) overnight, rinsing the cell twice with PBS buffer (pH 7.4), 400 µl of 1 µM calcein AM were added to each well and incubated for 45 min at room temperature in dark. The plates were read at excitation of 485 nm and emission of 530 nm by fluorescence microplate reader (Spectra Max M5e).

Neutrophil Apoptosis Assay: The percentage of hypodiploid DNA cells was measured by flow cytometry as the uptake of propidium iodide (PI) using a BD FACSCanto flow cytometer with BD FACSDiva software version 5.0.1 (BD Biosciences, San Jose, CA), as previously described (71). At least 10000 events were recorded for each sample.

Isolation of Thioglycollate Induced-Peritoneal Macrophages: 1.0mL of 4% Brewer’s thioglycollate broth (Sigma Aldrich, St. Louis, MO) was injected intra-peritoneally (i.p.) into both WT and DJ-1-/- (8 – 12 weeks) mice. Ninety-six hours after the injection, 10mL of cold 1x PBS was injected i.p. into the peritoneal cavity of the mice. The abdomen was gently massaged and peritoneal lavage fluid was aspirated. Red blood cells in the cell suspension were lysed using RBC lysis solution. Peritoneal macrophages were subsequently plated in 96 well plates and used for phagocytosis assay.

Pharmacological Inhibition Experiments in BMMs: WT and DJ-1-/- BMMs were incubated with 20µM Bay-11 7082 (Invivogen), or 500 µM mitoTEMPO (Invivogen) for 1 hour prior to incubation with pHrodo Bioparticles. Measurement of phagocytic function of WT and DJ-1-/- BMMs. Assays were conducted in 96 well plates in triplicate. Data were normalized to baseline
values at time 0 and presented as % fluorescence intensity with a defined max value. The fluorescence intensity was read using a plate reader. Results are presented as means ± SEM (*p≤0.05).

**Adoptive Transfer of Bone Marrow Derived Mononuclear Cells (BMCs):** Six hours after sham or CLP surgery, 2.5 x 10^5 BMCs derived from WT or DJ-1/- mice or saline were instilled through the jugular vein and survival and welfare was monitored as previously described.

**NADPH Oxidase Activity:** NADPH oxidase activity was measured in WT and DJ-1/- BMM lysates in the presence of NADPH (100mol/L) and lucigenin (10umol/L). Assays were performed in a 96 well plate and chemiluminescence was measured in a plate reader.
**Supplementary Table 1:** Mouse primer sequences for real-time PCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>5’ ACTCCCAGGTTGCCACAT 3’</td>
<td>5’ AAGCGACTCATGGTCATCTACAAA 3’</td>
</tr>
<tr>
<td>Hmox-1</td>
<td>5’ TAGCCCACCTCCTGTGGTTTCTTT 3’</td>
<td>5’ TGCTGGTTTCAAAGGTCAGGCCAC 3’</td>
</tr>
<tr>
<td>Gpx-1</td>
<td>5’ AGCGCTAGTGACGGATTCCACGT 3’</td>
<td>5’ ATTCTCAATGAGCAGCACCTTGCC 3’</td>
</tr>
<tr>
<td>MnSOD2</td>
<td>5’ ATGTAGCTGTCTCCAGCCACACCA 3’</td>
<td>5’ AAGGTCTGAGTTCAATTCGCCAGCA 3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’ ATCCAGTTGCTCTCTGGGACTGA 3’</td>
<td>5’ TAAGCTCCGACTTGTGAACTG 3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>5’ CAAAAAATGGGGCGATGGG 3’</td>
<td>5’ CCACTCGGCGCATCTGGTAG 3’</td>
</tr>
<tr>
<td>Arg1</td>
<td>5’ GGAACCAGAGAGGACATGA 3’</td>
<td>5’ AAGCTGGTCTGCTGGAAAAA 3’</td>
</tr>
<tr>
<td>Ym1</td>
<td>5’ GCCCACAGGAAAGTACACA 3’</td>
<td>5’ TTGAGCCACTGACGGCTTAAC 3’</td>
</tr>
<tr>
<td>DJ-1</td>
<td>5’ TGCCATCTGTCAGGTCTCATA 3’</td>
<td>5’ TGACCTGCTGGCCCAAAACC 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ AGAACCTGCAAGTATGATGACA 3’</td>
<td>5’ TGAAGTGGCAGGACACACCT 3’</td>
</tr>
<tr>
<td>Nox2</td>
<td>5’ CGGAGAGTTGGGAAAGGACATAA 3’</td>
<td>5’ GGTACTGGGCACCTTTATTA 3’</td>
</tr>
</tbody>
</table>
Supplementary Information

Figure Legends

Supplementary Fig 1: Effect of DJ-1 on Antioxidants and Cell Viability. A) Real-time PCR (RT-PCR) results for changes in the expression of messenger RNA for Nuclear factor (erythroid-derived 2)-like 2 (NRF2), Glutathione Peroxidase 1 (GPX1), Heme oxygenase 1 (Hmox1), and Manganese superoxide dismutase (MnSOD) normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in WT and DJ-1-/- BMMs exposed to saline or LPS (1µg/mL) over 24 hrs. Data are presented as means ± SEM (n=5 – 6). B) Cell Viability, as assessed using calcein AM assay, in WT and DJ-1-/- BMMs following 24 hours of 1µg/mL LPS treatment. Data are presented as means ± SEM (n=3).

Supplementary Fig 2: Effect of DJ-1 deficiency in cecal ligation and perforation model of sepsis. A) Change (Δ) in body weight, temperature and glucose from baseline (day 1) in WT and DJ-1-/- BMMs following sham (n=5) and CLP surgery (n=8). B) Percent survival at 48 hrs in WT and DJ-1-/- as a function of the ‘size’ of the perforation. DJ-1-/- and WT mice were randomized to CLP using either an 18 or 22 gauge needle. Mice were fluid resuscitated but were not given antibiotics. Survival in DJ-1-/- mice was higher irrespective of the size of the perforation (N=5/group; *p≤0.05, Log-rank/Mantel-Cox analysis). C) Bronchoalveolar lavage fluid (BALF) total cell count, total protein, IgM levels 24 and 48 hrs after sham (n=3) and CLP surgery (n=5). Data are presented as means ± SEM (*p≤0.05, two-way ANOVA).
**Supplementary Figure 3: Effect of DJ-1 deletion on polarization**

A) Real-time PCR (RT-PCR) results for changes in the expression of messenger RNA for inducible nitric oxide synthase (iNOS) normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in WT and DJ-1/- spleen and lung homogenates 24 hours after sham or CLP surgery.  

B) Representative western blot showing Nox2 and iNOS protein expression in WT and DJ-1/- spleen homogenates following 24 hrs of sham or CLP surgery. Gels are normalized to GAPDH expression.

**Supplementary Fig 4: Effect of DJ-1 deficiency on phagocytosis**

A) Representative hematoxylin & eosin photomicrographs of peritoneal lavage cytospins collected from WT and DJ-1/- animals 24 hrs after surgery (magnification 40x).  

B) Measurement of phagocytic function of WT and DJ-1 thioglycollate-induced peritoneal cells (PCs) using *E. coli* with no stimulation and 1 hour pre-stimulation of LPS using Vybrant Phagocytosis Kit. Bar graph represents average of triplicate wells per treatment condition.

**Supplementary Fig 5: Phagocytic function in the presence of inflammasome and ROS inhibitors**

A) Measurement of phagocytic function of WT and DJ-1/- BMMs exposed to 1 hour pretreatment of Bay-11 7082 (20μM) and B) mitoTempo (500μM). Assays were conducted in 96 well plates in triplicate and repeated twice. Data were normalized to baseline values at time 0 and presented as % fluorescence intensity with a defined max value. The fluorescence intensity was read using a plate reader. Results are presented as means ± SEM (*p≤0.05).

**Supplementary Figure 6: DJ-1 knockdown and overexpression in various cells**

A) Left panel – Representative western blot showing protein expression of DJ-1 in WT BMMs following
control or DJ-1 siRNA transfection with control saline or LPS treatment. Right panel - Representative western blot showing protein expression of DJ-1 in WT BMMs following control or DJ-1 adenovirus with control saline or LPS treatment B) Representative western blot showing DJ-1 expression in PMA activated THP-1 cells treated with control or DJ-1 siRNA for 48 hours followed by 4 hours of LPS or *E. coli* treatment. Gels are normalized to GAPDH expression. C) Representative western blot showing DJ-1 expression in septic PMN 24 hours after control or DJ-1 siRNA transfection. Gels are normalized to B-actin expression. Right panel - Percent propidium iodide (PI) uptake staining of control siRNA transfected or DJ-1 siRNA transfected PMNs at 24 hours. D) Percent propidium iodide uptake staining of non-transfected, non-transfected with LPS treatment, control siRNA transfected, DJ-1 siRNA transfected, control siRNA transfected with LPS treatment, and DJ-1 siRNA transfected with LPS treatment in healthy PMNs at 24 hours. Data is represented as means ± SEM.

**Supplementary Figure 7: Effect of DJ-1 deficiency on oxidative burst and NADPH oxidase complex**

A) Nox2 activity in DJ-1-/- BMMs was enhanced compared with WT BMMs with no stimulation (left panel) and 1 hour LPS (1µg/mL) pre-stimulation (right panel). Data is presented as relative light units (RLU) over amount of input protein (*p≤0.05, **p≤0.01). B) Levels of ROS in BMMs from WT or DJ-1-/- mice following 3 hours of treatment with LPS (1µg/mL) or *E. coli* bacteria (EB) treatment (1 x 10^6 CFU) with and without pretreatment with diphenyleneiodeneidium (10µM), presented as dihydrochlorofluorescein (DCF) levels per microgram of protein. Data are presented as means ± SEM (n=3 per group). C) Representative western blot showing anti-p47^phox^ immunoprecipitated from WT and DJ-1-/- BMMs and probed with anti-phosphoserine antibody in 24 hours LPS treated WT BMM samples with control or DJ-
1 siRNA. D) Representative western blot showing Nox2 protein expression at 4 hours with LPS or E. coli bacteria (EB) treated samples with and without pretreatment of cycloheximide (CHX, 10µg/mL). E) Representative western blot showing anti-ubiquitin protein ligase E3 component n-recognin 7 (UBR7) immunoprecipitated from WT and DJ-1-/ BMM lysates treated with LPS for 24 hrs and probed with anti-Nox2 antibody.

**Supplementary Figure 8: Genotyping results of WT and DJ-1-/ mice** A) Representative genotyping blot indicating 1 WT mouse (#4) and 5 DJ-1-/ mice (#1, 2, 3, 5, 6) bands as well as positive (P) and negative (N) controls. B) Chart summarizing the bacterial counts in basal WT and DJ-1-/ cecal (15mg) content, as determined by colony forming units/mL on selective agar plates.

**Supplementary Figure 9: DJ-1 levels in plasma of septic patients and correlation with organ dysfunction.** Correlation analysis of plasma DJ-1 levels with A) APACHE II, B) Creatinine, C) Bilirubin, D) Platelet Counts, E) Ang 2, F) Intercellular Adhesion Molecule 1 (ICAM1), G) E-selectin, and H) von Willebrand factor (vWF).
Supplementary Figure 4

A POST CLP PERITONEAL LAVAGE CELLS

WT

DJ-1-/-

B

Thioglycollate PCs
E. coli Fluor. Intensity

WT NS

WT LPS

DJ-1-/- NS

DJ-1-/- LPS

**
Supplementary Figure 5

A

B

- WT + Veh
- DJ-1/- + Veh
- WT + Bay-11 7082
- DJ-1/- + Bay-11 7082

- WT LPS + Veh
- DJ-1/- LPS + Veh
- wt LPS + mitoTEMPO
- DJ-1/- LPS + mitoTEMPO

Non-stimulated BMMS
% E. Coli Fluorescence Intensity

Time (mins)

Pre-stimulated BMMS
% E. Coli Fluorescence Intensity

Time (mins)
Supplemental Figure 6

(A) WT BMMs

<table>
<thead>
<tr>
<th>ctrl siRNA</th>
<th>DJ-1 siRNA</th>
<th>Ad-Ctrl</th>
<th>Ad-DJ-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>LPS</td>
<td>C</td>
<td>LPS</td>
</tr>
</tbody>
</table>

DJ-1

β-actin

(B) PMA-activated THP-1 cells

<table>
<thead>
<tr>
<th>ctrl siRNA</th>
<th>DJ-1 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>LPS</td>
</tr>
</tbody>
</table>

DJ-1

β-actin

(C) 24 hour SEPTIC PMNs

<table>
<thead>
<tr>
<th>DJ-1 siRNA</th>
</tr>
</thead>
</table>

24 hour 24 hour

% PI stain positive % PI stain positive

24 hour SEPTIC PMN 24 hour HEALTHY PMN

control siRNA DJ-1 siRNA

(D) 24 hour HEALTHY PMNs

<table>
<thead>
<tr>
<th>Non transfected + LPS</th>
<th>control siRNA + LPS</th>
<th>DJ-1 siRNA + LPS</th>
</tr>
</thead>
</table>

% PI stain positive % PI stain positive % PI stain positive

24 hour HEALTHY PMN
Supplementary Figure 8

A

WT 1kb
DJ-1/- 700bp

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>DJ-1/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Agar</td>
<td>3000 ± 152.8</td>
<td>2350 ± 652.6</td>
</tr>
<tr>
<td>Blood Agar (5% Sheep Blood)</td>
<td>9.584e+011 ± 5.571e+011</td>
<td>1.285e+012 ± 7.037e+011</td>
</tr>
<tr>
<td>MacConkey Agar (with Crystal Violet)</td>
<td>950 ± 104.1</td>
<td>1033 ± 60.09</td>
</tr>
<tr>
<td>Phenylethyl Alcohol Agar (5% Sheep Blood)</td>
<td>3.295e+007 ± 1.950e+006</td>
<td>1.614e+007 ± 1.286e+007</td>
</tr>
</tbody>
</table>