

BASIC RESEARCH PAPER

A novel PINK1- and PARK2-dependent protective neuroimmune pathway in lethal sepsis

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ABSTRACT

Although the PINK1-PARK2 pathway contributes to the pathogenesis of Parkinson disease, its roles in sepsis (a major challenge for critical care) were previously unknown. Here, we show that *pink1*^{-/-} and *park2*^{-/-} mice are more sensitive to polymicrobial sepsis-induced multiple organ failure and death. The decrease in the circulating level of the neurotransmitter dopamine in *pink1*^{-/-} and *park2*^{-/-} mice accelerates the release of a late sepsis mediator, HMGB1, via HIF1A-dependent anaerobic glycolysis and subsequent NLRP3-dependent inflammasome activation. Genetic depletion of *Nlrp3* or *Hif1a* in *pink1*^{-/-} and *park2*^{-/-} mice confers protection against lethal polymicrobial sepsis. Moreover, pharmacological administration of dopamine agonist (e.g., pramipexole), HMGB1-inhibitor (e.g., neutralizing antibody or glycyrrhizin), or NLRP3-inhibitor (e.g., MCC950) reduces septic death in *pink1*^{-/-} and *park2*^{-/-} mice. The mRNA expression of *HIF1A* and *NLRP3* is upregulated, whereas the mRNA expression of *PINK1* and *PARK2* is downregulated in peripheral blood mononuclear cells of patients with sepsis. Thus, an impaired PINK1-PARK2-mediated neuroimmunology pathway contributes to septic death and may represent a novel therapeutic target in critical care medicine.

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Introduction


Sepsis, including severe sepsis and septic shock, is a clinical condition occurring in patients following infection or injury.¹ It remains a major challenge in the intensive care unit despite advances in modern medicine. The pathogenesis of sepsis was partly attributable to dysregulated neuroimmune activation with altered production of neurotransmitters² and inflammatory mediators.³ Immunometabolism is an emerging field that intersects with key metabolic pathways involved in the immune response.⁴ Although a patient with sepsis often displays or exhibits symptoms complicated by encephalopathy and hyperlactatemia,⁵ the molecular mechanism underlying neurotransmitter-mediated regulation of immunometabolism has not yet been established.

Inflammation, the primary response of the innate immune system to damage and invasion, is associated with many neurodegenerative diseases, including Parkinson disease (PD). It has been well established that genetic deletion of the genes encoding PINK1 (PTEN-induced putative kinase 1) and PARK2 (parkin RBR E3 ubiquitin protein ligase) causes progressive mitochondrial damage and development of PD.⁶ Mitochondria play a

central role in energy metabolism and cell death. Although the PINK1-PARK2 pathway plays a major role in the maintenance of mitochondrial quality control by triggering mitophagy (a form of selective autophagy to remove damaged or superfluous mitochondria),^{7,8} its involvement in sepsis-induced immunometabolism dysregulation remain largely unknown.

In the current study, we show that *pink1*^{-/-} and *park2*^{-/-} mice are more sensitive to polymicrobial sepsis than wild-type (WT) mice partly due to impairment of immunometabolism. We demonstrate that the decrease in circulating levels of dopamine (a neurotransmitter released by the brain) in *pink1*^{-/-} and *park2*^{-/-} mice mediates HMGB1 (high mobility group box 1; a late mediator of lethal sepsis) release through activation of the NLRP3 (NLR family, pyrin domain containing 3) inflammasome (a platform for processing proinflammatory cytokines). Moreover, we demonstrate that HIF1A (hypoxia inducible factor 1 α subunit; a master transcriptional regulator of hypoxia)-mediated aerobic glycolysis contributes to NLRP3 inflammasome activation and lethal sepsis in *pink1*^{-/-} and *park2*^{-/-} mice. Genetic or pharmacological inhibition of *Hif1a*- and *Nlrp3*-dependent HMGB1 release confers protection against

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polymicrobial sepsis in *pink1*^{-/-} and *park2*^{-/-} mice. Thus, an impaired PINK1-PARK2 pathway occupies a previously unidentified pathogenic role in critical care medicine and may represent a novel target for patients with sepsis.

Results

pink1^{-/-} and *park2*^{-/-} mice are more sensitive to polymicrobial sepsis due to impaired dopamine production

Experimental sepsis can be induced in mice by cecal ligation and puncture (CLP) because it closely mimics many clinical features of polymicrobial infection observed in human sepsis.⁹ To determine the effect of PINK1 and PARK2 on polymicrobial sepsis, *pink1*^{-/-}, *park2*^{-/-}, and WT mice were subjected to moderate CLP using single punctures with 22-gauge syringe needles. The survival of *pink1*^{-/-} and *park2*^{-/-} mice was significantly lower than that of WT mice within 8 d of CLP (Fig. 1A). Histological analysis revealed more pronounced damage in multiple organs (e.g., liver, lung, kidney, heart, pancreas, brain, and small intestine) of the *pink1*^{-/-} and *park2*^{-/-} mice compared with age- and sex-matched control mice (Fig. 1B). Biochemical measurement of tissue enzymes also exhibited a greater elevation of liver (e.g., GPT/ALT [glutamic pyruvic transaminase, soluble]), heart (e.g., TNNI [troponin I]), kidney (e.g., creatinine), and pancreas (e.g., AMY2 [amylase]) enzymes in the *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 1C). Similarly, serum MPO (myeloperoxidase) and LDH (lactate dehydrogenase) activities were remarkably higher in *pink1*^{-/-} and *park2*^{-/-} mice than in WT mice (Fig. 1C), suggesting that sepsis-induced tissue injury is more significant in the *pink1*^{-/-} and *park2*^{-/-} mice.

Previous studies have suggested that loss of PINK1 or PARK2 results in a mitophagy deficiency-associated decrease of ATP synthesis and an increase of apoptosis in multiple cells, including neurons.^{6,10,11} Consistently, the tissue levels of ATP were significantly reduced in brains and livers (Fig. 1D) and CASP3/caspase-3 activity (an apoptosis marker) were increased in brain tissue neurons (Fig. 1E) from *pink1*^{-/-} and *park2*^{-/-} mice after CLP. The PINK1-PARK2 pathway also plays a role in xenophagy,¹² a selective autophagic pathway that delivers intracellular bacteria for degradation in lysosomes. However, the number of colony-forming units from blood and peritoneal lavage after CLP did not differ between *pink1*^{-/-}, *park2*^{-/-}, and WT mice (Fig. 1F), suggesting that the PINK1-PARK2 pathway might not influence bacterial elimination during experimental sepsis.

Given the essential role of PINK1 and PARK2 in dopaminergic neuronal function, we next compared the levels of dopamine between *pink1*^{-/-}, *park2*^{-/-}, and WT mice. Compared with WT mice, *pink1*^{-/-} and *park2*^{-/-} mice showed a significantly larger reduction in central (brain) and peripheral (serum) dopamine levels after CLP (Fig. 1G). The gene expression of the enzymes (e.g., *Pah* [phenylalanine hydroxylase], *Th* [tyrosine hydroxylase], and *Ddc* [dopa decarboxylase]) in the dopamine biosynthetic pathway did not significantly change in brain tissue neurons from *pink1*^{-/-} and *park2*^{-/-} mice after CLP (Fig. 1H). These findings suggest that decreases in neuron survival (but not in impaired dopamine biosynthesis) contribute to loss of dopamine production in *pink1*^{-/-} and *park2*^{-/-} mice.

To determine whether the reduced dopamine levels contribute to septic death, we supplemented (intraperitoneally) the septic mice with pramipexole (PRA; a Food and Drug Administration-approved dopamine agonist for treating PD patients¹³) at +2, +12, +24, and +48 h after CLP. Remarkably, administration of PRA restored both brain and serum dopamine levels in septic *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 1G) and cocurrently increased their survival rates (Fig. 1A). Consistently, PRA also conferred protection against tissue injury, as determined by tissue histology (Fig. 1B) and enzyme release (Fig. 1C), although it did not affect tissue bacterial loads in septic *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 1F). Thus, these findings suggest that the reduced circulating dopamine levels contribute to the exacerbated septic lethality in animals that are defective in the PINK1 and PARK2 pathways.

HMGB1 is a late mediator of septic death in *pink1*^{-/-} and *park2*^{-/-} mice

The pathogenesis of lethal sepsis is partly attributable to a dysregulated inflammatory response, as manifested by the overproduction of proinflammatory mediators. We next compared the circulating levels of inflammatory mediators between *pink1*^{-/-}, *park2*^{-/-}, and WT mice. Serum levels of early (IL [interleukin] 1B,¹⁴ TNF/TNF- α [tumor necrosis factor],¹⁵ and IL6¹⁶) and late (HMGB1¹⁷ and CIRBP [cold inducible RNA binding protein]¹⁸) mediators in *pink1*^{-/-} and *park2*^{-/-} mice were higher than those in the WT mice over a wide time period (6 to 48 h after CLP, Fig. 2A). However, treatment with PRA only markedly decreased the circulating levels of IL1B and HMGB1 (but not TNF, IL6, and CIRBP) in the septic *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 2A), suggesting that dopamine may specifically modulate the release of inflammasome-dependent cytokines during experimental sepsis.

To confirm whether increased IL1B or HMGB1 contribute to septic death in *pink1*^{-/-} and *park2*^{-/-} mice, we treated these mice with IL1B- or HMGB1-neutralizing antibodies in parallel experiments. Delayed administration of HMGB1- (but not IL1B-) specific neutralizing antibodies at +12, +24, and +48 h after CLP rescued *pink1*^{-/-} and *park2*^{-/-} mice from lethal sepsis (Fig. 2B), and attenuated tissue injury (Fig. 2C) and the release of tissue enzymes (Fig. 2D). Similarly, treatment with IL1B-neutralizing antibodies at +2, +6, and +12 h after CLP partly conferred protection against CLP-induced death in *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 2E). Moreover, delayed administration of glycyrrhizin (a direct HMGB1 inhibitor¹⁹) at +12, +24, and +48 h after CLP also promoted protection against CLP-induced animal lethality (Fig. 2B) and tissue injury (Fig. 2C and 2D) in *pink1*^{-/-} and *park2*^{-/-} mice. These findings suggest that excessive systemic accumulation of inflammasome-dependent cytokines (e.g., IL1B and HMGB1) contributes to exacerbated septic lethality in *pink1*^{-/-} and *park2*^{-/-} mice.

Activation of the NLRP3 inflammasome contributes to septic death in *pink1*^{-/-} and *park2*^{-/-} mice

Inflammasomes, the platforms that detect pathogens and sterile stressors, contribute to the release of proinflammatory mediators

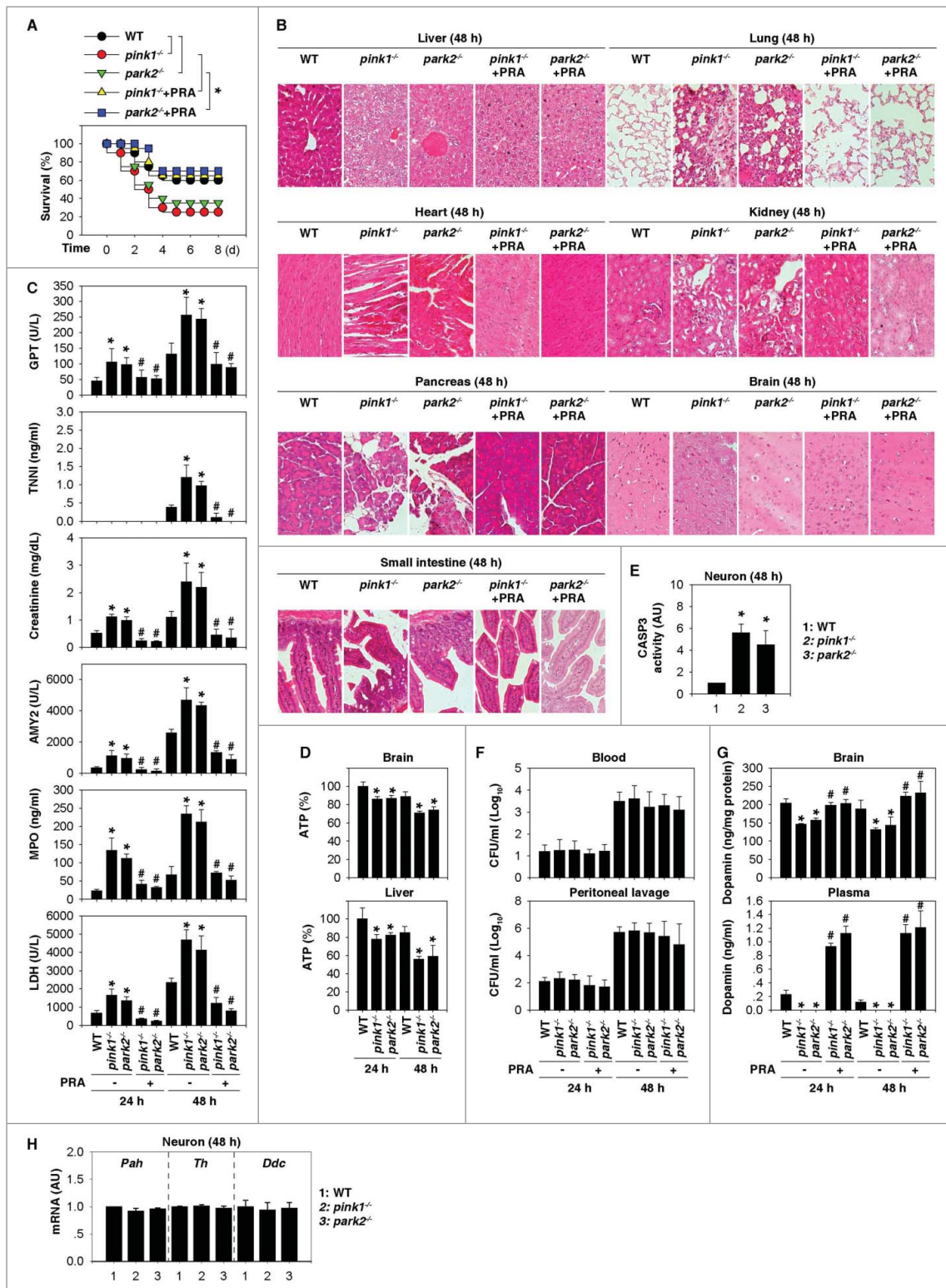
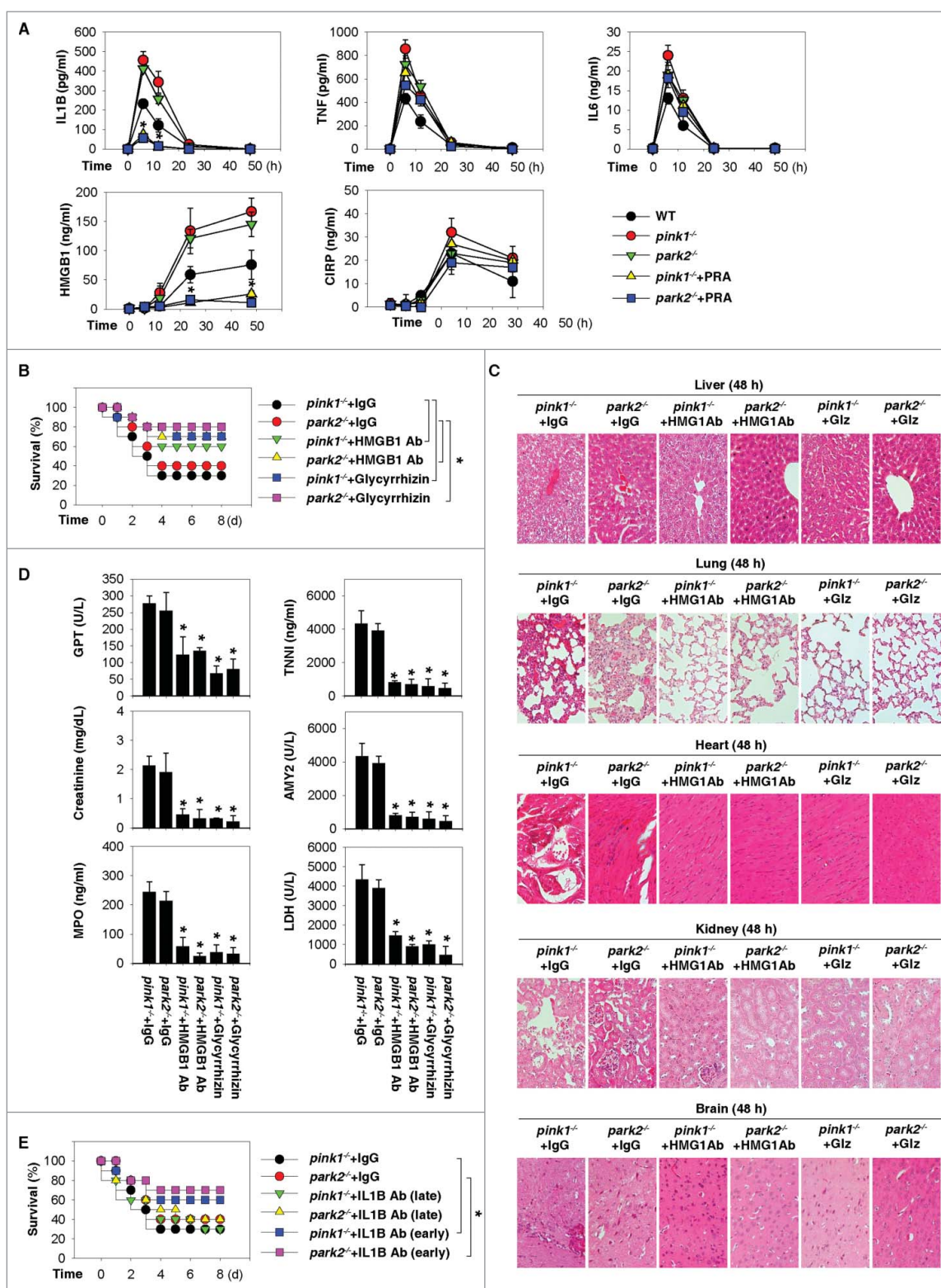


Figure 1. *pink1*^{-/-} and *park2*^{-/-} mice are more sensitive to polymicrobial sepsis due to impaired dopamine production. (A) Survival after moderate CLP in wild-type (WT), *pink1*^{-/-}, and *park2*^{-/-} mice with or without pramipexole (PRA; 1 mg/kg/i.p. at 2, 12, 24, and 48 h after CLP) treatment (n=20 mice/group; *, *P* < 0.05). (B to H) In parallel, tissue H&E staining (B), serum enzyme activity (C), ATP levels (D), CASP3 activity in neurons (E), bacterial loads (F), dopamine concentrations (G), and the indicated gene mRNA in neurons (H) were assayed (n = 3 to 5 mice/group; *, *P* < 0.05 versus WT group; # vs. the group without PRA).

such as IL1B and HMGB1 by activating CASP1/caspase-1 or CASP4/caspase-4 (formerly murine CASP11).^{20,21} Please note that murine CASP11 is no longer the primary acronym in the MGI database and both murine and human acronyms are now

CASP4; care should also be taken to prevent confusion with SCAF11/SFRS2IP, which is involved in pre-mRNA processing and is sometimes also termed CASP11. Dopamine has recently been identified as a negative regulator of NLRP3 inflammasome



activation.²² To assess the contribution of NLRP3 inflammasome activation to the elevated susceptibility of *pink1*^{-/-} and *park2*^{-/-} mice to septic insult, we generated double-knockout mice (*pink1*^{-/-} *nlrp3*^{-/-} and *park2*^{-/-} *nlrp3*^{-/-}). The disruption of *Nlrp3* attenuated the oversensitivity of *pink1*^{-/-} or

park2^{-/-} mice to lethal sepsis, as manifested by increased animal survival (Fig. 3A) and decreased tissue injury (Fig. 3B and 3C). Serum concentrations of IL1B and HMGB1 (but not IL6, TNF, and CIRBP) were also significantly reduced in these double-knockout mice compared with respective *pink1*^{-/-} or

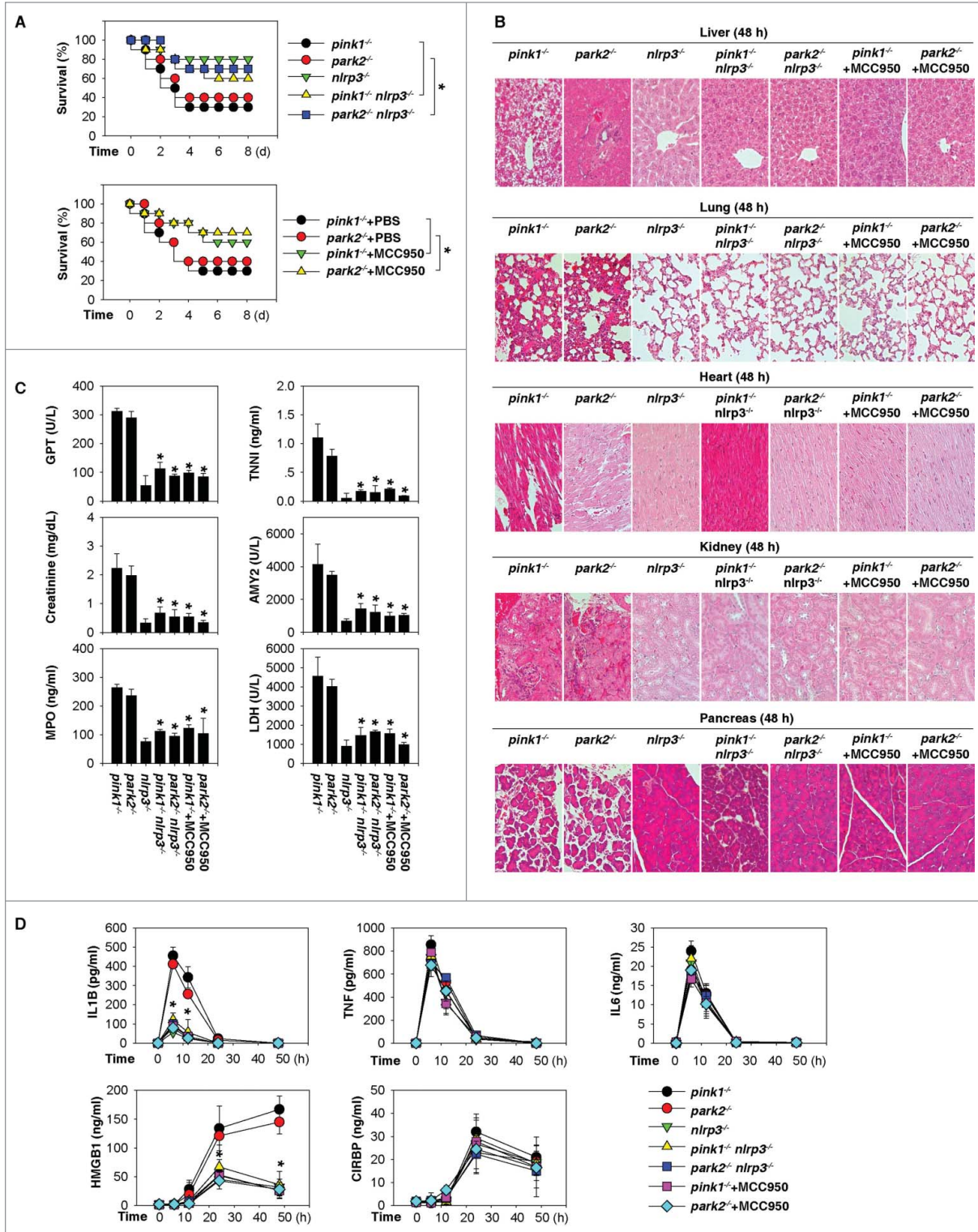


Figure 3. Activation of the NLRP3 inflammasome contributes to septic death in *pink1*^{-/-} and *park2*^{-/-} mice. (A) Survival after moderate CLP in the indicated knockout mice with or without MCC950 (20 mg/kg/i.p. at +2, +12, +24, and +48 h after CLP) or same volume phosphate-buffered saline treatment (n = 10 mice/group; *, P < 0.05). (B to D) In parallel, tissue H&E staining (B), serum enzyme activity (C), and serum proinflammatory mediators (D) were assayed (n = 3 to 5 mice/group; *, P < 0.05 versus *pink1*^{-/-} or *park2*^{-/-} group).

park2^{-/-} mice following CLP (Fig. 3D), suggesting that the sepsis-induced excessive accumulation of IL1B and HMGB1 in *pink1*^{-/-} and *park2*^{-/-} mice is regulated by NLRP3 inflammatory pathways.

To further test this possibility, we next determined whether pharmacological inhibition of NLRP3 inflammasomes by MCC950²³ affects the septic outcome in *pink1*^{-/-} and *park2*^{-/-} mice. As with knockout of *Nlrp3* in mice, administration of MCC950 at +2, +12, +24, and +48 h after CLP also increased animal survival and decreased tissue injury, as confirmed by histology (Fig. 3B) and enzyme assay (Fig. 3C) in *pink1*^{-/-} and *park2*^{-/-} mice. Consistently, MCC950 also reduced the serum levels of IL1B and HMGB1 (but not IL6, TNF, and CIRBP) in septic *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 3D). Thus, activation of the NLRP3 inflammasome contributes to septic death in *pink1*^{-/-} and *park2*^{-/-} mice by regulating IL1B and HMGB1 release.

HIF1A-mediated aerobic glycolysis contributes to inflammasome activation in *pink1*^{-/-} and *park2*^{-/-} mice

Others and we have demonstrated that aerobic glycolysis contributes to sepsis through promoting the release of proinflammatory mediators, including IL1B and HMGB1.^{24,25} We next analyzed the levels of circulating lactate (the end product of aerobic glycolysis) in *pink1*^{-/-} and *park2*^{-/-} mice. Compared with WT mice, the *pink1*^{-/-} and *park2*^{-/-} mice were slightly hyperlactatemic at baseline, and this phenotype was significantly enhanced following CLP surgery (Fig. 4A). The administration of a dopamine agonist, PRA, led to a reduction of circulating lactate levels in septic *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 4A), suggesting that dopamine may participate in the regulation of aerobic glycolysis and lactate production.

To test this possibility, we examined the impact of dopamine inhibition on the expression of HIF1A, a transcriptional activator responsible for regulating cellular and systemic aerobic glycolysis during inflammation.²⁶ Administration of PRA suppressed the sepsis-induced upregulation of *Hif1a* mRNA expression, as well as that of its target genes (e.g., *Ldha* and *Pdk1*) in the brain, skeletal muscle, and peritoneal macrophages from *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 4B). Reactive oxygen species have been implicated as important signaling molecules to enhance *Hif1a* mRNA expression and activity,²⁷ as well as inflammasome activation.²⁸ PRA displays antioxidant activity in neuroprotection through increased glutathione (GSH) production.²⁹ Similarly, administration of PRA suppressed the sepsis-induced GSH depletion in the brain from *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 4C), suggesting that dopamine inhibits HIF1A signaling through regulation of GSH production.

To confirm whether HIF1A-mediated aerobic glycolysis contributes to lethal sepsis in *pink1*^{-/-} and *park2*^{-/-} mice, we generated double-knockout mice (*pink1*^{-/-} *hif1a*^{-/-} and *park2*^{-/-} *hif1a*^{-/-}). The disruption of *Hif1a* attenuated the oversensitivity of *pink1*^{-/-} or *park2*^{-/-} mice to lethal sepsis, as manifested by increased animal survival (Fig. 4D) and decreased tissue injury judged by histological improvement (Fig. 4E) and reduction of tissue enzyme release (Fig. 4F). Importantly, serum levels of lactate, IL1B, and HMGB1 were significantly decreased in *pink1*^{-/-} *hif1a*^{-/-} and *park2*^{-/-}

hif1a^{-/-} mice after CLP as compared to respective *pink1*^{-/-} and *park2*^{-/-} mice (Fig. 4G). These data suggest that HIF1A is required for the enhanced glycolytic phenotype and inflammatory activation caused by the loss of *pink1* or *park2*.

Gene changes in the PINK1- and PARK2-dependent neuroimmune pathways in human sepsis

Although the murine CLP paradigm has some features that warrant its recommendation, this model of severe sepsis has some flaws and cannot fully mimic human sepsis.⁹ We next determined whether the PINK1-PARK2-HIF1A-NLRP3 pathway changes in peripheral blood mononuclear cells (PBMCs) of patients with sepsis. Compared with a healthy control group, the mRNA levels of *HIF1A* (Fig. 5A) and *NLRP3* (Fig. 5B) in PBMCs were increased in the sepsis group. In contrast, the mRNA levels of *PINK1* (Fig. 5C) and *PARK2* (Fig. 5D) in PBMCs were decreased in the sepsis group compared with the healthy control group (Table S1). These findings indicate a potential pathogenic role of the PINK1-PARK2-HIF1A-NLRP3 pathway in human sepsis.

Discussion

Increasing evidence has supported the important role of the nervous system and neurotransmitters in the regulation of the systemic inflammatory response to infection and injury.³⁰ Here, we demonstrate that disruption of the PINK1-PARK2 pathway exacerbates polymicrobial sepsis partly through impairing the release of the neurotransmitter dopamine. Conversely, dopamine limits the systemic inflammatory response through interfering with the immunometabolism pathway, including HIF1A-dependent anaerobic glycolysis and lactate production, which contributes to NLRP3-dependent inflammasome activation and subsequent HMGB1 release (Fig. 5E). Thus, neuroimmune signaling pathways could represent novel therapeutic targets for the treatment of sepsis and other lethal systemic inflammatory diseases.

As key regulators of the mitochondrial quality control pathway, PINK1 and PARK2 are critically involved in the regulation of mitochondrial dynamics in the brain and other organs. Genetic mutations of the *PINK1* and *PARK2* genes in humans are associated with early-onset of PD, a neurodegenerative disease characterized by the loss of dopaminergic neurons.⁶ In mice, *pink1* deficiency worsens aging-associated lung fibrosis, possibly through accelerating mitochondrial dysfunction.³¹ Given the high prevalence of PD and sepsis in elderly patients,³² as well as the currently observed exacerbation of septic lethality in *pink1*- and *park2*-deficient mice, it may be important to investigate whether PD patients also exhibit worse sepsis outcomes.

Current medical practice guidelines recommend the use of dopamine as a first-line drug to treat hypotension (low blood pressure), low cardiac output, and reduced perfusion of body organs due to shock, trauma, and sepsis.³³ However, dopamine therapy suffers from a relatively short half-life (<2 min in serum) and adverse side effects, particularly for patients with septic shock.³⁴ In contrast, dopamine

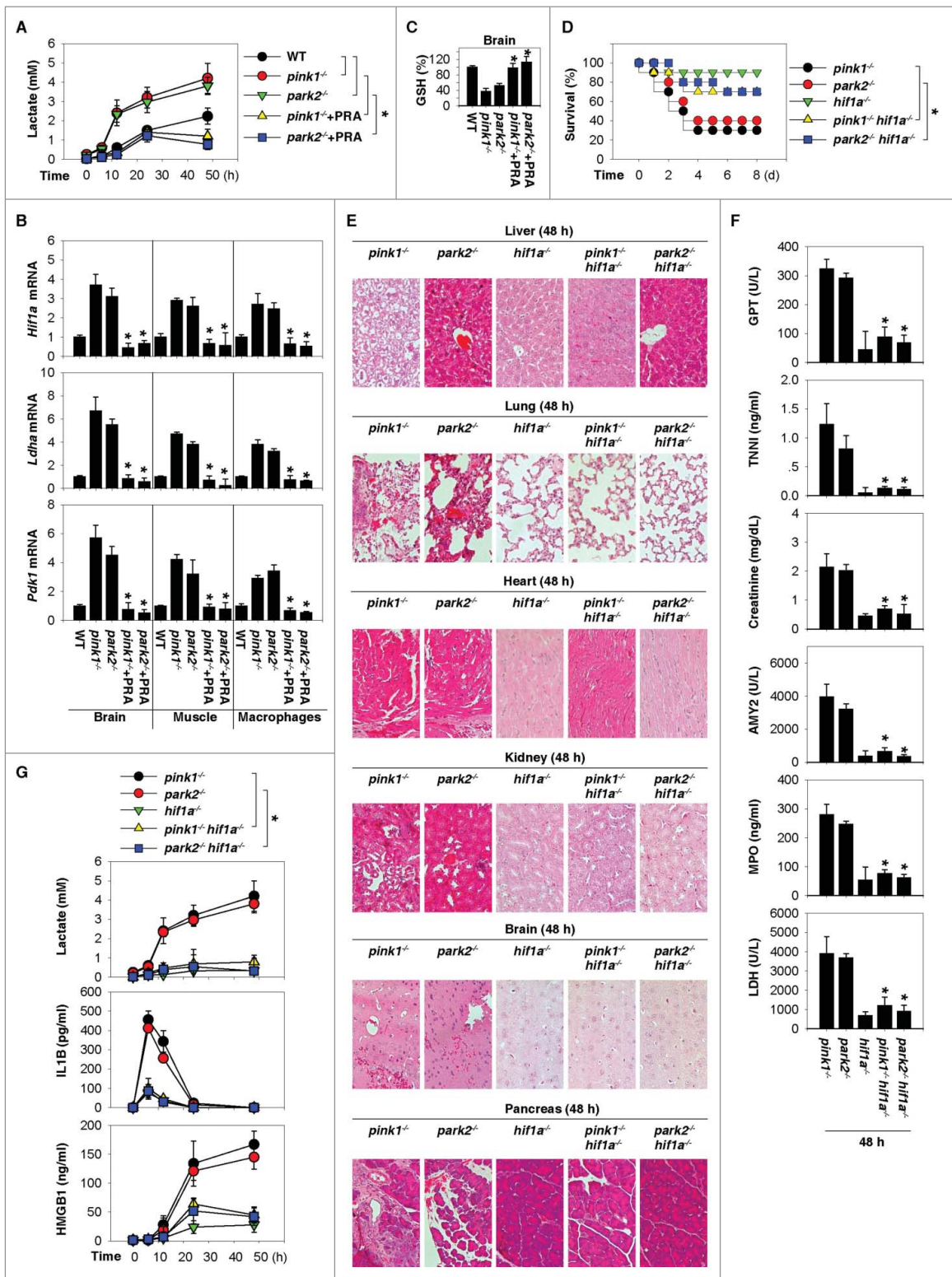


Figure 4. HIF1A-mediated aerobic glycolysis contributes to inflammasome activation in *pink1*^{-/-} and *park2*^{-/-} mice. (A, B) Time course of serum lactate levels (A) and the indicated gene expression at 48 h (B) in CLP-induced wild-type (WT), *pink1*^{-/-}, and *park2*^{-/-} mice with or without pramipexole (PRA; 1 mg/kg/i.p. at 2, 12, 24, and 48 h after CLP) treatment (n = 3 to 5 mice/group; *, P < 0.05 vs. the group without PRA). (C) In parallel, brain GSH levels at 48 h after CLP were assayed (n = 3 mice/group; *, P < 0.05 versus the group without PRA). (D) Survival after moderate CLP in the indicated knockout mice (n = 10 mice/group; *, P < 0.05). (E to G) In parallel, tissue H&E staining (E), serum enzyme activity (F), serum lactate (G), IL1B (G), and HMGB1 (G) were assayed (n = 3-5 mice/group; *, P < 0.05 vs. *pink1*^{-/-} or *park2*^{-/-} group).

agonists have much longer half-lives (usually 6 to 12 h in serum),¹³ but have not yet been tested for efficacy in experimental sepsis. In this study, we demonstrated that PRA, a dopamine agonist widely used to treat PD, confers

protection against lethal sepsis in *pink1*^{-/-} and *park2*^{-/-} mice. These findings support the potential use of dopamine agonist in the treatment of sepsis and other lethal systemic inflammatory diseases.

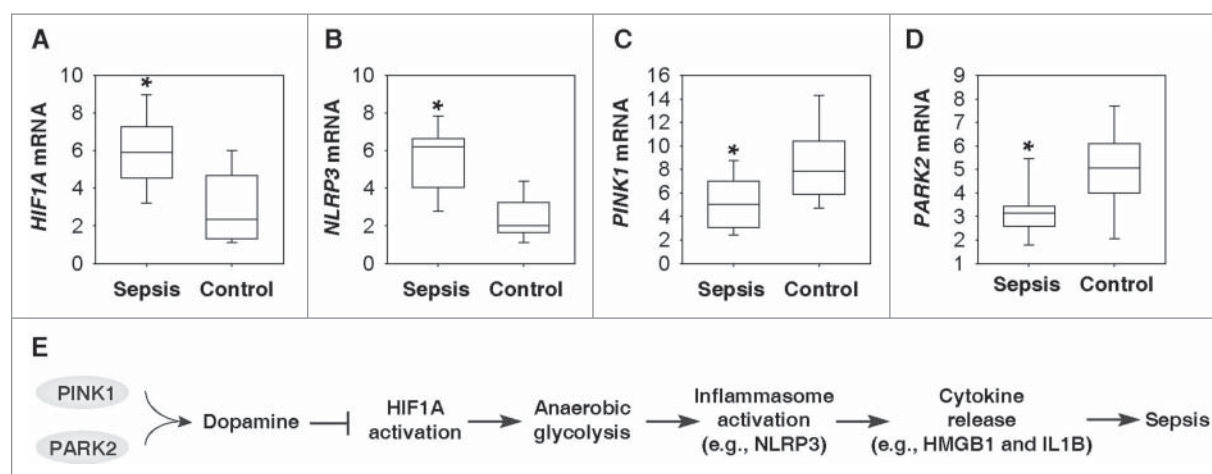


Figure 5. Gene changes in the PINK1- and PARK2-dependent neuroimmune pathways in human sepsis. (A to D) Box plots comparing measures of *HIF1A* (A), *NLRP3* (B), *PINK1* (C), and *PARK2* (D) mRNA levels in PBMC samples of sepsis patients ($n = 10$) and healthy controls ($n = 10$). The mRNA levels are presented as median value (black line), interquartile range (box), and minimum and maximum of all data (black line). *, $P < 0.05$ versus control group. (E) Schematic depicting PINK1- and PARK2-dependent protective neuroimmune pathways in lethal sepsis.

Dopamine does not cross the intact blood brain barrier and additional dopaminergic neurons, such as those in the adrenal medulla and mesentery, exist in the peripheral nervous system.³⁵ In addition to its central role in the regulation of motor function, peripheral dopamine may also serve as an endogenous regulator of immune functions. Consistent with a recent report,²² we found that the dopamine agonist PRA inhibited NLRP3 inflammasome activation. Although PRA can activate D2-like receptors with low affinity for the 5-hydroxytryptamine and $\alpha 2$ -adrenergic receptors, the functional contribution of dopamine D1-like receptors (DRD1 and DRD5) or D2-like receptors (DRD2, DRD3, and DRD4) to PRA-induced NLRP3 inflammasome inhibition remains unknown. Nevertheless, the essential role of NLRP3 inflammasome activation in worsening the septic lethality of *pink1*^{-/-} or *park2*^{-/-} mice has been supported by the results from the current study.

HMGB1 is actively secreted by activated innate immune cells during infection and passively released by necrotic cells during injury.³⁶ We observed that the release of HMGB1 is significantly increased in septic *pink1*^{-/-} or *park2*^{-/-} mice compared with septic WT controls. The regulatory role of the neuroimmune network in the regulation of HMGB1 release has been supported by our findings that pharmacological dopamine receptor activation (by PRA), NLRP3 inflammasome inactivation (by MCC950), or genetic *Nlrp3* deletion concurrently attenuated lethal sepsis and systemic HMGB1 accumulation in *pink1*^{-/-} or *park2*^{-/-} mice. Importantly, delayed administration of HMGB1-specific antibody or inhibitor (e.g., glycyrrhizin¹⁹) protects against animal death in CLP-induced *pink1*^{-/-} or *park2*^{-/-} mice, confirming HMGB1 as a late mediator of lethal systemic inflammation.³⁷

Elevated lactate production, hyperlactatemia, often occurs in patients with severe sepsis or septic shock partly due to anaerobic glycolysis.³⁸ The anaerobic glycolysis is regulated by a major transcription factor, HIF1A, which is upregulated during sepsis at both the mRNA and protein levels. We found that absence of dopamine in *pink1*^{-/-} or *park2*^{-/-} mice resulted in HIF1A-mediated aerobic glycolysis induction, although the

mechanisms remain unknown. In contrast, PRA impaired sepsis-induced upregulation of HIF1A and several glycolysis-associated genes in the brain, skeletal muscle, and peritoneal macrophages of *pink1*^{-/-} or *park2*^{-/-} mice, suggesting that neurotransmitters participate in the counter-regulation of systemic HIF1A expression during sepsis.

It has been shown that *Hif1a* deficiency renders mice resistant to lethal endotoxemia³⁹ and knockdown of *Hif1a* by RNAi inhibits glucose metabolism in fibroblasts from *pink1*^{-/-} mice.⁴⁰ Here, we provide evidence that HIF1A-mediated aerobic glycolysis may contribute to sepsis-induced NLRP3 inflammasome activation and HMGB1 release in *pink1*^{-/-} or *park2*^{-/-} mice. This observation was consistent with our previous findings that inhibition of PKM2 (pyruvate kinase, muscle), a transcriptional target and transcriptional coactivator of HIF1A, concomitantly reduced aerobic glycolysis, HMGB1 release, and septic lethality.^{24,41} Given the essential role of the PKM2-HIF1A metabolic pathway in controlling IL1B production by activated macrophages,⁴² it is possible that the HIF1A-dependent immunometabolic dysfunction might be a hallmark of sepsis.

In summary, understanding the complex pathogenesis of sepsis, a complex systemic response to injury and infection, may be an important first step in improving outcomes. Our study has revealed a novel PINK1- and PARK2-dependent neuroimmune pathway that regulates peripheral inflammation through controlling dopamine release, HIF1A and NLRP3 inflammasome activation, and HMGB1 secretion in a mouse model. This neuroimmune pathway orchestrates metabolic and inflammatory responses, leading to hyperlactatemia, tissue injury, and even organ dysfunction. Human monocytes from patients during sepsis display increased HIF1A and target gene expression, which cause immune suppression.⁴³ Persistent elevation of HMGB1 in patients with severe sepsis and septic shock may prolong inflammation and organ injury.⁴⁴ In addition, uncoupling protein-2 (the positive regulator of NLRP3 inflammasome activation) is significantly increased in myeloid cells of patients with sepsis.⁴⁵ We observed that the mRNA

expression of *HIF1A* and *NLRP3* is upregulated, whereas the mRNA expression of *PINK1* and *PARK2* is downregulated in PBMCs of patients with sepsis. Thus, the *PINK1-PARK2-HIF1A-NLRP3-HMGB1* pathway might represent a novel therapeutic target for future clinical management of sepsis and other lethal inflammatory diseases.

Materials and methods

Reagents

We purchased PRA (Selleck Chemicals, S2011), glycyrrhizin (Sigma, CDS020796), MCC950 (AdipoGen, AG-CR1-3615), and monoclonal IL1B/IL-1 β -neutralizing antibody (Clone B122; BioLegend, 503501) from the indicated manufacturers. Monoclonal HMGB1-neutralizing antibody (Clone 2G7) was generated as reported previously.⁴⁶

Mice

pink1^{-/-}, *park2*^{-/-}, *nlrp3*^{-/-}, and *hif1a*^{-/-} mice were all in the C57BL/6 background and purchased from Jackson Laboratories. These mice were crossed to generate the indicated double-knockout animals. All mice were housed on a 12-h light-dark cycle with controlled temperature (21°C to 23°C) and provided with standard rodent diet and water *ad libitum* throughout all experiments. Isolation and culture of adult mouse neurons were performed as previously described.⁴⁷ We conducted all animal care and experimentation in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines (<http://www.aaalac.org>) and with approval from the Institutional Animal Care and Use Committees from the University of Pittsburgh and Third Military Medical University.

CLP procedure

Sepsis was induced in male or female C57BL/6 mice (8- to 10-wk-old, 22 to 26 g weight) by CLP as previously described.⁴⁸ Briefly, anesthesia was induced with 5% halothane and maintained with 2% halothane. A small midline abdominal incision was made and the cecum was exteriorized and ligated with 4-0 silk immediately distal to the ileocecal valve without causing intestinal obstruction. The cecum was then punctured once with a 22-gauge needle. The abdomen was closed in 2 layers and the mice were injected subcutaneously with 1 ml Ringer solution including analgesia (0.05 mg/kg buprenorphine). After CLP, mice did not receive antibiotics. The mortality of WT mice typically was approximately 40% by d 7 post-CLP.

ELISA analysis

The GPT/ALT (Bioo Scientific Corporation, 3460-01), TNNI/troponin-I (Life Diagnostics, CTNI-1-US), creatinine (Bioo Scientific Corporation, 5606-01), AMY2/amylase (Abcam, ab102523), MPO (Abcam, ab155458), ATP (PerkinElmer, 6016736), LDH (Abcam, ab102526), dopamine (NOVUS,

KA1887), IL1B/IL-1 β (BioLegend, 433404), TNF/TNF α (BioLegend, 430907), IL6/IL-6 (BioLegend, 431307), HMGB1 (Shino-Test Corporation, 326054329), CIRBP/CIRP (CUSA-BIO, P60824), and lactate (Abcam, ab65331) concentrations in serum and/or supernatant fractions from the indicated tissue homogenate were measured using ELISA according to the manufacturer's protocol.

CASP3 activity assay

The activity of CASP3 in cell lysates was assayed by the CASP3 Activity Assay Kit (Cell Signaling Technology, 5723) according to the manufacturer's protocol. It contained a fluorogenic substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin or Ac-DEVDAMC) for CASP3. During the assay, activated CASP3 cleaved this substrate between DEVD and AMC, generating highly fluorescent AMC that can be detected using a fluorescence reader with excitation at 380 nm and emission between 420-460 nm.

GSH assay

The relative GSH concentration in tissue lysates was assessed using a Glutathione Assay Kit (Sigma, CS0260) according to the manufacturer's instructions.^{49,50} The measurement of GSH used a kinetic assay in which catalytic amounts (nmoles) of GSH caused a continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid and the GSSG formed was recycled by glutathione reductase and NADPH. The reaction rate was proportional to the concentration of glutathione up to 2 mM. The yellow product (5-thio-2-nitrobenzoic acid) was measured spectrophotometrically at 412 nm.

Measurement of bacterial counts

The peritoneal cavity was washed with 1 ml phosphate-buffered saline (Sigma, P3813) and the peritoneal lavage was collected under sterile conditions. Samples of blood and peritoneal lavage fluid were serially 10-fold diluted in sterile saline and cultured on tryptic soy agar pour plates (BD, BA-256665.02). Plates were incubated (37°C) for 24 to 48 h and colony counts were performed as described previously.⁵¹

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRI reagent (Sigma, T9424) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μ g of RNA using the iScript cDNA Synthesis kit (Bio-Rad, 1708890). Briefly, 20 μ l reactions were prepared by combining 4 μ l iScript Select reaction mix, 2 μ l gene-specific enhancer solution, 1 μ l reverse transcriptase, 1 μ l gene-specific assay pool (20 \times , 2 μ M), 12 μ l RNA diluted in RNase-free water. cDNA from various cell samples were then amplified by real-time quantitative PCR with specific primers (mouse *Hif1a*: 5'-ACCTTCATCGGAAACTCCAAAG-3' and 5'-CTGTTAGGCTGGGAAAAGTTAGG-3'; mouse *Ldha*: 5'-GCTCCCCAGAACAAGATTACAG-3' and 5'-TCGCCCT

TGAGTTTGTCTTC-3'; mouse *Pdk1*: 5'-CCACTGAGGAAGA TCGACAGAC-3' and 5'-AGAGGCGTGATATGGGCAAT CC-3'; mouse *Pah*: 5'-GCTGGACAGATTCCGCAATCAG-3' and 5'-CAGCAAAGTCTTTCGTCCTCGC-3'; mouse *Th*: 5'-T GCACACAGTACATCCGTCATGC-3' and 5'- GCAAATGT GCGTTCAGCCAACA-3'; mouse *Ddc*: 5'-GGAGCCAGAAAC ATACGAGGAC-3' and 5'- GCATGTCTGCAAGCATAG CTGG-3'; human *HIF1A*: 5'-TATGAGCCAGAAGAACTTTT AGGC-3' and 5'-CACCTCTTTTGGCAAGCATCCTG-3'; human *NLRP3*: 5'- GGACTGAAGCACCTGTTGTGCA-3' and 5'- TCCTGAGTCTCCCAAGGCATTC-3'; human *PINK1*: 5'- GTGGACCATCTGGTTCAACAGG-3' and 5'- GCAGCCAA AATCTGCGATCACC-3'; human *PARK2*: 5'- CCAGAG- GAAAGTCACCTGCGAA-3' and 5'- CTGAGGCTTCAAA- TACGGCACTG-3') and the data were normalized to mouse *Rn18s* rRNA (5'-CTTAGAGGGACAAGTGGCG-3' and 5'- ACGTGTGAGCCAGTCAAGTGTGTA-3') or human *GADPH* RNA (5'-GTCTCCTCTGACTTCAACAGCG-3' and 5'-ACCACCC TGTTGCTGTAGCCAA-3') using an CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, 1855195).

Patient samples

PBMCs from patients with sepsis and healthy controls were collected from Xiangya Hospital, Central South University. Collection of the samples was approved by Xiangya Hospital's Institutional Review Board. Sepsis was identified according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference guidelines.⁵²

Statistical analysis

Data are expressed as means ± SEM of 3 independent experiments. All data were analyzed using SigmaPlot11.0 software. One-way ANOVA was used for comparison among the different groups. When ANOVA was significant, post hoc testing of differences between groups was performed using a least significant difference test. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. A *P* value < 0.05 was considered statistically significant.

Abbreviations

AMY2	amylase
CIRBP	cold inducible RNA binding protein
CLP	cecal ligation and puncture
<i>Ddc</i>	dopa decarboxylase
DRD	dopamine receptor D1
GPT/ALT	glutamic pyruvic transaminase, soluble
GSH	glutathione
HIF1A	hypoxia inducible factor 1, α subunit
HMGB1	high mobility group box 1
IL	interleukin; LDH, lactate dehydrogenase
MPO	myeloperoxidase
NLRP3	NLR family, pyrin domain containing 3
<i>Pah</i>	phenylalanine hydroxylase
PARK2	parkin RBR E3 ubiquitin protein ligase
PBMC	peripheral blood mononuclear cell

PD	Parkinson disease
PINK1	PTEN induced putative kinase 1
PKM2	pyruvate kinase, muscle
PRA	pramipexole
<i>Th</i>	tyrosine hydroxylase
TNF/TNF- α	tumor necrosis factor
TNNI	troponin I
WT	wild type

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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