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Activation of AMPK to Diminish Sepsis-induced Lung Injury

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by

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ABSTRACT

Sepsis is the most frequent cause of death of hospitalized patients in modern ICUs. Severe infection, trauma, hemorrhage, burns, and surgery are significant causes of multi-organ injury and immune dysfunction that in turn primes for a high risk of secondary lung infections. In addition to detrimental inflammation, sepsis is linked to loss of metabolic plasticity due to mitochondrial dysfunction in immune cells and lung tissue. In particular, mitochondrial failure in lungs of critically ill septic patients is correlated with high mortality rates. We proposed that AMP-activated protein kinase (AMPK) activation, a major bioenergetic sensor and metabolic regulator, is a plausible target to diminish exaggerated immune pro-inflammatory activation, and also promote recovery of bioenergetic homeostasis, thus reducing severity of sepsis and acute lung injury (ALI). This possibility was explored in three subsequent studies that address (1) the effects of D1dopaminergic signaling on AMPK activity and endotoxin-induced ALI; (2) mechanistic insights into AMPK inactivation in sepsis and ALI, and use of new therapeutic interventions to recover AMPK activity; and (3) the impact of AMPK-autophagy signaling on immune cell regulation during ALI and microbial clearance. Our *first* studies revealed that the dopamine-D1R axis is linked to AMPK activation which resulted in reduced severity of ALI. We found in our *second* set of studies that severe sepsis caused phosphorylation and inhibition of AMPK by IKKβ and GSK3β. Notably, inhibition of GSK3β de-

creased inhibitory T479-AMPKα phosphorylation while T172-AMPKα activatory phosphorylation was increased. This event was associated with preservation of mitochondrial ETC components, improved bacterial clearance, and diminished sepsis related lung injury. In our *third* set of studies, we found that the autophagy-related protein Parkin is diminished in LPS treated immune cells and lungs of mice subjected to endotoxin-induced ALI. Importantly, AMPK activation was able to activate autophagy through a beclin-1 associated pathway, even in Parkin deficient mice. This resulted in diminished severity of ALI. Taken together, these studies provide substantial progress in understanding AMPK-bioenergetic maintenance of cellular homeostasis. Besides mechanistic insights, our studies indicate that AMPK activators may possibly be applied as therapies for sepsis and ARDS.

Keywords: ARDS, inflammation, bioenergetics, mitochondria, autophagy, Parkin.

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INTRODUCTION

Sepsis is a medical condition which has been around for millennia. In the time of Hippocrates it was described as “by which flesh rots, swamps generate foul airs, and wounds fester”[1]. The most recent Consensus Conference defined sepsis as "a life threatening organ dysfunction caused by a dysregulated host immune response to infection"[2]. Despite significant progress in understanding sepsis, this syndrome is the leading cause of death in hospitalized patients. The United States alone sees upwards of one million new cases and 200,000 deaths related to sepsis each year. Current treatments for this detrimental condition are limited, mostly involving supportive care; antibiotics, oxygen delivery, and fluid resuscitation. Clinical trials that attempt to target selective indices of sepsis (*e.g.* inflammation, immune function) have been largely unsuccessful, therefore developing new effective therapeutic approaches is needed[3].

Sepsis encompasses a continuum of aberrations in organ function[4]. Microbial infection is the most common cause of sepsis, although sepsis may also arise from noninfectious/sterile origins, including inflammatory organ disease, ischemia/reperfusion injury, and trauma and hemorrhage[5]. Sepsis is non discriminating in terms of age, race, geographic location, or health status of individuals who develops this life threatening condition. However, individuals who are very young or very old, individuals with compromised immune systems, those with preexisting injuries or wounds (both sterile and non-

sterile), and individuals with invasive devices have an increased risk of developing sepsis[1, 6-10].

Sepsis Definition and Diagnosis

Sepsis has a significant social and economic burden with an estimated $20 billion spent by the US healthcare system and is reportedly increasing[11-13]. Diagnosis of severe sepsis and shock in patients is difficult due to multiple factors that overlap with other conditions[14, 15]. Identification of the infection site and microbial pathogen are also time consuming. Another important issue is the lack of effective therapeutic interventions for severe sepsis and shock at the time of hospital admissions. Besides early antibiotic administration, vasopressors are used to maintain mean arterial pressure above 65 mmHg. Many patients require additional support, including fluid resuscitation and oxygen delivery, in particular when sepsis is associated with ARDS[2].

In 1992, American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee introduced the term "sepsis" and defined sepsis as "*a systemic inflammatory response to infections.*" Severe sepsis was then characterized by *sepsis associated with acute organ dysfunction,* whereas septic shock is defined by "*sepsis-induced hypotension, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction*"[16]. The Surviving Sepsis Campaign has implemented specific guidelines and provides updates for sepsis diagnosis that are solicited among the international community (Tables 1 and 2). In 2014, a task force comprised of 19 critical care, pulmonary, surgical, and infectious disease specialists

updated the definition and diagnosis of sepsis. Currently, sepsis is defined as "*life-threatening organ dysfunction caused by a dysregulated host response to infection*" with clinical diagnosis including an increase in Sequential Organ Failure Assessment (SOFA) score of 2 or more points (Table 2). Septic shock is recognized as "*a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than sepsis alon*e"[2]. In regards to metabolic abnormalities, increased levels of lactate in circulation is an established predictor of mortality among septic patients. However, it is important to note that recent clinical trials have shown that decreased lactate production had negligible or even an adverse impact on survival from sepsis[17, 18]. Clinical studies also suggest that lactate clearance may not be useful as a surrogate marker of microcirculatory blood flow[19].

Sepsis	*must exhibit at least two of the following*
	• Body temperature above 101°F (38.3°C) or below 96.8°F (36°C)
	• Heart rate higher than 90 beats a minute
	• Respiratory rate of more than 20 breaths per minute
	• Probable or confirmed infection
Severe Sepsis	*upgraded from sepsis if exhibiting indications of organ dysfunction*
	• Urine output significantly decreased
	• Abrupt change in mental status
	• Decrease in platelet count
	• Breathing difficulties
	• Abnormal heart pumping function
	• Abdominal pain
Septic Shock	*signs and symptoms of other categories*
	• Cytokine storm
	• Extremely low blood pressure
	• Nonresponsive to simple fluid replacement

Table 1. *Diagnostic criteria for sepsis subcategories*

		Score				
System		**0**	**1**	**2**	**3**	**4**
Respiratory	PaO_2/FiO_2 (mmHg)	>400	<400	<300	<200 with respiratory support	<100 with respiratory support
Cardiovascular	Blood pressure (mmHg) + Vasopressor (μg/kg/min)	MAP ≥70	MAP <70	MAP <70 dopamine <5 or dobutamine	MAP <70 dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1	MAP <70 dopamine 15 or epinephrine >0.1 or norepinephrine >0.1
Haematolgy	Platelets ($x10^3/\mu L$)	≥150	<150	<100	<50	<20
Gastrointestinal	Bilirubin (μmol/L)	<20	20-32	33-101	102-204	>204
Neurological	Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Renal	Creatinine (μmol/L) or urine output (mL/d)	<110	110-170	171-299	300-440 <500	>440 <200

***Table 2**. Sequential (Sepsis-Related) Organ Failure Assessment Score*[2]

Sepsis and Catecholamines

Catecholamines are naturally produced molecules which act as neurotransmitters in the nervous system and hormones in other organ systems. Catecholamines are released into circulation during the early phase of sepsis which is associated with regulation of the inflammatory response[20]. However, in established sepsis, maintaining blood pressure frequently requires catecholamine administration, in particular norepinephrine is the first-line vasopressor. It has been shown that norepinephrine increases mean arterial pressure (MAP) through vasoconstriction of blood vessels with minimal effect on heart rate, stroke volume, and cardiac output. Epinephrine is an additional intervention in cases of limited effect of norepinephrine to maintain MAP. Use of norepinephrine or epinephrine is relatively safe, however, epinephrine may increase lactate concentrations in circulation[21-23]. Dopamine is a precursor of norepinephrine and epinephrine. Dopamine is less frequently used to maintain MAP in septic shock, and is not recommended as a norepinephrine alternative with exception in patients with bradycardia and compromised systolic function. Adverse implications of dopamine administration are related to an increase in both heart rate and stroke volume and thus risk of tachyarrhythmias. Unlike norepinephrine, dopamine has been shown to increase the risk of death in patients[22-24]. It is important

to note that dopamine has multiple receptors and a variety of signaling that leads to beneficial or adverse effects in sepsis. Dopamine and norepinephrine both have anti-inflammatory effects, although activators of the β2 adrenergic axis had no effects in ARDS patients[25-29]. In contrast, use of D1 receptor agonists show significant protection in a murine model of sepsis[26]. It is possible that D1receptor agonists may provide protection beyond the effects on inflammation, perhaps by modulating metabolic and bioenergetic homeostasis, which we examined in our studies[30].

Sepsis and Immune Dysfunction

During sepsis, dysfunction occurs in multiple systems, including the immune responses. The innate immune system is responsible for immediate recognition and response to pathogens and other antigens. This occurs through identification of highly conserved patterns called pathogen-associated molecular patterns (PAMPs), such as LPS, peptidoglycan, flagellin, viral RNA, and glycolipids[31-36]. PAMPs are recognized by specific pattern recognition receptors (PRRs) which includes toll-like receptors (TLRs), retinoic acid inducible gene-1-like (RIG-1) receptors, nucleotide-binding oligomerization domain-like (NOD) receptors, and C-type lectin receptors[37]. In addition to PAMPs, PRRs also recognize damage-associated molecular patterns (DAMPs) which are molecules produced by the host in response to cellular damage, such as high-mobility group box 1 (HMGB1), extracellular DNA, and histones[38]. PRR activation results in series of intracellular signaling cascades leading to upregulation of pro-inflammatory cytokines, chemotactic signals, reactive oxygen species (ROS) intermediates, and iNOS,

the outcome of which is enhanced local immune cell function and recruitment of circulating innate immune cells to the site of infection or injury[39-41].

Dysfunction occurs in many immune cell types during sepsis. For example, neutrophils typically undergo apoptosis within 24 hours following their release from the bone marrow, however this event is delayed during sepsis[42]. These circulating neutrophils also exhibit impaired chemotactic abilities, bacterial clearance, and reduced ROS production[43, 44]. Reports also indicate impaired neutrophil function prior to acquiring nosocomial infections[45]. During sepsis, monocytes have a reduced capacity to release pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1α, IL-6, and IL-12, following exposure to bacterial compounds and TLR stimulation[46, 47]. LPS still has the ability to activate monocytes but activation is shifted towards an anti-inflammatory phenotype, e.g. IL-1RA and IL-10 production[46-48]. Depending on the magnitude and persistence of this state, patients exhibit increased mortality and acquisition of hospital acquired infections[49].

In order to clear infections and damaged cells/tissue as well as minimize damage to the surrounding cells and tissue, pro-inflammatory and anti-inflammatory responses must be delicately balanced. Excessive production of pro-inflammatory mediators may promote unnecessary tissue damage while disproportionate anti-inflammatory mediators can lead to the inability for the host to combat subsequent insults[3, 5, 50]. Both pro- and anti-inflammatory immune responses are simultaneously activated in the early stages of sepsis[51-53], however these antagonistic pathways do not always balance each other. Hyper pro-inflammatory cytokine release (i.e. cytokine storm) is a major contributor to deaths occurring during early sepsis. Two theories exist involving the progression of

inflammation and subsequent patient deaths. One theory outlines the idea that the release of high levels of pro-inflammatory cytokines by innate immune cells becomes persistent. The prolonged hyper pro-inflammatory environment results in organ injury which ultimately contributes to mortality. The second theory proposes that the initial hyper inflammatory phase (pro-inflammatory dominant) progresses into a prolonged immunosuppressive phase. In regards to the immunosuppressive phase, immune cells display a reduced capacity to properly respond to subsequent PAMPs and DAMPs, and thus contributing to late phase sepsis deaths[51-56].

Acute Respiratory Distress Syndrome

Dysregulation of the inflammatory response, such as in sepsis, is a significant cause of multiple organ injury, including the lungs, where alterations in endothelial, epithelial, and immune cell mechanics are leading to barrier permeability and edema. These events are directly responsible for the severity of lung injury and death among critically ill patients. In particular, acute respiratory distress syndrome (ARDS) is characterized by respiratory complications involving acute inflammatory lung injury associated with impairment of lung mechanics and gas exchange[57, 58]. Approximately 200,000 individuals are affected by ARDS every year in the United States[58, 59]. ARDS can be the result of either direct or indirect insults to the lungs with sepsis the most prevalent cause of lung injury and mortality in hospitals[58-60]. At the present time, only supportive care is available to attempt to improve survival, necessitating the development of therapeutic interventions[61-64].

The pathophysiology of ARDS is largely reliant on the exaggerated innate immune response and neutrophil accumulation in the lungs. Severe inflammation is associated with increased permeability due to endothelial and epithelial barrier dysfunction[57, 65, 66]. Besides neutrophils, alveolar macrophages are implicated in acute inflammatory conditions, but also have long lasting effects via adaptive immunity[67]. Alveolar macrophages interact with epithelial cells, lymphocytes, and mesenchymal stem cells through paracrine signaling to regulate the inflammatory response or lung injury[68-70]. Signaling between inflammatory and effector cells is necessary for regulating the balance between injurious and protective immune responses. Following stimulation, lung epithelial cells and alveolar macrophages release chemokines to recruit neutrophils and monocytes to sites of infection, a beneficial antimicrobial response; however, robust and lasting inflammatory responses are associated with collateral tissue damage. Bioactive mediators, including proteases and cytokines, produced by these cells may potentially induce endothelial and alveolar epithelial dysfunction. The impact of reactive oxygen species (ROS) in ALI is a more complex issue. For example, previous studies, including our own, have shown that H_2O_2 has anti-inflammatory effects on neutrophils and reduced the severity of LPS-induced ALI[71]. In turn, superoxide, hydroxyl radicals and reactive nitrogen species are implicated in lung tissue injury[72-74].

Alveolar type I epithelial cells comprise up to 90% of the surface of the lungs, including the alveolar spaces, and provide a highly permeable barrier for gases, anions, and fluid exchange. These cells are particularly susceptible to injury, as they are directly exposed to particulates and pathogens from constant exposure to the environment. Following injury, immune cells followed by protein-rich edema accumulate in the alveolar spac-

es[57, 75]. Mesenchymal cells also infiltrate the alveolar space which can lead to increased deposition of matrix components fibronectin and type I collagen. This is associated with a mechanism of repair, but may also lead to development of fibrosis[76, 77]. Neutrophils recruited to the lungs release molecules, including elastases, metalloproteases, other proteolytic enzymes, oxidants, reactive nitrogen species, and histones, which cause injury to the alveolar epithelial cells[78-80]. Neutrophils also have the ability to deploy neutrophil extracellular nets (NETs), which are comprised of DNA, to prevent dissemination of pathogens. However, excessive NET formation contributes to the severity of ALI, as observed in sterile inflammatory lung injury inflicted by trauma and hemorrhage[81-83]. In addition to neutrophils, circulating macrophages are recruited in a cytokine dependent manner, which can augment lung injury through inflammatory cytokines and release of apoptosis inducing molecules [84].

Bioenergetics and Metabolic Reprogramming in Sepsis and ARDS

Dysfunction in bioenergetics is observed during sepsis and is associated with organ injury and mortality[85-88]. The mitochondria serve as the primary site for energy production in the form of ATP generation. Cells can then utilize the energy released by hydrolysis of ATP to ADP to fuel many cellular functions including maintenance of ion pumps, protein synthesis, and carbohydrate and lipid tunover[89, 90]. A decrease in energy supply is detrimental to cell survival, especially upon increased energy demands under stress conditions[91, 92]. Thus mechanisms to maintain ATP levels within cells monitor these changes and adjust catabolic and anabolic processes accordingly.

After glucose enters the cell, it enters the glycolytic pathway where it undergoes a series of enzymatic reactions to produce pyruvate. This can occur under both aerobic and anaerobic conditions and produces NADH and a small amount of ATP for the cell. The pyruvate can then be converted into either lactate or acetyl CoA. Under normal conditions, the majority of the pyruvate gets converted into acetyl CoA and is shuttled into the mitochondria where it undergoes further biochemical reactions in the tricarboxylic acid cycle (Kreb's cycle) to generate NADH and $FADH_2$. The electron transport chain utilizes the NADH and $FADH_2$ to provide protons for the generation of an electrochemical gradient across the inner mitochondrial membrane. This gradient is used by ATP synthase of the electron transport chain to generate ATP. During inflammatory conditions, cells experience an increase in glycolysis related proteins, in particular in immune cells, and switch from oxidative phosphorylation to glycolysis for ATP generation. A significant shift from oxidative phosphorylation toward glycolysis was found during LPS-TLR4 engagement in macrophages and dendritic cells[93-95].

Activated macrophages are typically classified into two phenotypes, M1 and M2. M1 macrophages are characterized by their increased reliance on glycolysis, phagocytic ability, and augmented pro-inflammatory cytokines and ROS production, where as M2 macrophages rely heavily on oxidative phosphorylation and elicit anti-inflammatory functions and tissue repair mechanisms[96]. However, it has recently been suggested that activated macrophage phenotypes are not strictly M1 or M2 but exist in a spectrum with varying degrees of pro-inflammatory and anti-inflammatory functions[97, 98]. Metabolic/bioenergetic pathways contribute to development of specific phenotypes. In order to combat pathogens, classically activated macrophages must produce ROS and

other anti-microbial proteins. While NADPH oxidase is known for its contribution in producing ROS for the respiratory burst, mitochondrially produced ROS, as well as proteins, have been shown to be instrumental in macrophage function[99]. In order to maintain ATP production, the macrophages increase glycolytic flux through HIF-1α-dependent gene expression[100]. Studies have found that preservation of mitochondrial function is essential for macrophage phagocytic functions as well as neutrophil chemotaxis and respiratory burst[99, 101-103].

AMP-activated Protein Kinase

AMP-activated protein kinase (AMPK) is a highly conserved protein kinase, with orthologues found in almost all eukaryotic cells, which serves as a critical metabolic sensor and regulator of energy production and preservation at the cellular and whole organism levels. Functional AMPK is a heterotrimeric protein comprised of the α catalytic subunit and the β and ɣ regulatory subunits. Each subunit has multiple isoforms (mammalian α1, α2, β1, β2, ɣ1, ɣ2, ɣ3) which are encoded by specific PRKA genes (PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2, PRKAG3 respectively)[104]. The mechanism involved in sensing energy deficiency is coupled with alterations in cellular AMP and ADP levels[105, 106]. Conditions associated with limited nutrients and oxygen delivery typically lead to an increase in the AMP to ATP ratio leading to AMPK stimulation. In particular, binding of AMP and ADP to the AMPKɣ subunit induces allosteric activation of AMPK allowing for enhanced phosphorylation of the AMPKα1 Thr172 and protection from dephosphorylation, whereas ATP binding

reduces Thr172 phosphorylation[107-109]. While AMP increases activation of AMPK up to 10 fold, phosphorylation of Thr172 on the AMPK α subunit increases activity by 100-fold[110]. The primary upstream kinase responsible for Thr172 phosphorylation is liver kinase B1 (LKB1), however, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CAMKKβ) kinase is also known to phosphorylate Thr172 to a lesser extent[111-113].

Activation of AMPK is associated with bioenergetic stress, which is typically connected with nutrient deprivation, ischemia, hypoxia, oxidative stress, and exercise[114-117]. Pharmacologic agents have also been shown to activate AMPK including AICAR, metformin, berberine, A-769662, PT1, and 991[118-124]. One of the most essential functions of AMPK is related to recovery of metabolic homeostasis. This is possible by AMPK's ability to interact with downstream signaling components that promote catabolic processes while decreasing anabolic pathways. AMPK increases expression of genes responsible for oxidative metabolism, including PGC-1α dependent mitochondrial biogenesis, and down regulation of glycolytic genes through HIF-1α regulation[125-129]. A specific mechanism is implicated in inhibition of AMPK during sepsis. For example, AMPK activation is diminished following LPS through direct inhibitory phosphorylation by AKT, IKKβ, or GSK3β[130-132]. Diminished AMPK activity may also result from modification and inhibition of upstream kinases[133]. The initial decrease in AMPK activity may be beneficial in the early stages of inflammation and infection, however, prolonged inhibition could lead to problems in cellular bioenergetics and organ damage. Although inflammatory conditions cause a decrease in AMPK activity, studies have shown that preserving AMPK function is beneficial to diminish the severity of ALI in animal models. For example, AMPK activation diminished pro-inflammatory cytokine secretion

in LPS stimulated neutrophils and macrophages. Activation of AMPK also reduced the severity of murine endotoxin-induced ALI[134-136]. AMPK activation has been shown to provide a substantial benefit in experimental sepsis and ALI, however, pharmacological approaches to activate AMPK are not currently used in clinics.

In this dissertation we explore how AMPK activation through pharmacological activators affects the severity of lung injury associated with sepsis. We hypothesis that activation of AMPK will diminish inflammation and thus sepsis-induced lung injury.

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Abstract

Catecholamines, including β-adrenergic and dopaminergic neurotransmitters, have an essential role in regulating the "fight or flight" reflex and also affect immune cell pro-inflammatory action. However, little is known about whether catecholamines prevent dysfunction of metabolic pathways associated with inflammatory organ injury, including development of acute lung injury (ALI). We hypothesize that selected catecholamines may reduce metabolic alterations in LPS-stimulated macrophages and in the lungs of mice subjected to endotoxin-induced ALI, a situation characterized by diminished activity of AMP-activated protein kinase (AMPK). We found that activation of the dopamine 1 receptor (D1R) with fenoldopam, but not stimulation of adrenergic receptors with norepinephrine, resulted in a robust activation of AMPK in peritoneal macrophages, human monocytes, or alveolar epithelial cells (AECs). Such AMPK activation was mediated by a phospholipase C (PLC)–dependent mechanism. Unlike norepinephrine, D1R activation also prevented Thr172–AMPK dephosphorylation and kinase inactivation in LPS treated macrophages. Furthermore, we show that a culture of AECs with either fenoldopam or the AMPK activator metformin effectively diminished IL-1β–induced release of adverse paracrine signaling, which promotes the macrophage pro-inflammatory response. In vivo, fenoldopam reduced the severity of LPS-induced ALI, including development of pulmonary edema, lung permeability, and production of inflammatory cytokines TNF-α, MIP-2, or KC and HMGB1. Fenoldopam also prevented AMPK dephosphorylation in the lungs of LPS treated mice and prevented loss of mitochondrial complexes NDUFB8 (complex

I) and ATP synthase (complex V). Collectively, these results suggest that dopamine is coupled to AMPK activation, which provides a substantial anti-inflammatory and bioenergetic advantage and reduces the severity of endotoxin-induced ALI.

Introduction

Dopamine, norepinephrine, and epinephrine are the most abundant catecholamines. They are produced by enzymatic conversion of tyrosine predominantly in the adrenal medulla and central nervous system (1, 2). Besides a regulatory role in the "fight or flight" reflex, catecholamines are also affecting pro- and anti- inflammatory responses in immune cells and peripheral tissue, mediated by variety and bioavailability of adrenergic and dopaminergic receptors (2-4). For example, adrenergic pro- and anti- inflammatory signaling is dependent on the abundance of α and β receptors (5, 6). Besides adrenergic signaling, recent studies have underlined the importance of dopaminergic pathways in moderating inflammatory conditions in experimental models of organ injury (7, 8). The physiological effects of dopamine are mediated by dopamine receptors that consist of D1-like (D1, D5) and the D2-like (D2, D3, D4) subtypes. Recently, D1 dopaminergic signaling has been shown to diminish mortality in experimental sepsis, an important predisposition for development of acute respiratory distress syndrome (ARDS) (8-10). While anti-inflammatory action can be considered as a major effect of catecholamines, preservation of immune and peripheral tissue metabolic and bioenergetic homeostasis may have an equally important impact in organ injury. In particular, metabolic reprogramming and loss of bioenergetic plasticity of immune cells are related to mitochondrial dysfunction and contribute to development and likely insufficient resolution from inflammatory conditions (11). Recent studies suggest that the bioenergetic profile is differentially regulated by α or β -adrenergic receptor signaling pathways in human peripheral blood mononuclear cells (12). However, it is not known whether selective catecholamines affect function

of major bioenergetic sensors and metabolic regulators, such as AMP-activated protein kinase (AMPK).

The ability of AMPK to sense energy demand and preserve bioenergetic and redox homeostasis suggests that AMPK is a plausible target in sepsis, hemorrhage, or other inflammatory conditions associated with development of ARDS (13-15). AMPK is a serine/threonine protein kinase that consists of the α catalytic subunit and β and γ regulatory subunits. The AMPK α/β/γ heterotrimer has a unique mechanism of activation during bioenergetic imbalance that may be triggered by limited access to oxygen and nutrients (16). The activation process is initiated by binding of AMP and ADP to the γ subunit (17-19) that allows for phosphorylation of the AMPK α subunit by upstream kinases (16, 20, 21). Once activated, AMPK effectively preserves energy expenditure by switching from anabolic to catabolic metabolism (16). It is important to note that pharmacological AMPK activators, for example AICAR and metformin, have substantial protective effects on liver, kidney or heart in murine models of inflammatory organ injury (15, 22-25). Besides anti-inflammatory action, AMPK activation is linked to stimulation of autophagy/mitophagy, mitochondrial biogenesis, and normalization of mitochondrial redox status (26-29), pathways known to be dysregulated in sepsis and ALI.

Previous studies indicate that α or β adrenergic pathways are implicated in regulating AMPK activity and glucose homeostasis in skeletal muscle cells and adipocytes, respectively (30, 31). However, the potential impact of catecholamines on AMPK activity in immune cells and lung epithelial cells during inflammatory conditions remains to be determined. We hypothesize that activation of AMPK by specific adrenergic and/or dopaminergic systems may affect the severity of endotoxin-induced ALI.

Materials and methods

Mice

Male C57BL/6 mice were purchased from the National Cancer Institute-Frederick (Frederick, MD). Mice 10 to 12 weeks of age were used for experiments. Mice were given food and water ad libitum and kept on a 12-hours light-dark cycle. All experiments were conducted in accordance with approved protocols by the University of Alabama at Birmingham Animal Care and Use Committee.

Reagents and Antibodies

The dopamine D1 receptor agonist fenoldopam, m-3M3FBS, IL-1β and anti-HMGB1 antibody were purchased from R&D Systems (Minneapolis, MN). Histopaque, LPS, dopamine, norepinephrine, salbutamol, and phenylephrine were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies for phospho-Thr172-AMPK, total AMPK, and phospho-Ser79-ACC were obtained from Cell Signaling Technologies (Danvers, MA). HRP-conjugated β-actin antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Custom antibody mixtures and negative selection columns for mouse neutrophil isolation were obtained from Stem Cell Technologies (Vancouver, BC). Dispase, Anti-Mouse CD 16/32 and Anti-Mouse CD 45 were obtained from BD Bioscience (San Jose, CA). MACS human CD16 Microbeads was obtained from Miltenyi Biotec Inc. (Auburn, CA). PLC inhibitor U-73122 was purchased from Cayman Chemical (Ann Arbor, MI). DMEM was obtained from Corning Cellgro (Manassas, VA). RPMI 1640 was

purchased from Hyclone (Logan, UT). FBS was purchased from Atlanta Biologicals (Flowery Branch, GA). F12K medium was purchased from Gibco (Grand Island, NY).

Peritoneal Macrophages, Neutrophils and Monocytes Isolation and Culture

Peritoneal macrophages were isolated as previously described (32). Macrophages were elicited in 10- to 12- week-old mice by intraperitoneal application of Brewer thioglycollate. Cells were collected 4 days after thioglycollate injection. Peritoneal macrophages were cultured in RPMI 1640 media supplemented with 8% FBS at 37°C. In select experiments macrophages were incubated with RPMI 1640 media supplemented with 0.5% FBS for 2 hours prior to LPS exposure, as indicated in Figure legends. Bone marrow neutrophils were isolated using negative selection method as previously described (33). Neutrophil purity was consistently greater than 97%, as determined by Wright-Giemsa-stained cytospin preparations. Neutrophils were cultured in RPMI 1640 medium containing 8% FBS and treated as indicated in the figure legends. Neutrophil viability under experimental conditions was determined by Trypan blue staining and was consistently greater than 95%.

Monocyte isolation was processed according to UAB Institutional Review Board-approved protocols. Monocytes were isolated from blood samples using MACS Microbeads, selective for CD14+CD16+ cells, and accordingly with the manufacturers recommended protocol (Miltenyi Biotec, Auburn, CA). Monocytes were cultured in RPMI 1640 media supplemented with 8% FBS at 37°C.

Mouse Alveolar Lung Epithelial Cell Isolation and Culture

Epithelial cells were isolated as previously described [34]. Lungs were perfused through the heart using PBS. Dispase was instilled into the lungs through the trachea followed by digestion for 60 minutes at room temperature. Lungs were minced, incubated in DMEM containing DNase I, and then pipetted and passed through sequentially smaller pore size filters. Epithelial cells were collected from the cell suspension using the negative selection process and cell suspension plated overnight. Next, non-adherent epithelial cells were transferred to collagen coated culture plates and cultured in DMEM medium with 8% FBS. In selected experiments human lung epithelial cell line A549 cells (ATCC) were cultured and treated in F12K supplemented with 8% FBS.

Cytokine ELISA

ELISA was used to measure cytokine levels in culture media and bronchoalveolar lavage (BAL) fluids as previously described (33). Levels of TNF-α, MIP-2, and KC were determined using commercially available ELISA kits (R&D Systems) according to manufacturer's instructions.

Western Blot Analysis

Western Blot analysis was performed as described previously (35). Each experiment was carried out three or more times with cell populations obtained from separate groups of mice. In selected experiments, BAL fluids (30 μl) were mixed with Laemmli

sample buffer and boiled for 5 min followed by Western Blot analysis with anti-HMGB1 antibody.

A Mouse Model for Endotoxin-Induced Lung Injury

Lung injury was induced by intratracheal administration of LPS (2 mg/kg, i.t.) as previously described (22, 33). Characterization of lung injury is by neutrophil infiltration into the interstitium and airways of the lungs, interstitial edema development, and increased pro-inflammatory cytokine production. Besides mononuclear/neutrophilic infiltrates, cellular debris and proteinaceous material are present in alveolar space. Alveolar walls are also thickened, and the septa are edematous. To induce ALI, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was gently extended and LPS or PBS solution deposited into the pharynx followed by aspiration to the lungs (36). Fenoldopam (10 mg/kg, previously described (8)) in 0.5 ml of DMSO/saline or control vehicle (DMSO/saline) was injected intraperitoneally for 18 and 0.5 hour prior to LPS intratracheal instillation. Mice were euthanized 24 hours after LPS administration. Bronchoalveolar fluids (BALs) were obtained by lavaging the lungs three times with 1 ml PBS.

Statistical Analysis

Statistical analysis was performed using 3 or more independent experiments. Multigroup comparisons were performed using one-way ANOVA with Tukey's post hoc test.

Values were normally distributed. Statistical significance was determined by the Student's t-test for comparisons between two groups. A value of $p < 0.05$ was considered significant. Analyses were performed on SPSS version 16.0 (IBM, Armonk, NY) for Windows (Microsoft Corp., Redmond, WA).

Results

Dopaminergic and Adrenergic Signaling Have Distinct Effects on AMPK Activity in Macrophages and Neutrophils

In the first set of experiments, Thr172-AMPK phosphorylation (activation) was determined in murine peritoneal macrophages before and after stimulation of dopaminergic or adrenergic receptors. As shown in Figures 1A and B, exposure to dopamine or fenoldopam, a potent and highly selective D1R dopaminergic agonist, resulted in a robust increase in T172-AMPK phosphorylation. While the dopaminergic pathway increased AMPK activation, inclusion of adrenergic agonist norepinephrine or salbutamol (a β2 adrenergic agonist) had little or no effects (Figures 1D and E and Supplemental Figure 1). Consistent with results obtained from murine macrophages, culture of human monocytes with fenoldopam also increased T172-AMPK phosphorylation, whereas norepinephrine had no effects (Figure 1F). Next, we examined if similar mechanisms are operational in neutrophils. However, we found that AMPK phosphorylation was unaltered in fenoldopam treated neutrophils while norepinephrine caused a substantial T172-AMPK dephosphorylation (Supplemental Figure 2). These results suggest that the dopamine/D1R, but not norepinephrine/adrenergic, signaling pathway was coupled with AMPK activation in murine macrophages and human monocytes.

D1R and Phospholipase C Signaling Increases AMPK Activation in Fenoldopam Treated Macrophages

To determine the specific components of the D1R signaling implicated in AMPK activation, we examined the effects of adenylyl cyclase/cAMP, protein kinase A (PKA) and phospholipase C (PLC) in mouse peritoneal macrophages. As shown in Figure 2A, activation of adenylyl cyclase by forskolin or pretreatment with phosphodiesterase inhibitor IBMX to prevented cAMP degradation, did not activate AMPK. Of note, modest activation of AMPK was observed after inclusion of PKA inhibitor H-9 (Figure 2A). Next, we examined the effects of phospholipase C. As shown in Figure 2B, inclusion of PLC inhibitor U-73122 prevented the ability of fenoldopam to increase AMPK phosphorylation. In contrast to U-73122, culture cells with PLC activator m-3M3FBS resulted in activation of AMPK (Figure 2C). These results indicate that stimulation of D1 dopaminergic signaling and activation of PLC, but not adenylyl cyclase/cAMP or PKA, was implicated in activation of AMPK in peritoneal macrophages. It is important to note that such a mechanism of AMPK activation by PLC is likely cell population specific, as a recent study proposed that both cAMP/PKA and PLC may affect AMPK activity in skeletal muscle cells (37).

Stimulation of D1 Dopaminergic Pathway Prevents T172-AMPK De-Phosphorylation in LPS Treated Macrophages

Previous studies have shown that inflammatory conditions, including engagement of LPS/TLR4, are associated with T172-AMPK de-phosphorylation and decreased kinase

activity in macrophages and other cell populations (22, 25, 38, 39). As shown in Figure 3, exposure to LPS resulted in a time dependent decrease in T172-AMPK phosphorylation. Importantly, pre-treatment with fenoldopam diminished such LPS effects (Figures 3A and B). Of note, AMPK de-phosphorylation was not affected by norepinephrine (Figures 3C and D). These results suggest that selective activation of the D1 dopaminergic pathway preserved AMPK activity in LPS treated macrophages.

In additional experiments, we confirmed the anti-inflammatory effect of the D1R signaling pathway. In particular, pre-treatment with D1R agonist fenoldopam reduced TNF-α and MIP-2 production by LPS-stimulated macrophages (Figure 4). Because anti-inflammatory effects have been previously linked to cAMP-PKA signaling (40, 41), we tested if H-9 (PKA inhibitor) moderates fenoldopam action in LPS treated macrophages. However, PKA inhibitor did not diminish the ability of fenoldopam to reduce TNF-α production by LPS-stimulated macrophages (Supplemental Figure 3). These findings suggest that PLC, but not PKA, mediates anti-inflammatory action effects of fenoldopam.

Stimulation of D1R or Direct AMPK Activation Diminished the Adverse Epithelial Paracrine Signaling On Macrophage Pro-Inflammatory Response

In addition to the effects of dopaminergic signaling in macrophages and neutrophils, we examined if D1R stimulation affects AMPK activity in type II alveolar epithelial cells. Primary mouse alveolar epithelial cells (AECs) were treated with D1R agonist fenoldopam or AMPK activator metformin. As shown in Figures 5A and B, nearly a 3 fold increase of phosphoT172-AMPK was observed after treatment with fenoldopam for

4 hours, as compared to untreated (control) cells. Furthermore, we found that activation of AMPK also diminished the epithelial paracrine signaling which promotes macrophage pro-inflammatory response (Figures 5C and D). In these experiments, AECs were incubated with metformin (0 or 300 μM) or fenoldopam (0 or 1 μM) for 2 hours followed by inclusion of IL-1β (0 or 10 ng/ml) for an additional 4 hours. Next, cells were washed to remove AMPK activators and IL-1β, and then incubated for 24 hours. Conditioned media were further used to incubate peritoneal macrophages for 24 hours and the amounts of TNF-α determined by ELISA. These results suggest that AMPK activation in epithelial cells reduced the effects of paracrine signaling that caused macrophage pro-inflammatory activation.

Stimulation of D1R Dopaminergic Signaling Diminished the Severity of LPS-Induced Acute Lung Injury

Fenoldopam (10 mg/kg, i.p.) or vehicle (saline, i.p) was injected for 18 hours, and then a second dose of fenoldopam or saline was applied 1 hour prior to LPS intratracheal instillation (2 mg/kg; i.t.). The amount of inflammatory cytokines in BAL fluids, extent of pulmonary edema and lung permeability, as well as T172-AMPK phosphorylation status in lung homogenates were determined 24 hours after exposure to LPS. Representative images of lung sections (Figure 6A) demonstrate that fenoldopam effectively prevented LPS-mediated neutrophil accumulation (fenoldopam + LPS group) and overall preserved lung architecture, as compared to mice treated with LPS alone. Reduced pulmonary edema and vascular permeability was evidenced by a decrease in wet-to-dry ratios and BAL

protein content in fenoldopam and LPS mice, as compared to the LPS group (Figure 6B). Consistent with results obtained from peritoneal macrophages (Figure 4), fenoldopam also diminished BAL cytokines, including TNF-α, MIP-2, and KC (Figure 7A). High Mobility Group Box 1 (HMGB1), an important marker and mediator of lung injury, was significantly increased in BAL fluids of LPS treated mice. Importantly, fenoldopam effectively prevented such accumulation (Figure 7B and C). These results indicate that D1 dopaminergic agonist fenoldopam reduced the severity of endotoxin-induced ALI.

The D1R Signaling Pathway Preserved AMPK Activity Along With Major Components of ETC Complexes in Lung Tissue of Mice Subjected to LPS-Induced ALI

The amounts of phospho-T172-AMPK and phospho-S79- acetyl-CoA carboxylase (ACC), an AMPK downstream target, were measured in whole lung homogenates obtained from vehicle (saline), LPS, or fenoldopam and LPS treated mice. Western Blot analysis of lung homogenates shows a marked decrease in T172-AMPK and S79-ACC phosphorylation after intratracheal instillation of LPS, as compared to control (vehicle) group (Figures 8A and B). Importantly, application of fenoldopam was sufficient to prevent both AMPK and ACC de-phosphorylation in mice treated with LPS. These results suggest that stimulation of the D1R dopaminergic pathway preserved AMPK activity in lungs of mice subjected to LPS (Figure 9A).

As shown in Figures 8C and D, a substantial decrease in major components of the mitochondrial electron transport chain (ETC) complexes occurred in lungs of LPS treated mice, including NDUFB8 (Complex I) and ATP synthase alpha subunit (Complex V).

Importantly, fenoldopam effectively prevented such loss of NDUFB8 and ATP synthase alpha subunit. These findings are consistent with previous studies that preservation of mitochondrial structure and function reduces the severity of LPS-induced lung injury (42, 43).

Discussion

In this study, we found that dopamine-mediated stimulation of the D1R pathway is associated with AMPK activation in peritoneal macrophages, monocytes and alveolar epithelial cells. Activation of the D1R signaling prevented T172-AMPK de-phosphorylation (inactivation) when fenoldopam was applied prior to LPS exposure. We also found that fenoldopam-dependent activation of AMPK in AECs diminished IL-1β -induced adverse paracrine signaling that promoted macrophage pro-inflammatory response. Furthermore, our results indicate that administration of fenoldopam effectively preserved AMPK activity along with major mitochondrial ETC components in lungs of mice subjected to intratracheal instillation of LPS. Importantly, activation of the D1R-AMPK signaling axis prior to LPS administration reduced the severity of ALI.

The ability of D1R to preserve AMPK activity is likely an important event to reduce exaggerated macrophage pro-inflammatory activation and therefore diminish the extent of ALI in LPS treated mice. This possibility is supported by recent studies that demonstrate the effects of AMPK activators metformin or AICAR to diminish LPS-mediated organ injury, including lung, heart, kidney or liver injury (33, 44-48). Besides anti-inflammatory effects associated with inhibition of the NF-κB signaling cascade, AMPK activation may also accelerate endothelial barrier recovery, and decrease lung permeability, processes known to be dysregulated in sepsis and ARDS (22, 48, 49). In addition to dopamine and AMPK anti-inflammatory effects, dopamine signaling has also been implicated in increasing a liquid clearance in lung epithelium (7, 50). Of note, dopamine/D1R signaling may also improve lung mechanics due to airway smooth muscle

relaxation (51). Furthermore, the dopaminergic-vagal axis has been recently shown to increase survival in a murine model of polymicrobial sepsis (8).

Although dopamine-D1R signaling are linked to the cAMP/PKA anti-inflammatory signaling cascade (40, 41), we also observed cAMP/PKA independent, but PLC-dependent AMPK activation in macrophages (Figure 9A). Given the importance of AMPK to diminish macrophage pro-inflammatory activation, our results suggest that both cAMP/PKA and PLC/AMPK pathways may synergistically lower a detrimental inflammation in LPS treated mice. Furthermore, the PLC/AMPK pathway may also prevent bioenergetic dysfunction of immune cells and in the lung tissue of mice subjected to ALI. It is important to note that while AMPK activation in peritoneal macrophages occurs after stimulation of D1R-PLC axis, but not adenylyl cyclase/cAMP or PKA, such a mechanism of AMPK activation is likely cell type specific. For example, both cAMP/PKA and PLC were implicated in AMPK activation in skeletal muscle cells (37). Despite mechanistic differences and synergy between dopamine receptors, our findings suggest that AMPK activation is a plausible target to prevent development of ARDS and possibly other inflammatory conditions associated with organ dysfunction.

Our results show a benefit of fenoldopam/D1-AMPK axis, though previous studies have also demonstrated the importance of D2 receptors in LPS-induced ALI (7). The exact crosstalk between D1 and D2 receptors, including implication of D2 deficiency on D1 signaling is not known. Although D1R and D2R have distinct signaling cascades, dopamine likely has a beneficial synergistic impact on ALI through utilization of both receptors. For example, D1R-mediated activation promotes the AMPK-bioenergetic pathway while engagement of dopamine-D2R diminishes vascular lung permeability (7). Of

note, recent studies indicate a possible formation of D1R-D2R heteromer receptors and downstream effects mediated by phospholipase C-mediated calcium signaling (52, 53).

Understanding the relationship between inflammation and metabolism creates an opportunity to develop effective pharmacological interventions, including the possibility of repurposing AMPK activators to reduce adverse inflammation in conjunction with preservation of metabolic homeostasis. Although both D1R and β-adrenergic pathways have a potent anti-inflammatory effect, D1R signaling may provide an advantage due to activation of AMPK in immune cells and peripheral lung tissue. In addition to reducing LPS/TLR4-mediated neutrophil and macrophage pro-inflammatory activation (13, 22, 39), AMPK has much broader implications in regulating cellular bioenergetics and redox homeostasis in immune cells and lung tissue (16, 54). Given that mitochondrial dysfunction is associated with unfavorable outcome of sepsis and sepsis-related ARDS (55, 56), and that AMPK affects mitochondrial quality control (autophagy/mitophagy) and biogenesis (26), AMPK activation is likely an important target to diminish the severity of organ injury (26, 57). Indeed, we found that fenoldopam effectively prevented the decrease of mitochondrial complex I (NDUFB8) and complex V (ATP synthase α-subunit) in mice subjected to intratracheal instillation of LPS (Figure 8C and D). This validates recently published findings that preservation of AMPK activity reduces the extent of mitochondrial dysfunction in lungs of septic mice (43), or due to renal injury in a murine model of diabetes (29).

Mortality rates from ARDS remain high (20-30%) as described in recent studies (58-61). Despite progress in understanding mechanisms associated with development and perpetuation of acute lung injury, no pharmacological approach to diminish the severity

or to improve survival from this condition is available for critically ill patients. Although selective catecholamines have been shown to prevent lung injury in experimental models, clinical trials with β-adrenergic agonists provided no benefit to patients with ARDS (62, 63). The exact mechanism related to such limited efficacy of β-adrenergic agonists is not well understood. It is possible that inflammatory conditions, including hemorrhagic shock or sepsis are associated with desensitization of adrenergic pathways (64-67). Another possibility is that β-adrenergic signaling alone is not preserving AMPK function in inflammatory settings and therefore not preventing metabolic and bioenergetic dysfunction of immune and stromal cells. In contrast, dopamine appears to have both anti-inflammatory effects and the capacity to stimulate AMPK activity in macrophages and in alveolar epithelial cells. It is important to note that further studies should also delineate if bioavailability of D1R is affected in critically ill patients.

Taken together, our results suggest that engagement of dopamine signaling followed by AMPK activation may provide a substantial advantage linked to both AMPK anti-inflammatory and bioenergetic function. These findings also suggest that activation of the dopamine-AMPK signaling pathway effectively protects the lungs in a murine model of endotoxin-induced ALI.

Authorship

N.B. and Z.L. performed the experiments. N.B., J.-F.P., and J.W.Z. interpreted the results and drafted the manuscript. NB and J.W.Z revised and edited the final version of manuscript.

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Abbreviations

ACC – acetyl-CoA carboxylase

AEC – alveolar epithelial cell

ALI- acute lung injury

AMPK – AMP-activated protein kinase

ARDS –acute respiratory distress syndrome

BAL – bronchoalveolar lavage

D1R – dopamine 1 receptor

D2R – dopamine 2 receptor

ETC – electron transport chain

HMGB1 – high mobility group box 1

IBMX - Isobutylmethylxanthine

KC – keratinocyte-derived chemokine

PKA – protein kinase A

PLC – phospholipase C

References

1. Kopin, I. J. (1968) Biosynthesis and metabolism of catecholamines. Anesthesiology 29, 654-60.

2. Barnes, M. A., Carson, M. J., Nair, M. G. (2015) Non-traditional cytokines: How catecholamines and adipokines influence macrophages in immunity, metabolism and the central nervous system. Cytokine 72, 210-9.

3. Schulze, J., Vogelgesang, A., Dressel, A. (2014) Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases. Aging and disease 5, 327-39.

4. Flierl, M. A., Rittirsch, D., Nadeau, B. A., Chen, A. J., Sarma, J. V., Zetoune, F. S., McGuire, S. R., List, R. P., Day, D. E., Hoesel, L. M., Gao, H., Van Rooijen, N., Huber-Lang, M. S., Neubig, R. R., Ward, P. A. (2007) Phagocyte-derived catecholamines enhance acute inflammatory injury. Nature 449, 721-5.

5. Arcaroli, J., Yang, K. Y., Yum, H. K., Kupfner, J., Pitts, T. M., Park, J. S., Strassheim, D., Abraham, E. (2002) Effects of catecholamines on kinase activation in lung neutrophils after hemorrhage or endotoxemia. J Leukoc Biol 72, 571-9.

6. Stanojevic, S., Dimitrijevic, M., Kustrimovic, N., Mitic, K., Vujic, V., Leposavic, G. (2013) Adrenal hormone deprivation affects macrophage catecholamine metabolism and beta2-adrenoceptor density, but not propranolol stimulation of tumour necrosis factor-alpha production. Experimental physiology 98, 665-78.

7. Vohra, P. K., Hoeppner, L. H., Sagar, G., Dutta, S. K., Misra, S., Hubmayr, R. D., Mukhopadhyay, D. (2012) Dopamine inhibits pulmonary edema through the VEGF-VEGFR2 axis in a murine model of acute lung injury. Am J Physiol Lung Cell Mol Physiol 302, L185-92.

8. Torres-Rosas, R., Yehia, G., Pena, G., Mishra, P., del Rocio Thompson-Bonilla, M., Moreno-Eutimio, M. A., Arriaga-Pizano, L. A., Isibasi, A., Ulloa, L. (2014) Dopamine mediates vagal modulation of the immune system by electroacupuncture. Nat Med 20, 291-5.

9. Yan, Y., Jiang, W., Liu, L., Wang, X., Ding, C., Tian, Z., Zhou, R. (2015) Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. Cell 160, 62-73.

10. Sheu, C. C., Gong, M. N., Zhai, R., Chen, F., Bajwa, E. K., Clardy, P. F., Gallagher, D. C., Thompson, B. T., Christiani, D. C. (2010) Clinical characteristics and outcomes of sepsis-related vs non-sepsis-related ARDS. Chest 138, 559-67.

11. Deutschman, C. S. and Tracey, K. J. (2014) Sepsis: current dogma and new perspectives. Immunity 40, 463-75.

12. Lunemann, J. D., Buttgereit, F., Tripmacher, R., Baerwald, C. G., Burmester, G. R., Krause, A. (2001) Norepinephrine inhibits energy metabolism of human peripheral blood mononuclear cells via adrenergic receptors. Bioscience reports 21, 627-35.

13. O'Neill, L. A. and Hardie, D. G. (2013) Metabolism of inflammation limited by AMPK and pseudo-starvation. Nature 493, 346-55.

14. Wu, S. B., Wu, Y. T., Wu, T. P., Wei, Y. H. (2014) Role of AMPK-mediated adaptive responses in human cells with mitochondrial dysfunction to oxidative stress. Biochim Biophys Acta 1840, 1331-44.

15. Liu, T. F., Brown, C. M., El Gazzar, M., McPhail, L., Millet, P., Rao, A., Vachharajani, V. T., Yoza, B. K., McCall, C. E. (2012) Fueling the flame: bioenergy couples metabolism and inflammation. J Leukoc Biol 92, 499-507.

16. Hardie, D. G., Ross, F. A., Hawley, S. A. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 13, 251-62.

17. Woods, A., Cheung, P. C., Smith, F. C., Davison, M. D., Scott, J., Beri, R. K., Carling, D. (1996) Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. J Biol Chem 271, 10282-90.

18. Song, R., Peng, W., Zhang, Y., Lv, F., Wu, H. K., Guo, J., Cao, Y., Pi, Y., Zhang, X., Jin, L., Zhang, M., Jiang, P., Liu, F., Meng, S., Zhang, X., Jiang, P., Cao, C. M., Xiao, R. P. (2013) Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. Nature 494, 375-9.

19. Xiao, B., Heath, R., Saiu, P., Leiper, F. C., Leone, P., Jing, C., Walker, P. A., Haire, L., Eccleston, J. F., Davis, C. T., Martin, S. R., Carling, D., Gamblin, S. J. (2007) Structural basis for AMP binding to mammalian AMP-activated protein kinase. Nature 449, 496-500.

20. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., Hardie, D. G. (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab 2, 9-19.

21. Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., Carling, D. (2003) LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Current biology: CB 13, 2004-8.

22. Zhao, X., Zmijewski, J. W., Lorne, E., Liu, G., Park, Y. J., Tsuruta, Y., Abraham, E. (2008) Activation of AMPK attenuates neutrophil proinflammatory activity and de-

creases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 295, L497-504.

23. Bae, H. B., Zmijewski, J. W., Deshane, J. S., Tadie, J. M., Chaplin, D. D., Takashima, S., Abraham, E. (2011) AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. Faseb J 25, 4358-68.

24. Hoogendijk, A. J., Pinhancos, S. S., van der Poll, T., Wieland, C. W. (2013) AMP activated protein kinase activation by AICAR reduces lipoteichoic acid induced lung inflammation. J Biol Chem.

25. Meares, G. P., Qin, H., Liu, Y., Holdbrooks, A. T., Benveniste, E. N. (2013) AMP-activated protein kinase restricts IFN-gamma signaling. J Immunol 190, 372-80.

26. Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S., Kohnz, R. A., Mair, W., Vasquez, D. S., Joshi, A., Gwinn, D. M., Taylor, R., Asara, J. M., Fitzpatrick, J., Dillin, A., Viollet, B., Kundu, M., Hansen, M., Shaw, R. J. (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331, 456-61.

27. Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Matsumura, T., Araki, E. (2006) Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. Diabetes 55, 120-7.

28. Eid, A. A., Lee, D. Y., Roman, L. J., Khazim, K., Gorin, Y. (2013) Sestrin 2 and AMPK connect hyperglycemia to Nox4-dependent endothelial nitric oxide synthase uncoupling and matrix protein expression. Mol Cell Biol 33, 3439-60.

29. Dugan, L. L., You, Y. H., Ali, S. S., Diamond-Stanic, M., Miyamoto, S., DeCleves, A. E., Andreyev, A., Quach, T., Ly, S., Shekhtman, G., Nguyen, W., Chepetan, A., Le, T. P., Wang, L., Xu, M., Paik, K. P., Fogo, A., Viollet, B., Murphy, A., Brosius, F., Naviaux, R. K., Sharma, K. (2013) AMPK dysregulation promotes diabetes-related reduction of superoxide and mitochondrial function. J Clin Invest 123, 4888-99.

30. Hutchinson, D. S. and Bengtsson, T. (2006) AMP-activated protein kinase activation by adrenoceptors in L6 skeletal muscle cells: mediation by alpha1-adrenoceptors causing glucose uptake. Diabetes 55, 682-90.

31. Hutchinson, D. S., Chernogubova, E., Dallner, O. S., Cannon, B., Bengtsson, T. (2005) Beta-adrenoceptors, but not alpha-adrenoceptors, stimulate AMP-activated protein kinase in brown adipocytes independently of uncoupling protein-1. Diabetologia 48, 2386-95.

32. Jiang, S., Park, D. W., Gao, Y., Ravi, S., Darley-Usmar, V., Abraham, E., Zmijewski, J. W. (2015) Participation of proteasome-ubiquitin protein degradation in autophagy and the activation of AMP-activated protein kinase. Cell Signal 27, 1186-97.

33. Zmijewski, J. W., Lorne, E., Zhao, X., Tsuruta, Y., Sha, Y., Liu, G., Siegal, G. P., Abraham, E. (2008) Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. Am J Respir Crit Care Med 178, 168-79.

34. Corti, M., Brody, A. R., Harrison, J. H. (1996) Isolation and primary culture of murine alveolar type II cells. Am J Respir Cell Mol Biol 14, 309-15.

35. Park, D. W., Jiang, S., Tadie, J. M., Stigler, W. S., Gao, Y., Deshane, J., Abraham, E., Zmijewski, J. W. (2013) Activation of AMPK Enhances Neutrophil Chemotaxis and Bacterial Killing. Mol Med 19, 387-98.

36. Zhang, M., Dong, Y., Xu, J., Xie, Z., Wu, Y., Song, P., Guzman, M., Wu, J., Zou, M. H. (2008) Thromboxane receptor activates the AMP-activated protein kinase in vascular smooth muscle cells via hydrogen peroxide. Circ Res 102, 328-37.

37. Park, S. J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., Ke, H., Rehmann, H., Taussig, R., Brown, A. L., Kim, M. K., Beaven, M. A., Burgin, A. B., Manganiello, V., Chung, J. H. (2012) Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. Cell 148, 421-33.

38. Hattori, Y., Suzuki, K., Hattori, S., Kasai, K. (2006) Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension 47, 1183-8.

39. Sag, D., Carling, D., Stout, R. D., Suttles, J. (2008) Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. J Immunol 181, 8633-41.

40. Seldon, P. M., Meja, K. K., Giembycz, M. A. (2005) Rolipram, salbutamol and prostaglandin E2 suppress TNFalpha release from human monocytes by activating Type II cAMP-dependent protein kinase. Pulmonary pharmacology & therapeutics 18, 277-84.

41. Seldon, P. M., Barnes, P. J., Meja, K., Giembycz, M. A. (1995) Suppression of lipopolysaccharide-induced tumor necrosis factor-alpha generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase. Molecular pharmacology 48, 747-57.

42. Srivastava, A., McGinniss, J., Wong, Y., Shinn, A. S., Lam, T. T., Lee, P. J., Mannam, P. (2015) MKK3 deletion improves mitochondrial quality. Free Radic Biol Med.

43. Liu, Z., Bone, N., Jiang, S., Park, D. W., Tadie, J. M., Deshane, J., Rodriguez, C. A., Pittet, J. F., Abraham, E., Zmijewski, J. W. (2015) AMP-activated protein kinase and Glycogen Synthase Kinase 3beta modulate the severity of sepsis-induced lung injury. Mol Med.

44. Decleves, A. E., Sharma, K., Satriano, J. (2014) Beneficial Effects of AMP-Activated Protein Kinase Agonists in Kidney Ischemia-Reperfusion: Autophagy and Cellular Stress Markers. Nephron. Experimental nephrology.

45. Russell, R. R., 3rd, Li, J., Coven, D. L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F. J., Mu, J., Birnbaum, M. J., Young, L. H. (2004) AMP-activated protein kinase mediates ischemic glucose uptake and prevents post ischemic cardiac dysfunction, apoptosis, and injury. J Clin Invest 114, 495-503.

46. Kim, A. S., Miller, E. J., Wright, T. M., Li, J., Qi, D., Atsina, K., Zaha, V., Sakamoto, K., Young, L. H. (2011) A small molecule AMPK activator protects the heart against ischemia-reperfusion injury. Journal of molecular and cellular cardiology 51, 24-32.

47. Bergheim, I., Luyendyk, J. P., Steele, C., Russell, G. K., Guo, L., Roth, R. A., Arteel, G. E. (2006) Metformin prevents endotoxin-induced liver injury after partial hepatectomy. J Pharmacol Exp Ther 316, 1053-61.

48. Jian, M. Y., Alexeyev, M. F., Wolkowicz, P. E., Zmijewski, J. W., Creighton, J. R. (2013) Metformin-stimulated AMPK-alpha1 promotes microvascular repair in acute lung injury. Am J Physiol Lung Cell Mol Physiol 305, L844-55.

49. Xing, J., Wang, Q., Coughlan, K., Viollet, B., Moriasi, C., Zou, M. H. (2013) Inhibition of AMP-activated protein kinase accentuates lipopolysaccharide-induced lung endothelial barrier dysfunction and lung injury in vivo. Am J Pathol 182, 1021-30.

50. Barnard, M. L., Olivera, W. G., Rutschman, D. M., Bertorello, A. M., Katz, A. I., Sznajder, J. I. (1997) Dopamine stimulates sodium transport and liquid clearance in rat lung epithelium. Am J Respir Crit Care Med 156, 709-14.

51. Mizuta, K., Zhang, Y., Xu, D., Mizuta, F., D'Ovidio, F., Masaki, E., Emala, C. W. (2013) The dopamine D1 receptor is expressed and facilitates relaxation in airway smooth muscle. Respiratory research 14, 89.

52. Chun, L. S., Free, R. B., Doyle, T. B., Huang, X. P., Rankin, M. L., Sibley, D. R. (2013) D1-D2 dopamine receptor synergy promotes calcium signaling via multiple mechanisms. Molecular pharmacology 84, 190-200.

53. Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lanca, A. J., O'Dowd, B. F., George, S. R. (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. The Journal of biological chemistry 279, 35671-8.

54. Colombo, S. L. and Moncada, S. (2009) AMPKalpha1 regulates the antioxidant status of vascular endothelial cells. Biochem J 421, 163-9.

55. Brealey, D., Karyampudi, S., Jacques, T. S., Novelli, M., Stidwill, R., Taylor, V., Smolenski, R. T., Singer, M. (2004) Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am J Physiol Regul Integr Comp Physiol 286, R491-7.

56. Singer, M. (2007) Mitochondrial function in sepsis: acute phase versus multiple organ failure. Crit Care Med 35, S441-8.

57. Kim, J., Kundu, M., Viollet, B., Guan, K. L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13, 132-41.

58. Rubenfeld, G. D., Caldwell, E., Peabody, E., Weaver, J., Martin, D. P., Neff, M., Stern, E. J., Hudson, L. D. (2005) Incidence and outcomes of acute lung injury. N Engl J Med 353, 1685-93.

59. Erickson, S. E., Martin, G. S., Davis, J. L., Matthay, M. A., Eisner, M. D. (2009) Recent trends in acute lung injury mortality: 1996-2005. Crit Care Med 37, 1574-9.

60. Hudson, L. D., Milberg, J. A., Anardi, D., Maunder, R. J. (1995) Clinical risks for development of the acute respiratory distress syndrome. Am J Respir Crit Care Med 151, 293-301.

61. Nuckton, T. J., Alonso, J. A., Kallet, R. H., Daniel, B. M., Pittet, J. F., Eisner, M. D., Matthay, M. A. (2002) Pulmonary dead-space fraction as a risk factor for death in the acute respiratory distress syndrome. N Engl J Med 346, 1281-6.

62. Bosmann, M., Grailer, J. J., Zhu, K., Matthay, M. A., Sarma, J. V., Zetoune, F. S., Ward, P. A. (2012) Anti-inflammatory effects of beta2 adrenergic receptor agonists in experimental acute lung injury. FASEB J 26, 2137-44.

63. Perkins, G. D., Gates, S., Park, D., Gao, F., Knox, C., Holloway, B., McAuley, D. F., Ryan, J., Marzouk, J., Cooke, M. W., Lamb, S. E., Thickett, D. R., Collaborators, B. A.-P. (2014) The beta agonist lung injury trial prevention. A randomized controlled trial. Am J Respir Crit Care Med 189, 674-83.

64. Wagener, B. M., Roux, J., Carles, M., Pittet, J. F. (2015) Synergistic Inhibition of beta2-adrenergic Receptor-mediated Alveolar Epithelial Fluid Transport by Interleukin-8 and Transforming Growth Factor-beta. Anesthesiology 122, 1084-92.

65. Bernardin, G., Strosberg, A. D., Bernard, A., Mattei, M., Marullo, S. (1998) Beta-adrenergic receptor-dependent and -independent stimulation of adenylate cyclase is impaired during severe sepsis in humans. Intensive Care Med 24, 1315-22.

66. Modelska, K., Matthay, M. A., Brown, L. A., Deutch, E., Lu, L. N., Pittet, J. F. (1999) Inhibition of beta-adrenergic-dependent alveolar epithelial clearance by oxidant mechanisms after hemorrhagic shock. Am J Physiol 276, L844-57.

67. Pittet, J. F., Lu, L. N., Morris, D. G., Modelska, K., Welch, W. J., Carey, H. V., Roux, J., Matthay, M. A. (2001) Reactive nitrogen species inhibit alveolar epithelial fluid transport after hemorrhagic shock in rats. J Immunol 166, 6301-10.

Figures

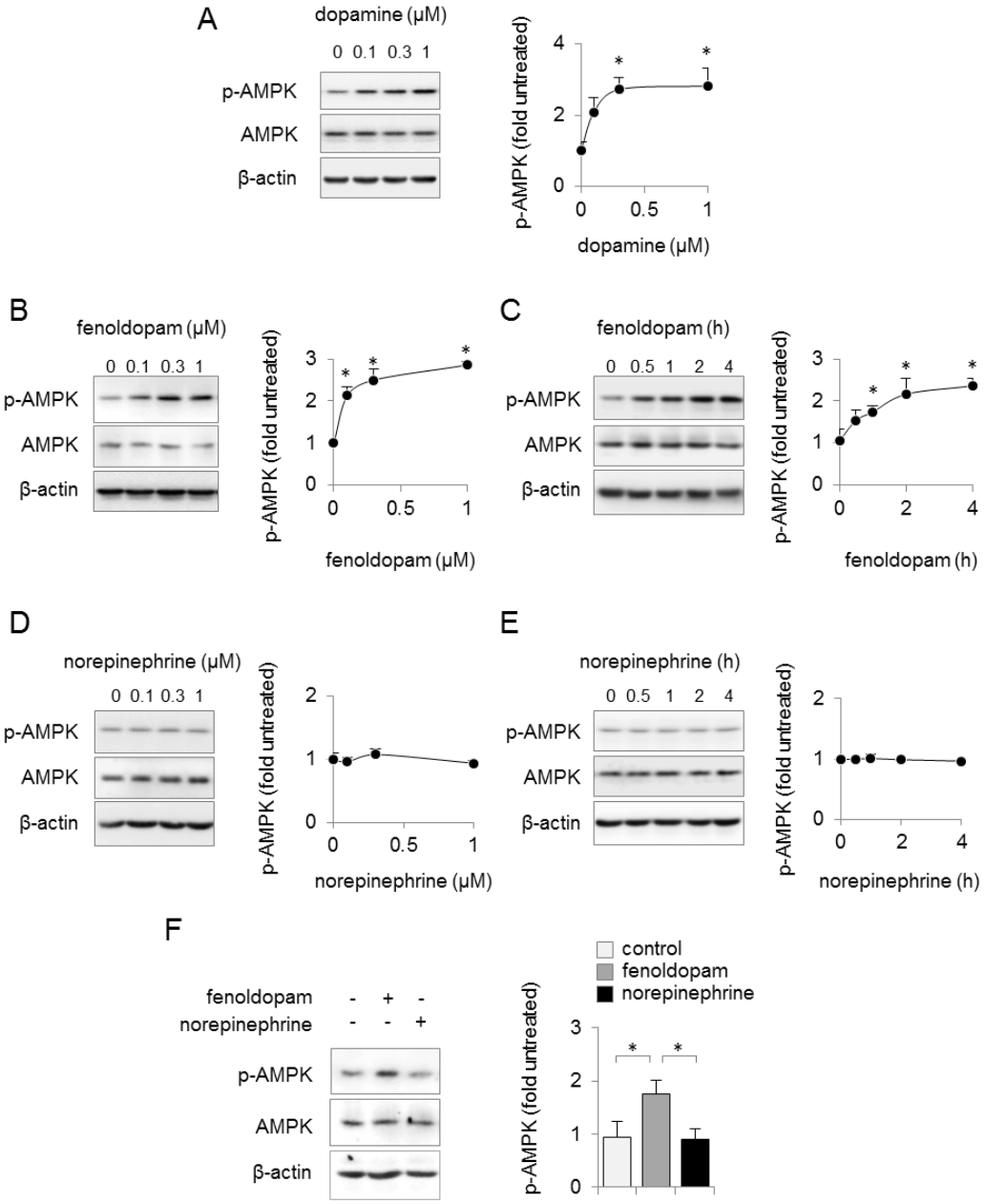


Figure 1. Dopaminergic and adrenergic signaling have distinct effects on AMPK activity in murine macrophages and human monocytes. Western blot and quantitative analysis show the amounts of pT172–AMPK, total AMPK, and β-actin in mouse peritoneal macrophages treated dose dependently with dopamine (A), D1R agonist fenoldopam (B), or norepinephrine (D) for 2 h. Cells were also incubated with fenoldopam (C) (1 μM) or norepinephrine (E) (1 μM) for the indicated time. (F) Human blood monocytes were treated with fenoldopam (0 or 1 μM) or norepinephrine (0 or 1 μM) for 2 h followed by Western blot analysis of pT172–AMPK, total AMPK, and β-actin. Means ± sd, n = 3, *P < 0.05.

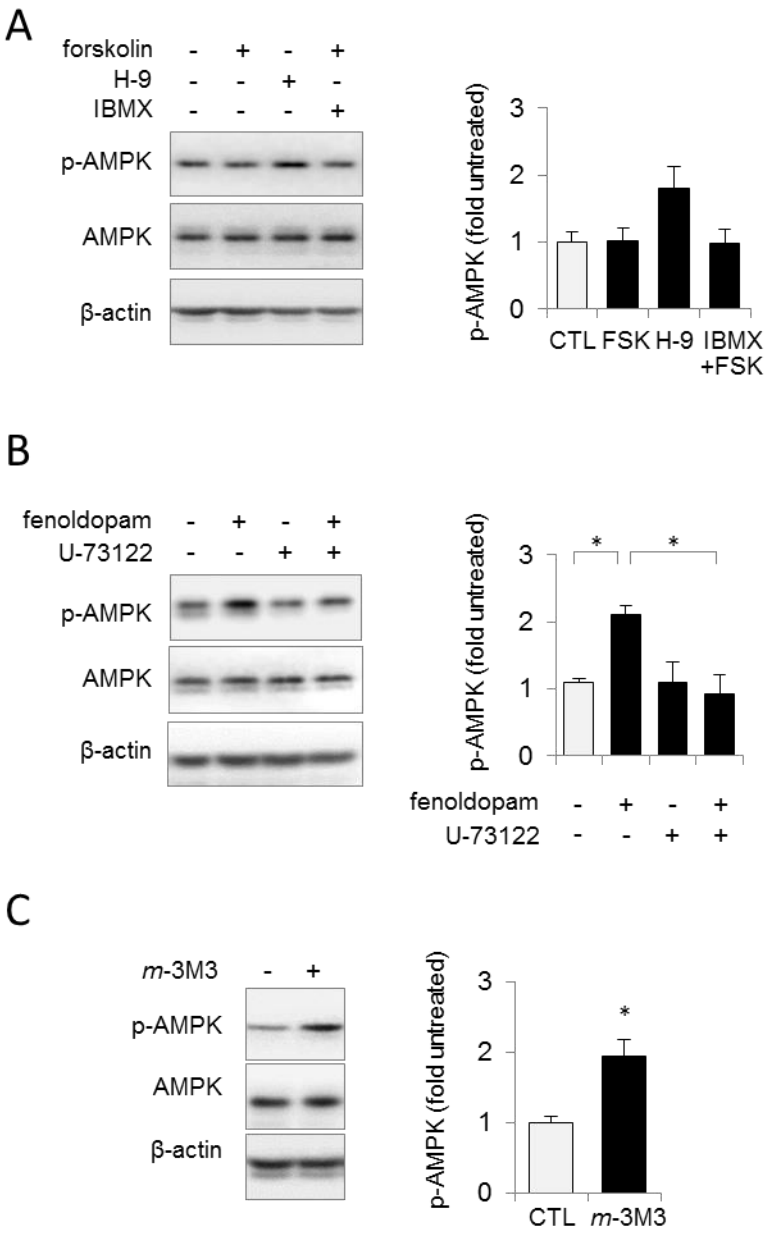


Figure 2. The D1R–PLC signaling axis, but not cAMP–PKA, induces AMPK activation in peritoneal macrophages. (A) Cells were pre-incubated with forskolin (0 or 10 μM), H-9 (0 or 10 μM), or a combination of IBMX (100 μM) and forskolin (10 μM) for 60 min. Next, cells were treated with fenoldopam (0 or 1 μM) for an additional 2 h. pT172–AMPK, total AMPK, and β-actin are shown. (B) Macrophages were pretreated with PLC inhibitor U-73122 (0 or 5 μM) for 60 min and then incubated with fenoldopam (1 μM) for 2 h. (C) Macrophages were treated with PLC activator m-3M3FBS (0 or 5 μM) for 2 h followed by Western blot analysis of AMPK. Means ± sd, n = 3, *P < 0.05.

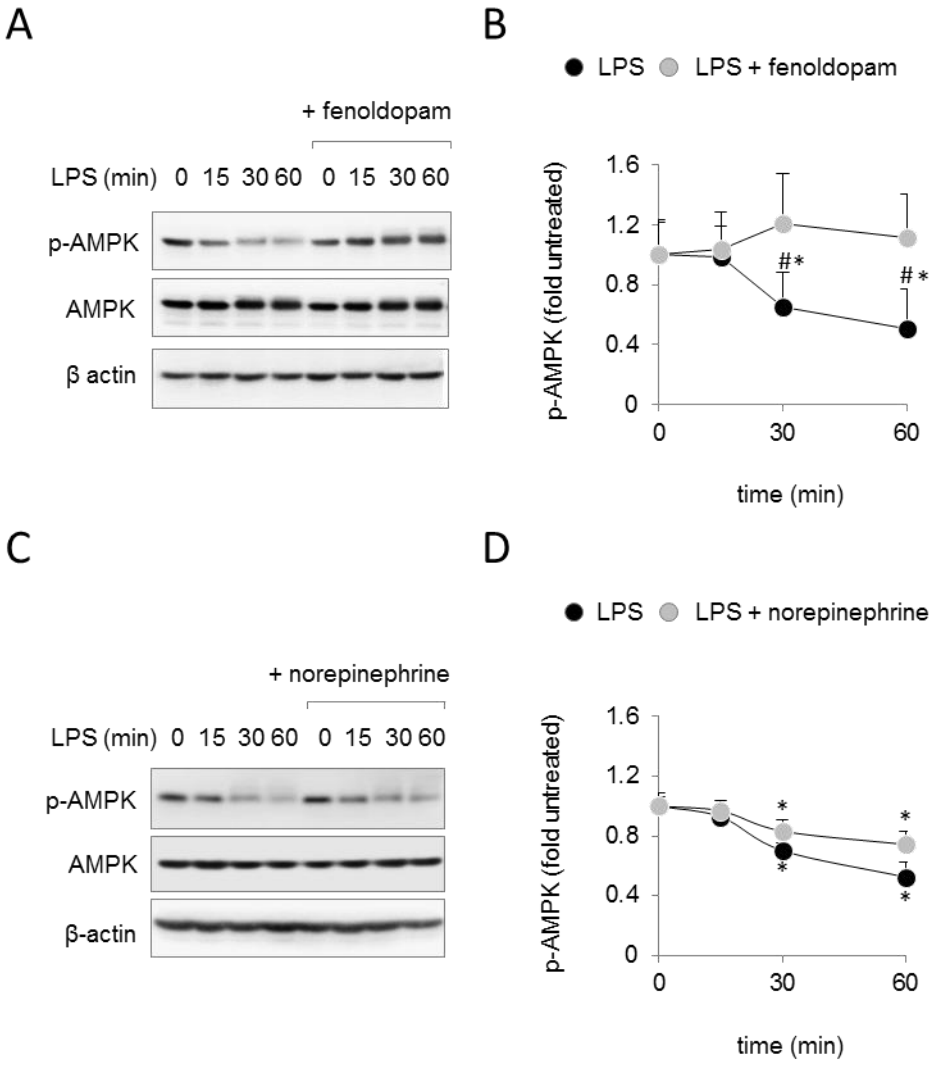


Figure 3. Engagement of fenoldopam/D1R signaling prevents AMPK dephosphorylation in LPS treated macrophages. Peritoneal macrophages were cultured with fenoldopam (A and B) (0 or 1 μM) or norepinephrine (C and D) (0 or 1 μM) for 60 min followed by exposure to LPS (300 ng/ml) for the indicated time. Representative Western blots show the amounts of pT172-AMPK, total AMPK, and β-actin. Means ± sd, n = 3, *P < 0.05 compared with the control (untreated); #P < 0.05 compared with fenoldopam + LPS treatment for 30 or 60 min with LPS alone (30 or 60 min).

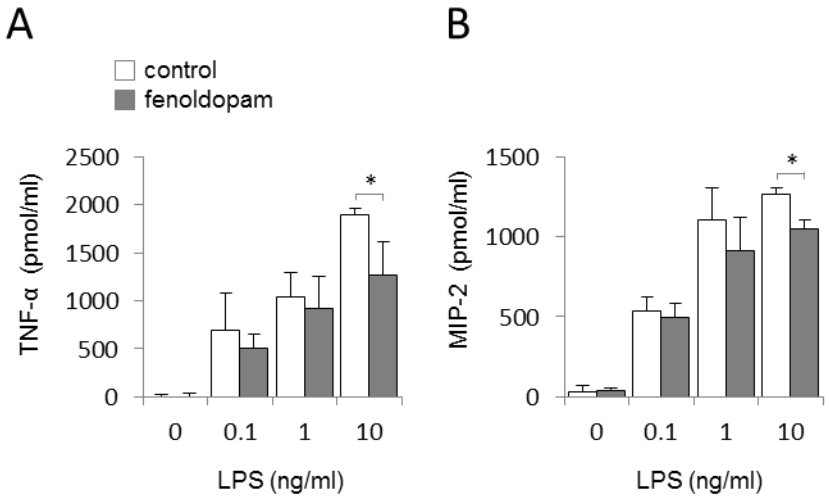


Figure 4. Activation of the D1 dopaminergic pathway reduces LPS-mediated macrophage pro-inflammatory cytokine production. Macrophages obtained from 3 mice were pretreated with dopaminergic agonist fenoldopam (0 or 1 μM) for 60 min and then treated with LPS (0, 0.1, 1, or 10 ng/ml) for an additional 4.5 h. The amounts of TNF-α (A) and MIP-2 (B) cytokines in culture media were determined using ELISA. Means ± sd, n = 3 independent experiments, each with 4 biologic replicates. *P < 0.05.

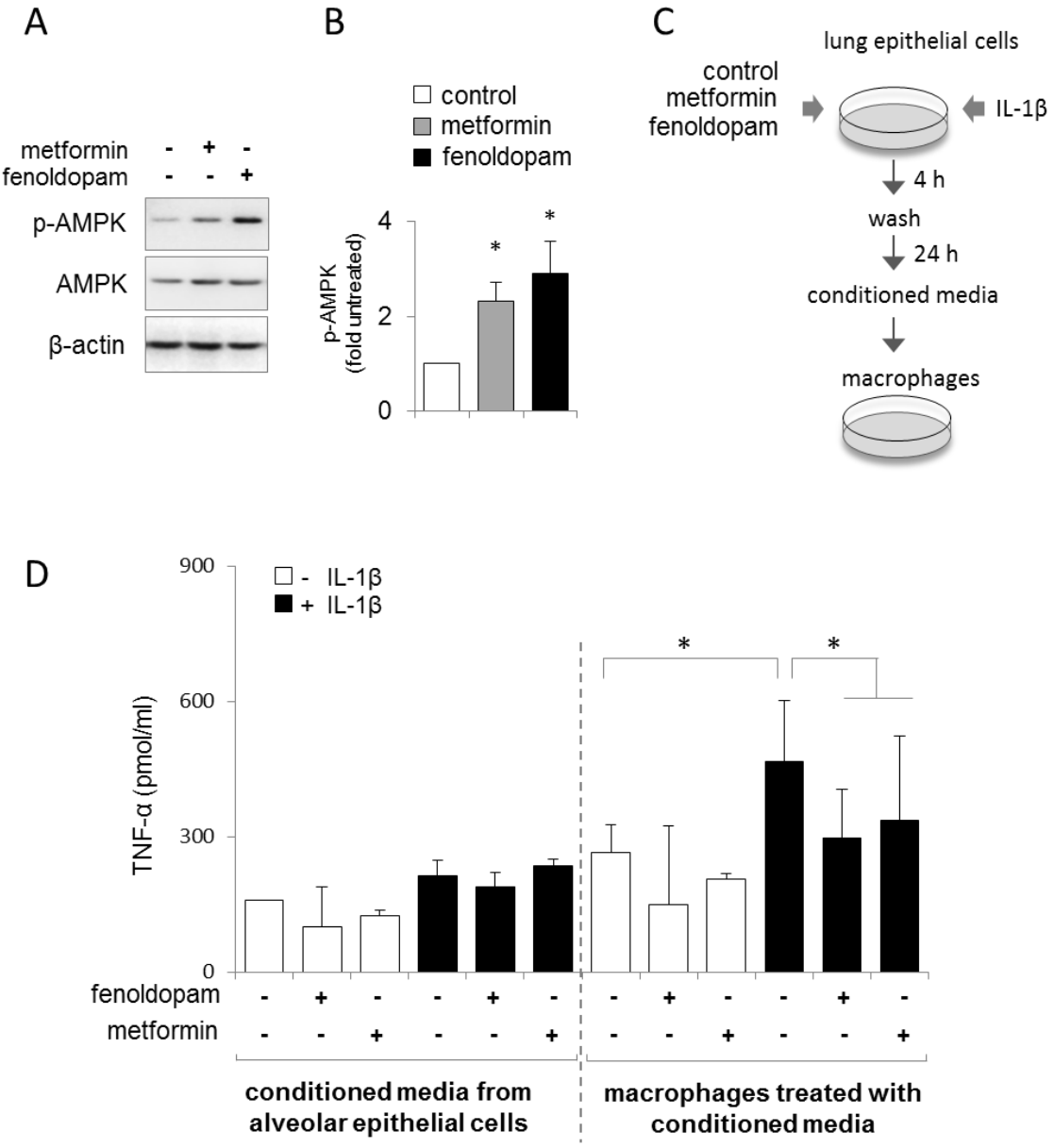


Figure 5. D1R–AMPK axis diminished the adverse epithelial paracrine signaling that promotes macrophage pro-inflammatory response. (A and B) Primary type II AECs were incubated with metformin (0 or 300 μM) or fenoldopam (0 or 1 μM) for 4 h followed by Western blot analysis of phospho-T172-AMPK and total AMPK. Means ± sd, n = 3, *P < 0.05 compared with the control (untreated). (C and D) AECs were pretreated with metformin or fenoldopam for 4 h followed by inclusion of IL-1β (0 or 10 ng/ml) for an additional 4 h. Cells were then washed, and the medium was collected after incubation for 24 h. Conditioned medium (1:1 normal:conditioned medium) was used to treat peritoneal macrophages for 24 h followed by TNF-α ELISA. Means ± sd, n = 3, *P < 0.05 compared with the control (untreated cells).

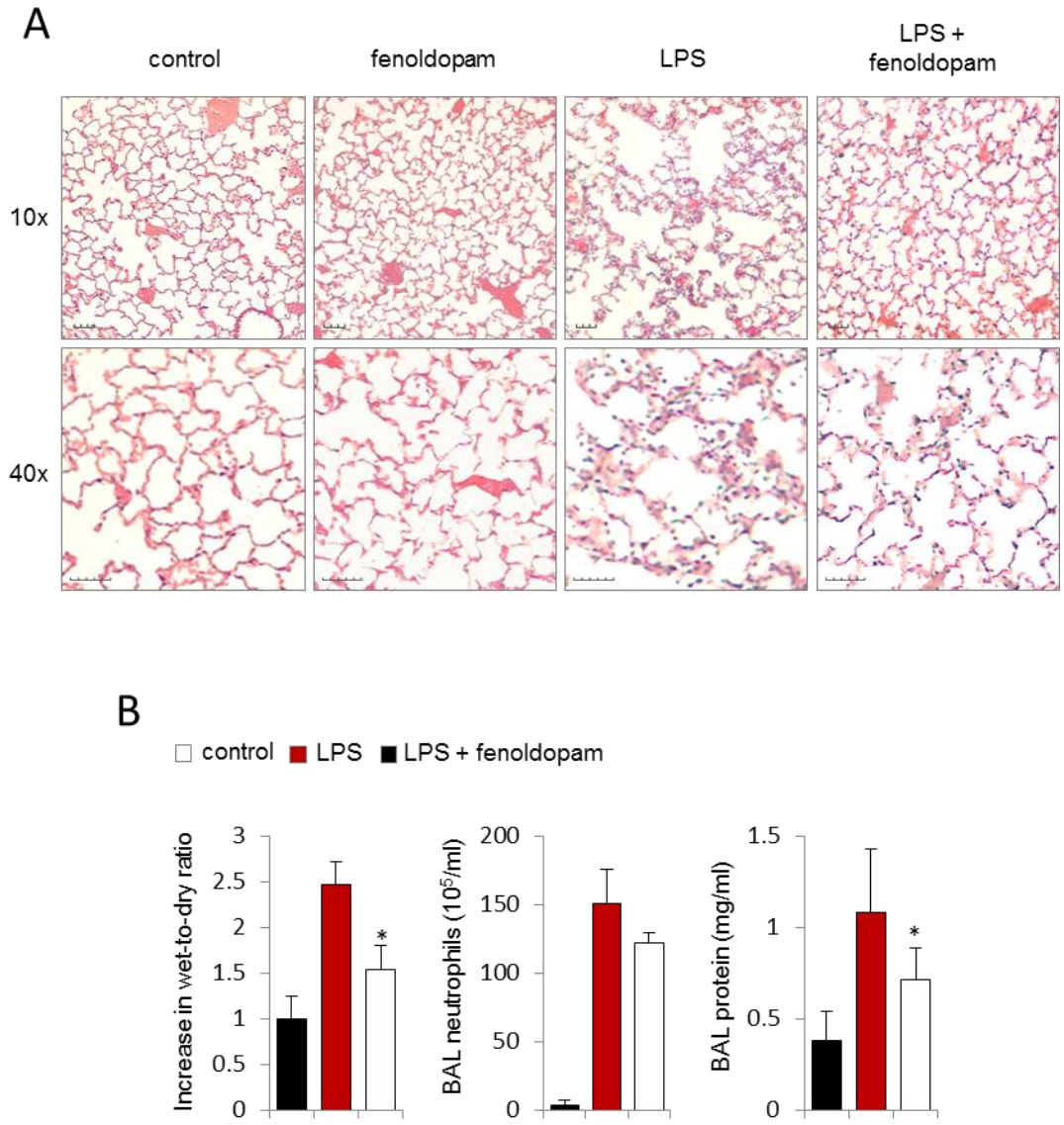


Figure 6. Activation of the D1R dopaminergic signaling pathway diminished severity of LPS-mediated ALI. Mice were first treated with vehicle (control; saline 500 µl, i.p.) or fenoldopam (10 mg/kg; 500 µl, i.p.) for 18 h and a second dose of saline or fenoldopam was applied 15 min before i.t. instillation of LPS (saline or 2 mg/kg; 50 µl, i.t.). (A) Representative images show H&E-stained lung sections obtained from control mice or mice treated with fenoldopam, LPS, or a combination of fenoldopam and LPS. Scale bars, 100 µm (10×) or 1000 µm (40×). (B) Increase in lung wet-to-dry ratios, number of lung neutrophils in BAL fluids, and BAL proteins were obtained 24 h after exposure to LPS alone or a combination of LPS and fenoldopam. Means ± sd, n = 4, *P < 0.05 comparing fenoldopam + LPS to mice treated with LPS alone.

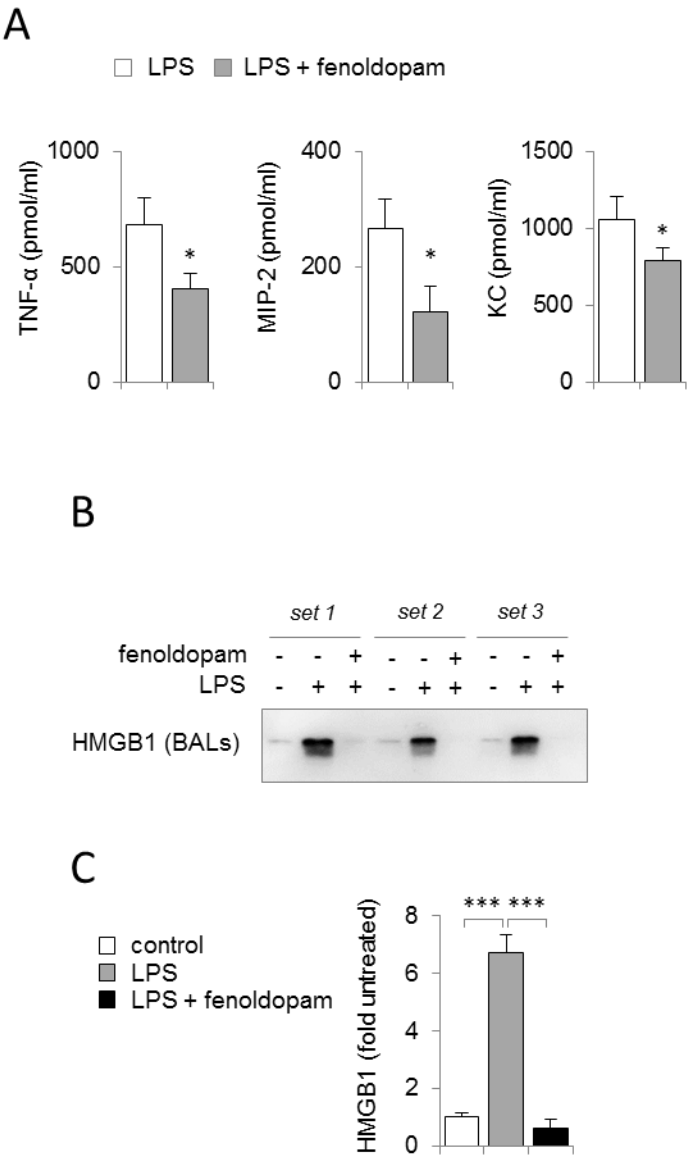


Figure 7. The D1R dopaminergic signaling agonist fenoldopam diminished production of pro-inflammatory cytokines in lungs of mice subjected to LPS-induced ALI. (A) TNF-α, MIP-2, and KC were measured in BAL fluids obtained from control (saline), LPS, or fenoldopam and LPS–treated mice (as described in Fig. 6). Means ± sd, n = 5, *P < 0.05. Representative Western blot (B) and quantitative analysis (C) show the amounts of HMGB1 in BALs obtained from 3 sets of mice: control, LPS alone, or treated with a combination of fenoldopam and LPS. Means ± sd; n = 3; ***P < 0.001.

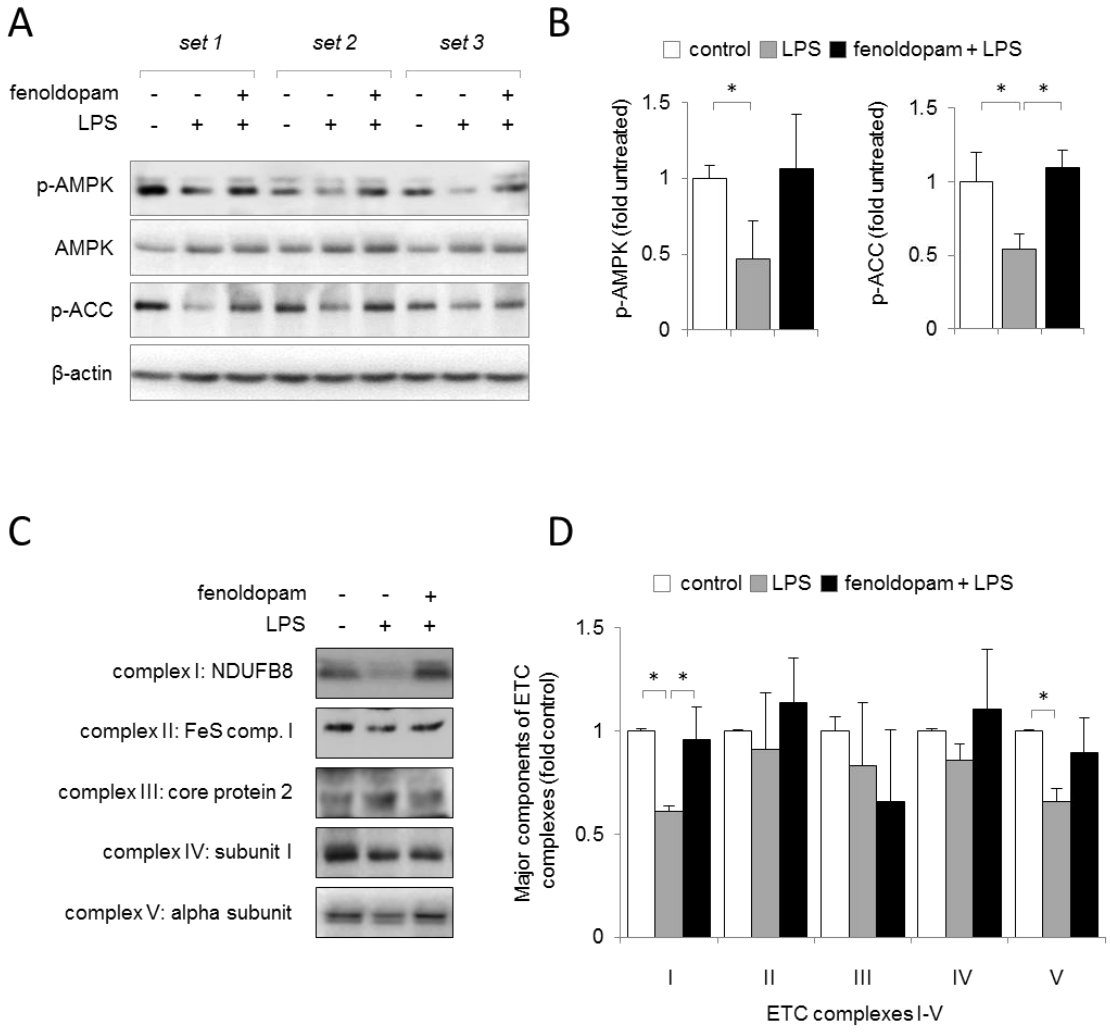


Figure 8. The D1R dopaminergic agonist fenoldopam prevented T172-AMPK dephosphorylation in lungs of mice subjected to LPS-induced ALI. Mice were treated with LPS or fenoldopam and LPS for 24 h (as described in Fig. 6). Representative Western blots (A) and quantitative analysis (B) show the extent of T172-AMPK and S79-ACC phosphorylation in 3 sets of lung homogenates. (C and D) The extent of major components of ETC complexes was determined in lung homogenates. Means ± sd, n = 3–4 mice/group, *P < 0.05.

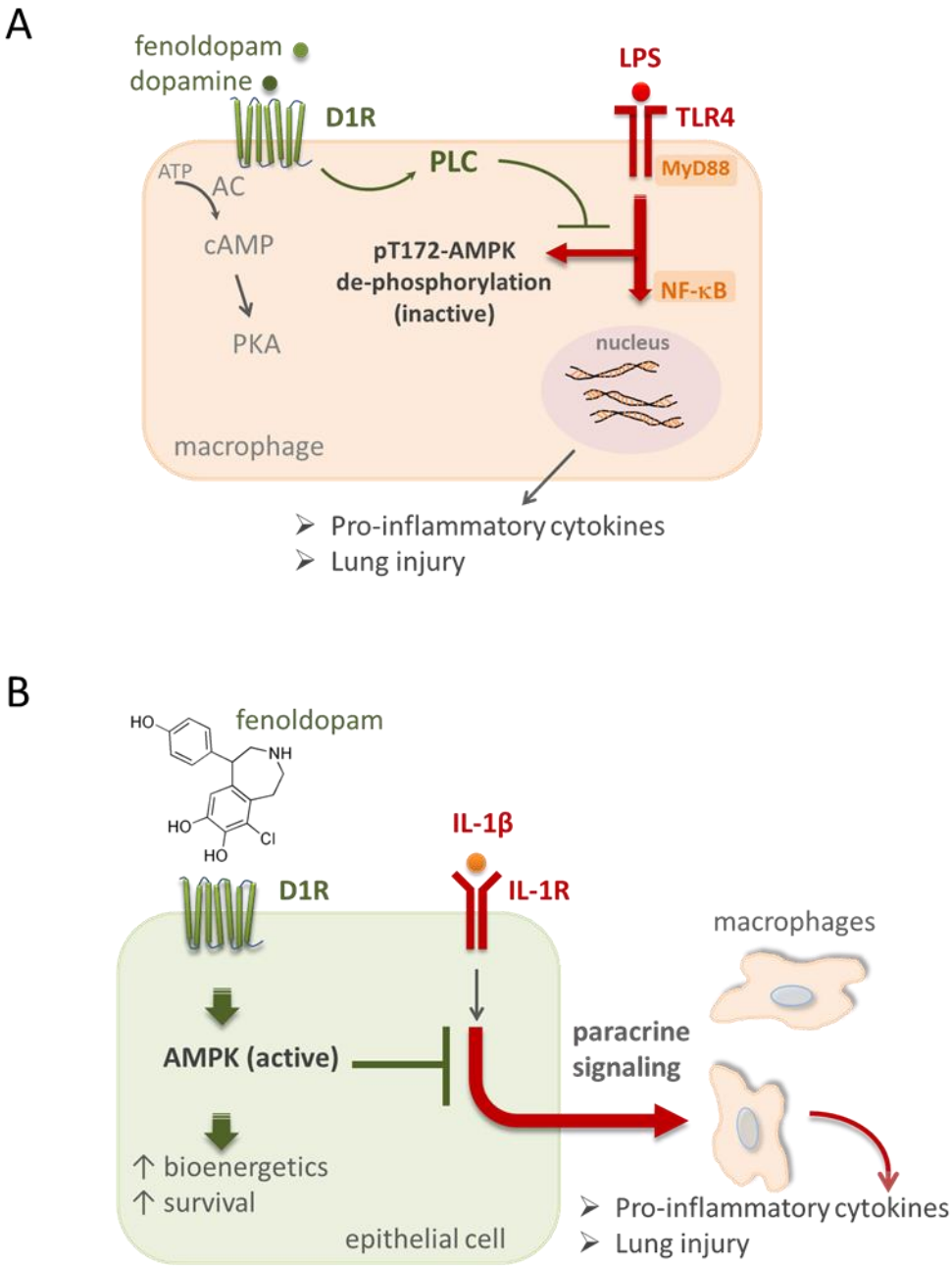
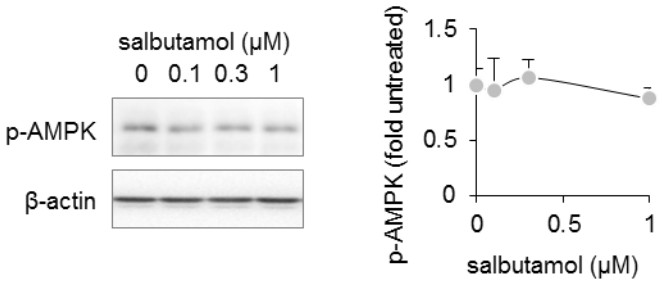
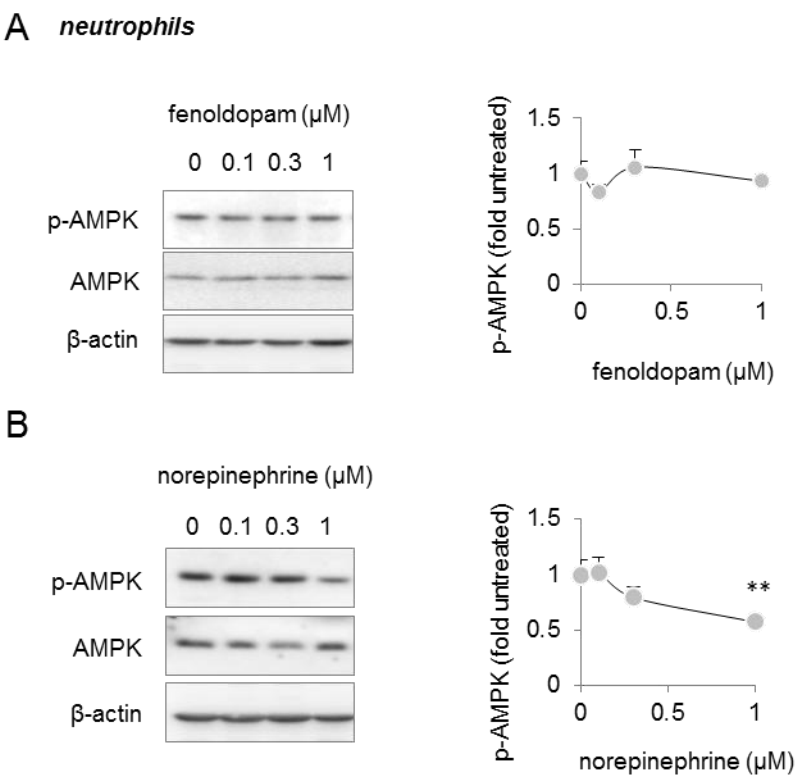


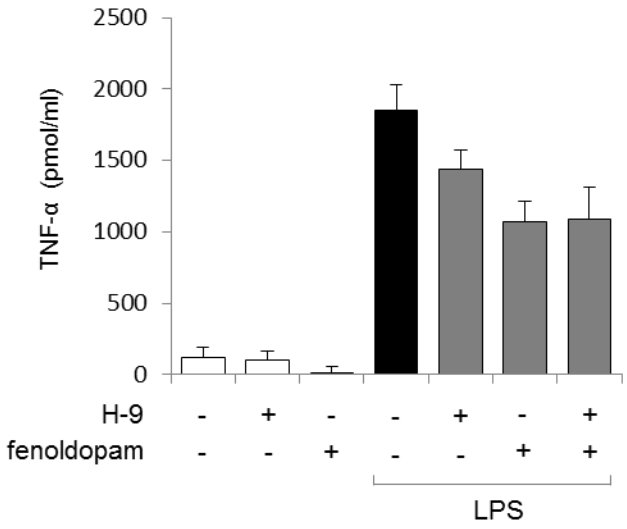
Figure 9. Schematic of D1R signaling in macrophages and lung epithelial cells. (A) LPS/TLR4 engagement promotes AMPK dephosphorylation and decreased kinase activity in peritoneal macrophages. (A) In turn, stimulation of dopaminergic D1R signaling with dopamine or fenoldopam followed by PLC activation prevented LPS-mediated dephosphorylation of AMPK. (B) Stimulation of D1R with fenoldopam and subsequent activation of AMPK diminished the adverse paracrine signaling from IL-1β–treated AECs and, therefore, prevented macrophage pro-inflammatory activation and reduced the severity of LPS-induced ALI.



Supplemental Digital Content 1. The effects of salbutamol on AMPK activation in macrophages. Representative Western blots for treatment with salbutamol showing Thr172 phosphorylation in response to dose (0, 0.1, 0.3, or 1 μM) in macrophages. Means ± SD (n = 3), ** $p < 0.01$.



Supplemental Digital Content 2. The effects of D1-dopaminergic or β-adrenergic agonists on T172-AMPK phosphorylation and pro-inflammatory cytokine production in LPS-stimulated neutrophils. (A and B) Bone marrow neutrophils were treated dose dependently with fenoldopam or norepinephrine for 2 hours followed by Western Blot analysis of pT172-AMPK, AMPK and β-actin. Means ± SD (n = 3), ** $p < 0.01$.



Supplemental Digital Content 3. The anti-inflammatory effects of fenoldopam are independent of the cAMP-PKA pathway. Macrophages were pre-treated with H-9 (0 or 10 μM) for 60 minutes followed by dopaminergic agonist fenoldopam (0 or 1 μM) for an additional 60 minutes and then treated with LPS (0 or 1 ng/ml) for an additional 4.5 hours. The amounts of TNF-α cytokine in culture media were then determined using ELISA. Means ± SD, n = 4, * $p < 0.05$.

CHANGES IN CELLULAR BIOENERGETICS CONTRIBUTES TO THE SEVERITY OF SEPSIS

In spite of significant progress in understanding the mechanisms taking place during sepsis, specific therapeutic interventions for critically ill patients are not available. Clinical trials aimed at targeting various aspects of sepsis, e.g. LPS/TLR4, TNF, IL-1, platelet activating factor, coagulation, and immune function, appeared to have modest effects at best. Currently, only supportive interventions with vasopressors, antibiotics, and ventilators are used for sepsis or ARDS patients. In the previous chapter we examined the impact of catecholamines, i.e. norepinephrine and dopamine, on immune cell bioenergetics. We found that both norepinephrine and dopamine have anti-inflammatory effects, however, stimulation of the D1R signaling pathway via fenoldopam activates the bioenergetic regulator AMPK in macrophages and lung tissue and diminishes the severity of lung injury[26, 30, 137].

The mitochondria are known for their involvement in generating ATP for the cell, but also produce ROS and metabolic intermediates which can be used by other cellular processes. Mitochondria consist of an inner (cristae) and outer membrane that allows for the generation of an electrochemical gradient within the mitochondria which is utilized to generate ATP. During sepsis, mitochondrial dysfunction (possibly via nitrosylation of electron transport chain complexes) is followed by bioenergetic reprogramming to glycolytic metabolism[95, 138-140]. While this switch is linked to bioenergetic adaptation for the inflammatory response, a prolonged loss of oxidative phosphorylation has a detrimental

impact and is correlated with the severity of organ injury and failure. Thus mitochondria/bioenergetic dysfunction has emerged as an essential target in sepsis. One possible approach to restore mitochondrial function is activation of AMPK signaling, which includes mitochondrial biogenesis and autophagy. However, our initial studies revealed that AMPK activity is significantly diminished in LPS treated immune cells and in the lung of mice subjected to endotoxin-induced ALI. We hypothesize that specific mechanisms involved in AMPK inactivation during sepsis are associated with loss of bioenergetic plasticity of immune and lung parenchyma cells. Identification of inhibitory mechanism(s) is a crucial step to develop new therapeutic interventions for bioenergetic dysfunction in sepsis and ARDS.

AMP-ACTIVATED PROTEIN KINASE AND GLYCOGEN SYNTHASE KINASE 3β MODULATE THE SEVERITY OF SEPSIS-INDUCED LUNG INJURY

by

NATHANIEL B. BONE, ZHONGYU LIU, SHAONING JIANG, DAE WON PARK, JEAN-MARC TADIE, JESSY DESHANE, CILINA ANN RODRIGUEZ, JEAN-FRANCOIS PITTET, EDWARD ABRAHAM, AND JAROSLAW W. ZMIJEWSKI

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Format adapted and errata corrected for dissertation

Abstract

Alterations in metabolic and bioenergetic homeostasis contribute to sepsis-mediated organ injury. However, how AMP-activated protein kinase (AMPK), a major sensor and regulator of energy expenditure and production, affects development of organ injury and loss of innate capacity during polymicrobial sepsis remains unclear. In the present experiments, we found that cross-talk between the AMPK and GSK3β signaling pathways controls chemotaxis and the ability of neutrophils and macrophages to kill bacteria ex vivo. In mice with polymicrobial abdominal sepsis or more severe sepsis induced by the combination of hemorrhage and intra-abdominal infection, administration of the AMPK activator metformin or the GSK3β inhibitor SB216763 reduced the severity of acute lung injury (ALI). Improved survival in metformin treated septic mice was correlated with preservation of mitochondrial complex V (ATP synthase) function and increased amounts of ETC complex III and IV. Although immunosuppression is a consequence of sepsis, metformin effectively increased innate immune capacity to eradicate *P. aeruginosa* in the lungs of septic mice. We also found that AMPK activation diminished accumulation of the immunosuppressive transcriptional factor HIF-1α as well as the development of endotoxin tolerance in LPS treated macrophages. Furthermore, AMPK-dependent preservation of mitochondrial membrane potential also prevented LPS-mediated dysfunction of neutrophil chemotaxis. These results indicate that AMPK activation reduces the severity of polymicrobial sepsis-induced lung injury and prevents the development of sepsis-associated immunosuppression.

Introduction

Severe infection accompanied initially by an overly exuberant inflammatory response and later by immunosuppression is frequently associated with dysfunction of vital organs and has a direct impact on morbidity and mortality in critically ill patients (1). Sepsis is the most frequent cause of death in hospitalized patients (2). Sterile inflammatory conditions linked to hemorrhage, trauma or burns worsen organ dysfunction in polymicrobial sepsis (3,4). Acute respiratory distress syndrome (ARDS) (5,6) frequently accompanies sepsis, and is associated with higher mortality rates in this setting (7). Effective pharmacologic interventions are not available for sepsis, a condition that affects more than a million patients each year in the United States (8). Similarly, there is no available pharmacologic intervention that improves the outcome from ARDS (9).

Excessive production of inflammatory mediators, including cytokines such as IL-1β and IL-17, disruption of endothelial and epithelial barriers with increased permeability, along with alterations in cellular bioenergetics and immunosuppression appear to contribute to organ dysfunction and mortality in sepsis (1, 10–12). While innate immune cells play a central role in host response to infection, exaggerated macrophage and neutrophil pro-inflammatory activation is also implicated in increased severity of sepsis-induced organ injury (13–15). The late or adaptive phase of sepsis is associated with apoptosis of lymphocytes and with epithelial and endothelial cell dysfunction as well as with diminished activation of neutrophils, macrophages and other cell populations involved in innate immunity. Such late phase immunosuppression appears to contribute to enhanced susceptibility to secondary infections that result in increased mortality (16, 17). Previous experiments have shown that loss of mitochondrial structure and function in

immune cells is implicated in organ failure in sepsis (18, 19). Of note, the extent of mitochondrial dysfunction in the lungs has been shown to correlate with mortality in sepsis (19, 20). Approaches to prevent mitochondrial dysfunction or to restore mitochondrial bioenergetics may diminish the severity of sepsis-associated lung injury (21–23).

The ability of the AMP-activated protein kinase (AMPK) to detect metabolic alterations and to modulate cellular bioenergetic and redox states appears to contribute to mortality and organ dysfunction in sepsis as well as to recovery from this life-threatening condition (24–26). AMPK is a heterotrimer that consists of one catalytic α and two regulatory β and γ subunits (27). This serine/threonine kinase has a unique mechanism of activation that is coupled to increases in energy demand, typically either due to excessive energy expenditure and/or deficient energy production. Such situations are associated with increased AMP-to-ATP ratios followed by AMP-dependent binding to the AMPKγ subunit, allosteric domain rearrangement and phosphorylation of T172-AMPKα by upstream kinases (28). Both AMP binding and phosphorylation of T172 are required for optimal AMPK activation. Activated AMPK participates in limiting energy expenditure while promoting pathways of energy production, including fatty acid oxidation, glycolysis and enhanced oxidative phosphorylation (24). Although enhanced AMPK activation induced by pharmacologic agents, such as metformin, an important therapeutic approach to type 2 diabetes, recent studies also show that administration of metformin can retard aging in experimental models and has been suggested to be associated with an increased lifespan of diabetic patients (29, 30). In addition to the effects of AMPK activation on glucose and lipid metabolism, studies, including those from our laboratory, indicate that activated AMPK has anti-inflammatory effects in TLR4-activated neutrophils and mac-

rophages, and also diminishes the severity of endotoxin-induced lung injury in preclinical models (31–33).

Although sepsis is accompanied by alterations in bioenergetics of immune and parenchyma cells, as well as an increase in generation reactive oxygen and nitrogen species (ROS/RNS), that should result in AMPK activation, activation of AMPK is often not found in such settings (34–37). More recently, we have shown that the IκB kinase beta (IKKβ)/glycogen synthase kinase beta (GSK3β) signaling axis contributes to preventing AMPK activation both after TLR4 engagement in neutrophils and macrophages, and in the lungs of mice subjected to sterile inflammatory conditions, including endotoxemia (38). However, it is not known whether this mechanism is operational during polymicrobial inter-abdominal infection, a clinically relevant issue in sepsis-induced ARDS. Because polymicrobial sepsis is linked to harmful inflammation and diminished capacity of the innate system for bacterial eradication, we also determined if AMPK activation contributes to subsequent development of immunosuppression. In particular, we examined whether AMPK activation affects clearance of *P. aeruginosa* lung infection following polymicrobial abdominal sepsis.

Materials and Methods

Mice

Male C57BL/6 mice were purchased from the National Cancer Institute in Frederick, Maryland. Mice 10 to 12 weeks of age were used for experiments. The mice were housed in the animal facility at the University of Alabama at Birmingham. All experiments were conducted in accordance with protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Reagents and Antibodies

The GSK3β inhibitor BIO (6-bromoindirubin-3′-oxime) was purchased from R&D Systems whereas SB216763 was from Sigma-Aldrich. PS-1145 (IKK1/2 inhibitor), metformin (AMPK activator), LPS (TLR4 agonist) and brewer thioglycollate medium were obtained from Sigma-Aldrich. W-peptide (chemoattractant) was purchased from Phoenix Pharmaceuticals. Antibodies for phospho Thr172-AMPK, phospho Ser485-AMPK, total AMPK, phospho Ser79-ACC and total ACC were purchased from Cell Signaling Technology. HRP-conjugated β-actin antibody was from Santa Cruz Biotechnology. Anti-phospho-Thr479-AMPK antibody was generated as described previously (39) and was a gift from Ken Inoki of the University of Michigan. Anti-HMGB1 antibody was purchased from R&D Systems. Total OXPHOS Rodent WB Antibody Cocktail was obtained from Abcam. Hoechst dye and JC-1 probe were from Life Technologies. siRNA to the AMPKα1 subunit, scrambled siRNA and Accell medium were purchased from Thermo Scientific/Dharmacon.

Neutrophil and Peritoneal Macrophage Isolation

Bone marrow neutrophils were purified using a negative selection column (31,38). In brief, bone marrow cell suspensions were isolated from the femur and tibia of mice by flushing with RPMI 1640 medium with 5% fetal bovine serum (FBS). The cell suspension was passed through a glass wool column and collected by washing with phosphate buffer solution (PBS) containing 5% FBS. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary Abs specific for the cell-surface markers F4/80, cluster of differentiation 4 (CD4), CD45R, CD5 and TER119 (Stem Cell Technologies) for 15 min at 4°C followed by subsequent incubation with anti-biotin tetrameric Abs (100 μL; Stem Cell Technologies) for 15 min. The complex of anti-tetrameric antibodies and cells was then incubated with colloidal magnetic dextran iron particles (60 μL; Stem Cell Technologies) for an additional 15 min at 4°C. The T cells, B cells, RBCs, monocytes and macrophages were captured in a column surrounded by a magnet, allowing the neutrophils to pass through. Neutrophil purity, as determined by Wright-Giemsa-stained cytospin preparations, was consistently >98%.

Peritoneal macrophages were isolated as described previously (38). Macrophages were elicited in 10- to 12-wk-old mice by use of Brewer thioglycollate. Cells were collected 4 d after intraperitoneal (IP) injection of thioglycollate, cultured for 3 d ex vivo and then treated as described in the figure legends.

Transwell Migration Assay

Bone marrow neutrophils (106 cells/well) were incubated with PS1145 (0 or 10 μmol/L), BIO (0 or 5 μmol/L), SB216763 (0 or 30 μmol/L) or metformin (0 or 500 μmol/L) for 2 h followed by exposure to LPS (300 ng/mL) for an additional 60 min. Transwell migration assay was performed using 24-well cell plate BD Falcon cell culture inserts (pore size 3 μm) (Translucent PET Membrane, BD Biosciences). Briefly, bone marrow neutrophils (106/mL) were placed into the upper reservoir, and chemotaxis initiated by inclusion of W-peptide (50 nmol/L) in the lower reservoir of transmigration chamber. Chemotaxis was determined after neutrophils were allowed to migrate for 60 min in RPMI media supplemented with FBS (5%). Cells in capillary structures of transmigrating chamber were subjected to Wright-Giemsa-staining followed by image acquisition using light microscopy. Each condition was tested three or more times using independent cell populations.

In Vitro Killing Assay

Neutrophils (5 × 106 cells/mL) cultured in RPMI 1640 (0.5% FBS) were pretreated with PS1145 (0 or 10 μmol/L) or SB216763 (0 or 30 μmol/L) for 60 min or metformin (0 or 500 μmol/L) for 2 h, followed by inclusion of *Pseudomonas aeruginosa* (PAK; 5 × 107/mL; 1:10 ratio neutrophil/PAK) for an additional 90 min. Similar to neutrophils, PS1145, SB216763 or metformin treated peritoneal macrophages (5 × 106 cells/mL) were incubated with PAK (macrophage/PAK; 1:10 ratio) for 90 min. The cell/bacterial solutions were centrifuged at 375g for 5 min, and then the cell pellets were lysed by add-

ing 100 μL of Triton-X 100 (0.1%). The lysates were then plated on agar plates with ampicillin and incubated overnight at 37°C. Colony-forming units (CFUs) were calculated by counting bacterial colonies grown on agar plates using colony counter software (Bio-Rad) and expressed as a percentage of colonies obtained from untreated neutrophils or macrophages.

siRNA Knockdown of AMPKα1

Peritoneal macrophages were incubated with scramble (1 μmol/L) or AMPKα1-specific siRNA (1 μmol/L), as described previously (40). Briefly, cells (5 × 105/well) in 12-well plates were incubated in Accell medium (serum free) containing siRNA (1 μmol/L) for AMPKα1 for 72 h. Cells were then subjected to AMPK Western Blot analysis or exposure to GSK3β inhibitor followed by TNF-α enzyme-linked immunosorbent assay (ELISA).

Cecal Ligation and Puncture (CLP)-Induced Sepsis

CLP was performed in 10- to 12-wk-old male C57BL/6 mice as described before (41). Briefly, the cecum was ligated 1.0 cm from the tip of cecum, which was an approximately 50% cecum ligation. A through-and-through puncture was performed with a 21-gauge needle and then a drop of feces was extruded to the peritoneal cavity. Saline (0.9%; 500 μL) was then applied into the peritoneal cavity and the abdominal wall inci-

sion was closed in two layers. The control group of mice (sham) underwent surgery without CLP.

A Mouse Model of Hemorrhage and Resuscitation

Hemorrhage was performed using the previously described method (42). C57BL/6 male mice were anesthetized by inhalation of isoflurane (5%), and then both femoral arteries were cannulated with catheters (Braintree Scientific). The systemic arterial pressure line was continuously measured, independently from the hemorrhage/resuscitation catheter line. Blood withdrawal was performed for 60 minutes with a 25 ± 5 mmHg mean arterial pressure (MAP), typically a resultant of nearly 60% (~800 μL) blood loss. Next, mice were fully resuscitated with Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich) for 30 min. CLP procedure was conducted within 24 h, as described above.

Application of Metformin or GSK3β Inhibitor SB216763 in Mice Subjected to Sepsis-Induced Lung Injury

Mice were treated with metformin (100 mg/kg) or control (saline) IP applications in three doses; 48 h, 24 h and 30 min prior to CLP. In selected experiments, the second dose of metformin was given before hemorrhage. Mice were given the GSK3β inhibitor SB216763 (20 mg/kg) dissolved in 500 μL of DMSO/saline (1:40) or control vehicle (DMSO/saline 1:40) IP three times, that is, 48 h, 24 h and 30 min prior to CLP. Mice

were euthanized 24 h after CLP, followed by preparation of lung homogenates for Western blot analysis, lung sections for H&E staining and collection of BAL fluids for cytokine ELISA. In particular, BAL fluids were collected by lavaging the lungs three times with 1 mL of PBS followed by measurement of inflammatory cytokines and protein content. Independent groups of mice were used to measure wet-to-dry ratios to determine the extent of pulmonary edema. In particular, after measuring the weight of freshly harvested (wet) lungs, the lungs were kept in an incubator for 7 d at 80°C. Next, the weight of dry lungs was measured followed by calculation of wet-to-dry ratio. Independent groups of mice were used to prepare lung homogenates in RIPA buffer (Sigma-Aldrich) followed by Western blot analysis of phosphorylated and total amounts of AMPK.

Cytokine ELISA

ELISA was used to measure cytokines in bronchoalveolar lavage (BAL) fluids. The amounts of tumor necrosis factor alpha (TNF-α), MIP-2, IL-6 and KC were determined by using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions and as previously described (38,43).

Macrophage Endotoxin Tolerance Assay

Peritoneal macrophages (3×10^5/well) were first treated with LPS (0 or 10 ng/mL) for 24 h then media washed three times followed by incubation for an additional 60 min. Next, cells were exposed to a second stimulation with LPS (10 ng/mL) for 4 h. In

selected experiments, macrophages were also treated with metformin (1 mmol/L), AICAR (500 μmol/L) or SB216763 (30 μmol/L) for 60 min followed by incubation with LPS (first stimulation) for an additional 24 h.

Measurement of Mitochondrial Membrane Potential (mΔΨ)

Bone marrow neutrophils were seeded 80% (confluent) in a 4-well chambered coverslip coated with fibronectin (40 μg/mL). The cells were left unaltered or treated with AICAR (250 μmol/L), metformin (500 μmol/L) or BIO (20 μmol/L) for 60 min followed by inclusion of LPS (300 ng/mL) for an additional 60 min. The JC-1 probe (100 ng/mL) and Hoechst (1 μg/mL) were applied 30 min before image acquisition. Microscopy was performed using a confocal laser scanning microscope (model LSM 710 confocal microscope; Carl Zeiss MicroImaging). Quantitative fluorescent intensity (red/green pixel intensity) of the images was processed using IPLab Spectrum. In an additional experiment, mitochondrial membrane potential was also measured after mitochondrial depolarization with FCCP (100 nmol/L).

In Vivo Bacterial Killing Assay

Briefly, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was gently extended and wild-type PAK strain of *Pseudomonas aeruginosa* (2.5 × 107/mouse) suspension in PBS (50 μL) or PBS alone (control; 50 μL) was deposited into the pharynx followed by bacterial aspiration

into the lungs, similar to the method that was described previously (32). Lung homogenates were prepared 4 h after P. aeruginosa instillation and serial dilutions used to determine CFUs/mL. The number of bacterial colonies grown on agar plates (CFUs) was measured using colony counter software (Bio-Rad).

Protein Concentration and Cell Counts in BAL Fluid

Briefly, protein concentration in BAL fluid was determined by Bradford method with Bio-Rad protein assay dye reagent concentrate (Bio-Rad). The numbers of neutrophils in BAL fluid were determined after cytospin and Wright-Giemsa staining followed by image acquisition using light microscopy.

Western Blot Analysis

Western blot analysis was performed as described previously (34,38). Each experiment was carried out three or more times with peritoneal macrophages or lung homogenates obtained from separate groups of mice. In selected experiments, BAL fluids (30 μL) were mixed with Laemmli sample buffer and boiled for 5 min followed by Western blot analysis of HMGB1.

Statistical Analysis

Multigroup comparisons were performed using one-way analysis of variance (ANOVA) with Tukey post hoc test. Statistical significance was determined by the Student t test for comparisons between two groups. A value of $P < 0.05$ was considered significant. Analyses were performed on SPSS version 16.0 (IBM) for Windows (Microsoft).

Results

Participation of AMPK and GSK3β Signaling Pathways in Neutrophil- and Macrophage-Dependent Bacterial Killing

We have recently found that in LPS/TLR4 stimulated macrophages had diminished Thr172-AMPK phosphorylation and increased GSK3β-dependent Thr479-AMPK inhibitory phosphorylation (Supplementary Figure 1) (38). However, it is not known whether similar mechanisms are operational during infection, including development of lung injury due to polymicrobial intra-abdominal infection. Therefore, we investigated if the clearance of bacteria ex vivo may be affected by AMPK activation, and specifically examined the role of IKKβ/GSK3β signaling pathways in this process. Neutrophils or peritoneal macrophages were incubated with or without the AMPK activator metformin (500 μmol/L), the IKK1/2 inhibitor PS1145 (10 μmol/L) or the GSK3β inhibitor SB216763 (30 μmol/L), and then were cultured with *P. aeruginosa* (PAK; 5 × 107) for 90 min followed by measuring bacterial survival using CFU assays. As shown in Figures 1A, B and C, inclusion of metformin, IKK1/2 or GSK3β inhibitors had no adverse effects on neutrophil- or macrophage-dependent killing of bacteria. Indeed, the numbers of CFUs (an indicator of bacteria survival) were reduced after incubation of the macrophages or neutrophils with metformin or PS1145. Exposure of neutrophils to SB216763 reduced the amounts of bacteria, though such treatment was less effective in macrophages (Figures 1B, C). Both metformin and the AMPK activator AICAR, as well as the GSK3β inhibitor SB216763, resulted in degradation of NLRP3, a major regulator of cell death, inflammatory organ injury and production of IL-1β in stimulated macrophages (44). These results suggest that in spite of the anti-inflammatory effects associated with

AMPKα1 activation during sterile inflammatory processes, metformin-enhanced AMPK activity does not diminish neutrophil- and macrophage-dependent bacterial killing. Of note, while AMPKα1 and AMPKα2 subunits are typically found in stromal cells, neutrophils and macrophages exclusively express the AMPKα1 subunit (40).

AMPK Activation by Metformin or through GSK3β Inhibition Decreased the Severity of Lung Injury following Polymicrobial Sepsis

Given the ability of AMPK to modulate pro-inflammatory activation of macrophages and neutrophils, but without adverse effects on bacterial killing, we next examined if AMPK activation diminishes the severity of lung injury in mice with intra-abdominal sepsis. Mice were treated with either metformin (100 mg/kg; IP) or SB216763 (20 mg/kg; IP) once a day for 2 d, followed by cecal ligation and puncture (CLP). All mice subjected to CLP (50% cecum ligation and double puncture) or combined CLP and metformin survived 24 h. Neutrophil accumulation, increased lung permeability and significantly increased amounts of TNF-α, MIP-2, IL-6 and KC were found in bronchoalveolar lavages of CLP treated mice (Figure 2). However, pretreatment with the AMPK activators metformin or SB216763 prevented such adverse effects of CLP (Figures 2A–E).

AMPK Activation Diminishes the Severity of Lung Injury following Hemorrhage and Intra-abdominal Sepsis

Because the model of CLP used in our experiments resulted in modestly severe ALI and a lack of mortality at 24 h, we examined the effects of metformin-induced AMPK activation in a more severe model of sepsis produced by the combination of hemorrhage and CLP (45). In particular, mice were first subjected to hemorrhage (~60% total blood volume) followed 60 min later by resuscitation with buffered saline. CLP was performed 24 h after hemorrhage. As compared with the lack of mortality 24 h after CLP alone, hemorrhage and subsequent CLP resulted in a 30% mortality rate (Figure 3B). Pretreatment with metformin prevented mortality in hemorrhage/CLP group, and also diminished the severity of pulmonary edema and increased lung permeability, as determined by lung wet-to-dry ratios and BAL protein content (Figures 3B, C). Consistent with the anti-inflammatory effects of AMPK activation, reduced amounts of TNF-α, MIP-2, IL-6 and KC were found in the BALs of mice subjected to hemorrhage/CLP and pretreated with metformin, as compared with control hemorrhage/CLP mice (Figure 3D). Western blot analysis showed accumulation in BAL fluid of high mobility group box 1 (HMGB1), an important marker and mediator of organ injury, in mice subjected to hemorrhage and CLP, and diminished levels of pulmonary HMGB1 accumulation in metformin-pretreated mice (Figure 3E).

AMPK Activation Prevents Dissipation of Mitochondrial ATP Synthase (Complex V) and Increases the Amounts of ETC Complexes III and IV in the Lungs of Septic Mice

Severe sepsis is associated with mitochondrial dysfunction in critically ill patients (18). Consistent with this, Western blot analysis of mitochondrial ETCs demonstrated a substantial decrease in ATP synthase (complex V) in the lungs of mice with sepsis, as compared with controls (Figures 4A, B). We also observed similar decreases of ATP synthase in the lungs of mice subjected to intratracheal (i.t.) instillation of LPS (2 mg/kg, i.t.) 24 h previously (data not shown). Treatment of mice with metformin partially prevented sepsis-induced depletion of complex V and also increased the total amounts of ETC complex III and IV (Figures 4A, B).

GSK3β Regulates AMPK Activity in the Lungs of Septic Mice

Western blot analysis showed that phosphorylation of Thr172-AMPK and S79-ACC (a downstream target of AMPK) was diminished in the lungs of mice with sepsis, as compared with control animals (Figures 5A, B). In contrast, increased T172-AMPK and S79-ACC phosphorylation was found when metformin was administered before CLP. Of note, metformin increased T172-AMPK phosphorylation in mice with sepsis to levels similar to those found in control groups. In addition to the modest increases in phospho-T172-AMPK detected in the metformin/sepsis group, increased phosphorylation of S485-AMPK and T479-AMPK, events associated with inhibition of AMPK activation, were present in the lungs of septic mice pretreated with metformin (Figures 5A, ,6C).6C). These results suggest that metformin might interact with a limited pool of AMPK, and

particularly AMPK that was not affected by IKKβ and GSK3β inhibitory phosphorylation of S485 and T479 in AMPK. To examine this issue, AMPK phosphorylation was determined in lung homogenates obtained from mice treated with the GSK3β inhibitor SB216763 before the induction of sepsis. Western blot analysis demonstrated that SB216763 increased phosphorylation of T172-AMPK and its downstream target S79-ACC (Figures 5D, E). Of note, exposure to SB216763 resulted in higher levels of phospho T172-AMPK in the lungs of septic mice as compared with that present in controls (Figures 5D, E). As anticipated, SB216763 diminished GSK3β-mediated T479-AMPK phosphorylation, but had no effect on IKKβ-dependent phosphorylation of S485-AMPK (Figures 5D, F). These results suggest that metformin activates a specific AMPK pool not affected by GSK3β-dependent inhibition in the lungs of septic mice (Figure 5G).

AMPK Activation Prevents Macrophage Reprogramming into an Endotoxin Tolerogenic Phenotype

Recent studies indicate that pro-inflammatory stimulation, including LPS/TLR4 engagement, is associated with reprogramming of monocytes that results in diminished responses to second challenge with LPS, a condition known as endotoxin tolerance (46,47). AMPK activation has been shown to reduce pro-inflammatory cytokine production effectively in LPS-stimulated macrophages (38,48,49). However, it is not clear whether AMPK activation participates in the development of endotoxin tolerance. To examine this question, macrophages were first pretreated with LPS (0 or 10 ng/mL), LPS and metformin (1 mmol/L) or LPS and SB216763 (30 μmol/L per mL) for 24 h (Figure

6A). Next, the cells were washed and incubated for an additional 60 min prior to exposure to second dose of LPS (0 or 10 ng/mL). As shown in Figure 6B, robust pro-inflammatory activation was found in LPS treated macrophages, whereas pre-exposure to LPS-induced endotoxin tolerance. In contrast, we observed more than a 50% increase of LPS-stimulated TNF-α production in macrophages that were pretreated with the combination of LPS and metformin or LPS and SB216763. These results indicate that AMPK activation inhibits the development of LPS-induced endotoxin tolerance.

AMPK Activation Diminishes HIF-1α Production in Macrophages

Recent studies have shown that enhanced expression of hypoxia-inducible factor 1α (HIF-1α) participates in sepsis-induced immunosuppression (50). Thus, we investigated whether AMPK activation affects HIF-1α accumulation in LPS treated macrophages. As shown in Figures 6C and D, incubation of macrophages with metformin, AICAR or the GSK3β inhibitor SB216763 resulted in diminished LPS-induced accumulation of HIF-1α. These results suggest that cross-talk between AMPK and GSK3β may be implicated in regulating development of macrophage immunosuppressive phenotypes.

AMPK and GSK3β Signaling Pathways Participate in Modulating Neutrophil Chemotaxis

Innate immune activation is the host's first-line defense against microbial infection. However, sepsis-induced accumulation of inflammatory mediators and microbial

products in the circulation has adverse effects on chemotaxis of neutrophils (51,52). Given the importance of mitochondria in regulating neutrophil chemotaxis (53) we used a JC-1 probe to measure if LPS affected mitochondrial membrane potential. As shown in Figures 7A and B, culture neutrophils with LPS resulted in significant mitochondrial membrane depolarization. Furthermore, inclusion of metformin or GSK3β inhibitor (BIO) prevented the effects of LPS. Of note, BIO was used because SB216763 produced substantial fluorescence and interfered with JC-1 fluorescence. Next, we examined if the AMPK and/or GSK3β signaling axis also participate(s) in modulating neutrophil migration after LPS/TLR4 engagement. Neutrophils were pretreated with the AMPK activator metformin (0 or 500 μmol/L), GSK3β inhibitors SB216763 (0 or 30 μmol/L) or BIO (0 or 5 μmol/L) for 60 min. To establish the effects LPS/nuclear factor kappa B (NF-κB) signaling, cells were also pretreated with IKK1/2 inhibitor PS1145 (0 or 10 μmol/L). Next, cells were incubated with LPS (0 or 300 ng/mL) for 60 min, washed and then added into the upper reservoir of transmigration chambers. Neutrophil migration through pore structures was initiated by inclusion of the chemoattractant W-peptide (50 nmol/L, an equivalent of human IL-8) into the lower reservoir. As shown in Figure 7C, exposure to LPS nearly completely immobilized neutrophils, whereas pretreatment with metformin, PS1145, SB216763 or BIO diminished such inhibitory effects of LPS. These results indicate that both AMPK and GSK3β are involved in regulating LPS-dependent mitochondrial membrane depolarization and inhibition of neutrophil chemotaxis.

AMPK Activation Diminished the Onset of Immunosuppression in Mice with Sepsis

P. aeruginosa is frequently associated with sepsis and ventilator-associated secondary infection, including *P. aeruginosa* (16,54). Given the ability of AMPK activators to preserve neutrophil and macrophage function, we examined if metformin improves immune homeostasis and efficient killing of bacterial in vivo. Mice were treated with two doses of metformin (100 mg/kg; IP), given 24 h and 30 min prior to i.t. instillation of *P. aeruginosa* (PAK; 2.5 × 107). As shown in Figures 8A and B, there was significant decrease in CFUs in lung homogenates of PAK infected mice treated with metformin, compared with mice treated with PAK alone. Next, we determined if metformin affects bacterial clearance in the lungs of mice subjected to sepsis. As compared with control mice, CLP-induced sepsis resulted in a robust increase in CFUs (~10 fold) in lung homogenates. Metformin partially prevented such adverse effects of sepsis (Figures 8C, D). In additional experiments, we found that metformin also diminished the amounts of bacteria in samples obtained from peritoneal lavages of CLP mice (Figure 8E).

Discussion

In this study, we found that cross-talk between AMPK and GSK3β was involved in regulating lung inflammation and development of lung injury in experimental models of polymicrobial abdominal sepsis or by the more severe combination of hemorrhage and abdominal sepsis. The AMPK activator metformin and the GSK3β inhibitor SB216763 prevented the decrease in neutrophil chemotaxis induced by LPS, and also enhanced the ability of neutrophils and macrophages to kill bacteria. In vivo treatment with metformin improved survival of mice with polymicrobial abdominal sepsis stabilized mitochondrial complex V and increased the amounts of mitochondrial complexes III and IV. Although activated AMPK diminished production of pro-inflammatory mediators in LPS treated macrophages, this event was not associated with diminished bacterial killing. Indeed, metformin or SB216763 effectively prevented development of LPS-induced macrophage immunosuppressive phenotypes. Similarly, metformin increased bacterial clearance in the lungs of mice with sepsis.

In spite of increases in AMP-to-ATP ratios and ROS formation, which normally result in AMPK activation, there was no increase in AMPK activity in the experimental models of ARDS or in critically ill patients (35–37). Recent studies demonstrated that interactions between PI3K/AKT and GSK3β as well as between IKKβ and GSK3β promoted direct phosphorylation and inactivation of AMPK, thereby suggesting potential mechanisms for the lack of AMPK activation in preclinical models of sepsis and in patients with critical illness (38, 39). Such findings are consistent with the ability of GSK3β inhibitors to diminish monocyte proinflammatory activation and to reduce mortality in

experimental sepsis, ischemic organ injury and endotoxin-induced lung injury (38, 55, 56).

Previous studies, including results obtained in our laboratory, have shown that enhanced AMPK activation diminished the severity of lung injury in experimental models of sterile inflammation, such as after exposure of the lungs to LPS or peptidoglycan (PGN) (31, 32, 36). Our new data indicate that AMPK activation also decreases pulmonary injury in the setting of polymicrobial sepsis. Of note, despite the inhibitory activity of AMPK activation on pro-inflammatory cytokine release in the lungs, bacterial clearance was increased in the lungs of septic mice treated with metformin and in mice with *P. aeruginosa* pneumonia. It is important to note that while moderate inflammation is necessary to initiate the accumulation of immune cells to sites of infection, diminishing exaggerated and deleterious pro-inflammatory activation is not necessarily associated with loss of innate immune function (1, 8, 17). For example, a recent study has shown that mice treated with the specific NF-κB inhibitor BMS-345541 had reduced severity of lung injury following CLP-induced sepsis (57). Similarly, we found that treatment with an IKK1/2 inhibitor or activation of AMPK by metformin or GSK-3β inhibitors had no adverse effects on bacterial killing by neutrophils or macrophages ex vivo. Of note, activation of AMPK has been shown to increase phagocytosis in neutrophils and macrophages (58–61).

Our results show that AMPK activation provided substantial protection against sepsis-induced lung injury. However, the exact role of AMPK that plays during recovery of immune and peripheral tissue homeostasis needs to be further examined. A possible mechanism by which AMPK activation may modulate acute inflammatory responses,

such as sepsis-induced lung injury, is linked to cellular bioenergetics. Previous studies have shown that sepsis-mediated organ injury was associated with alterations in mitochondrial structure and function (19). Mitochondrial impairment in peripheral tissues and in immune cells has been correlated with morbidity and mortality associated with sepsis (18, 62). For example, significant loss of ATP synthase (complex V) has been found in circulating monocytes in patients with sepsis, and is likely to participate in disrupted immune bioenergetic homeostasis (63). Our results showed that AMPK activation effectively prevented loss of ATP synthase (ETC complex V) in the lungs of septic mice. Of note, besides direct immunoregulatory action, AMPK activation is also involved in preservation of epithelial and endothelial bioenergetics and in recovery of lung tissue homeostasis, including restoration of intercellular connections (33, 36). A recent study indicates that AMPK activation in the brain also diminished LPS-mediated development of sepsis-ALI, evidence for more diverse mechanism of AMPK action (64). Of note, while AMPK is an established metabolic sensor and regulator, MKK3 signaling axis has been also shown to affect mitochondrial function in sepsis/ALI (65).

Diminished macrophage and neutrophil pro-inflammatory activation, as well as T-cell exhaustion, is characteristic of the immunosuppression described in late sepsis (16, 66). While engagement of TLR4 in macrophages and neutrophils diminishes AMPK activity (36, 38), HIF-1α has been shown to promote an immunosuppressive status in monocytes during human sepsis (50). Our results indicate that AMPK activation prevented both accumulation of HIF-1α and development of endotoxin tolerance in LPS treated macrophages. These new findings are similar to the ability of AMPK activation to inhibit

HIF-1α expression in cancer cells and insulin- and IGF-1-induced expression of HIF-1α in endothelial cells (67, 68).

Our data suggest that therapeutic interventions that induce AMPK activation may be beneficial in diminishing organ dysfunction, enhancing bacterial clearance and improving survival in severe polymicrobial sepsis. Although this hypothesis is primarily supported through the use of metformin as an AMPK activator, similar results were found using other AMPK activators, including GSK-3β inhibitors, as performed in the present experiments. Previous studies have suggested that metformin can be used safely in patients with critical illness, COPD or asthma (48–50). More than 50 million type 2 diabetics are taking metformin daily worldwide, and recently metformin was selected for a clinical trial to evaluate its effect on human longevity (ClinicalTrials.gov NCT02432287) (69). Given the safety of metformin in many humans with diverse pathophysiologic conditions, and the suggestion that metformin may have beneficial effects in diminishing inflammation-associated organ dysfunction, it may be appropriate to consider its use in clinical trials enrolling severely ill septic patients with organ dysfunction.

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Disclosure

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

References

1. Deutschman CS, Tracey KJ. Sepsis: current dogma and new perspectives. Immunity. 2014;40:463–75.

2. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med. 2013;369:2063.

3. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect Dis. 2013;13:260–8.

4. Lord JM, et al. The systemic immune response to trauma: an overview of pathophysiology and treatment. Lancet. 2014;384:1455–65.

5. Wheeler AP, Bernard GR. Acute lung injury and the acute respiratory distress syndrome: a clinical review. Lancet. 2007;369:1553–64.

6. Force ADT, et al. Acute respiratory distress syndrome: the Berlin Definition. JAMA. 2012;307:2526–33.

7. Sheu CC, et al. Clinical characteristics and outcomes of sepsis-related vs non-sepsis-related ARDS. Chest. 2010;138:559–67.

8. Fink MP, Warren HS. Strategies to improve drug development for sepsis. Nat Rev Drug Discov. 2014;13:741–58.

9. Standiford TJ, Ward PA. Therapeutic targeting of acute lung injury and acute respiratory distress syndrome. Transl Res. 2016;167:183–91.

10. Flierl MA, et al. Adverse functions of IL-17A in experimental sepsis. FASEB J. 2008;22:2198–205.

11. Bosmann M, Ward PA. Therapeutic potential of targeting IL-17 and IL-23 in sepsis. Clin Transl Med. 2012;1:4.

12. Mariathasan S, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature. 2004;430:213–8.

13. Brown KA, et al. Neutrophils in development of multiple organ failure in sepsis. Lancet. 2006;368:157–69.

14. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13:159–75.

15. Abraham E. Neutrophils and acute lung injury. Crit Care Med. 2003;31:S195–9.

16. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol. 2013;13:862–74.

17. Gentile LF, et al. Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. J Trauma Acute Care Surg. 2012;72:1491–501.

18. Singer M. The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. Virulence. 2014;5:66–72.

19. Brealey D, et al. Association between mitochondrial dysfunction and severity and outcome of septic shock. Lancet. 2002;360:219–23.

20. Singer M. Mitochondrial function in sepsis: acute phase versus multiple organ failure. Crit Care Med. 2007;35:S441–8.

21. Srivastava A, et al. MKK3 deletion improves mitochondrial quality. Free Radic Biol Med. 2015;87:373–84.

22. Matthay MA. Therapeutic potential of mesenchymal stromal cells for acute respiratory distress syndrome. Ann Am Thorac Soc. 2015;12(Suppl 1):S54–7.

23. Rocha M, Herance R, Rovira S, Hernandez-Mijares A, Victor VM. Mitochondrial dysfunction and antioxidant therapy in sepsis. Infect Disord Drug Targets. 2012;12:161–78.

24. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol. 2012;13:251–62.

25. Eid AA, Lee DY, Roman LJ, Khazim K, Gorin Y. Sestrin 2 and AMPK connect hyperglycemia to Nox4-dependent endothelial nitric oxide synthase uncoupling and matrix protein expression. Mol Cell Biol. 2013;33:3439–60.

26. Colombo SL, Moncada S. AMPKalpha1 regulates the antioxidant status of vascular endothelial cells. Biochem J. 2009;421:163–9.

27. Chen L, et al. Structural insight into the autoinhibition mechanism of AMP-activated protein kinase. Nature. 2009;459:1146–9.

28. Woods A, et al. Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. J Biol Chem. 2003;278:28434–42.

29. Martin-Montalvo A, et al. Metformin improves healthspan and lifespan in mice. Nat Commun. 2013;4:2192.

30. Bannister CA, et al. Can people with type 2 diabetes live longer than those without? A comparison of mortality in people initiated with metformin or sulphonylurea monotherapy and matched, non-diabetic controls. Diabetes Obes Metab. 2014;16:1165–73.

31. Zhao X, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2008;295:L497–504.

32. Zmijewski JW, et al. Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. Am J Respir Crit Care Med. 2008;178:168–79.

33. Jian MY, Alexeyev MF, Wolkowicz PE, Zmijewski JW, Creighton JR. Metformin-stimulated AMPK-α1 promotes microvascular repair in acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2013;305:L844–55.

34. Jiang S, et al. Human resistin promotes neutrophil proinflammatory activation and neutrophil extracellular trap formation and increases severity of acute lung injury. J Immunol. 2014;192:4795–803.

35. Tadie JM, et al. Toll-like receptor 4 engagement inhibits adenosine 5′-monophosphate-activated protein kinase activation through a high mobility group box 1 protein-dependent mechanism. Mol Med. 2012;18:659–68.

36. Xing J, et al. Inhibition of AMP-activated protein kinase accentuates lipopolysaccharide-induced lung endothelial barrier dysfunction and lung injury in vivo. Am J Pathol. 2013;182:1021–30.

37. Meares GP, Qin H, Liu Y, Holdbrooks AT, Benveniste EN. AMP-activated protein kinase restricts IFN-gamma signaling. J Immunol. 2013;190:372–80.

38. Park DW, et al. GSK3β-dependent inhibition of AMPK potentiates activation of neutrophils and macrophages and enhances severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2014;307:L735–45.

39. Suzuki T, et al. Inhibition of AMPK catabolic action by GSK3. Mol Cell. 2013;50:407–19.

40. Jiang S, et al. Mitochondria and AMP-activated protein kinase-dependent mechanism of efferocytosis. J Biol Chem. 2013;288:26013–26.

41. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc. 2009;4:31–6.

42. Rodriguez C, et al. Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction. Biochem J. 2009;422:423–32.

43. Zmijewski JW, et al. Antiinflammatory effects of hydrogen peroxide in neutrophil activation and acute lung injury. Am J Respir Crit Care Med. 2009;179:694–704.

44. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nat Rev Immunol. 2013;13:397–411.

45. Ayala A, et al. Shock-induced neutrophil mediated priming for acute lung injury in mice: divergent effects of TLR-4 and TLR-4/FasL deficiency. Am J Pathol. 2002;161:2283–94.

46. LaRue KE, McCall CE. A labile transcriptional repressor modulates endotoxin tolerance. J Exp Med. 1994;180:2269–75.

47. McClure C, Brudecki L, Yao ZQ, McCall CE, El Gazzar M. Processing body formation limits proinflammatory cytokine synthesis in endotoxin-tolerant monocytes and murine septic macrophages. J Innate Immun. 2015;7:572–83.

48. Sag D, Carling D, Stout RD, Suttles J. Adenosine 5′-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. J Immunol. 2008;181:8633–41.

49. Jeong HW, et al. Berberine suppresses proinflammatory responses through AMPK activation in macrophages. Am J Physiol Endocrinol Metab. 2009;296:E955–64.

50. Shalova IN, et al. Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1α Immunity. 2015;42:484–98.

51. van der Poll T, Opal SM. Host-pathogen interactions in sepsis. Lancet Infect Dis. 2008;8:32–43.

52. Tavares-Murta BM, et al. Failure of neutrophil chemotactic function in septic patients. Crit Care Med. 2002;30:1056–61.

53. Fossati G, et al. The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. J Immunol. 2003;170:1964–72.

54. Barbier F, Andremont A, Wolff M, Bouadma L. Hospital-acquired pneumonia and ventilator-associated pneumonia: recent advances in epidemiology and management. Curr Opin Pulm Med. 2013;19:216–28.

55. Martin M, Rehani K, Jope RS, Michalek SM. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol. 2005;6:777–84.

56. Rocha J, et al. Inhibition of glycogen synthase kinase-3β attenuates organ injury and dysfunction associated with liver ischemia-reperfusion and thermal injury in the rat. Shock. 2015;43:369–78.

57. Li H, et al. NF-κB inhibition after cecal ligation and puncture reduces sepsis-associated lung injury without altering bacterial host defense. Mediators Inflamm. 2013;2013;503213.

58. Park DW, et al. Activation of AMPK enhances neutrophil chemotaxis and bacterial killing. Mol Med. 2013;19:387–98.

59. Bae HB, et al. AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. FASEB J. 2011;25:4358–68.

60. Mounier R, et al. AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. Cell Metab. 2013;18:251–64.

61. Labuzek K, Liber S, Gabryel B, Adamczyk J, Okopien B. Metformin increases phagocytosis and acidifies lysosomal/endosomal compartments in AMPK-dependent manner in rat primary microglia. Naunyn Schmiedebergs Arch Pharmacol. 381:171–86.

62. Zolfaghari PS, et al. Skeletal muscle dysfunction is associated with derangements in mitochondrial bioenergetics (but not UCP3) in a rodent model of sepsis. Am J Physiol Endocrinol Metab. 2015;308:E713–25.

63. Japiassu AM, et al. Bioenergetic failure of human peripheral blood monocytes in patients with septic shock is mediated by reduced F1Fo adenosine-5′-triphosphate synthase activity. Crit Care Med. 2011;39:1056–63.

64. Mulchandani N, et al. Stimulation of brain AMP-activated protein kinase attenuates inflammation and acute lung injury in sepsis. Mol Med. 2015;21:637–44.

65. Mannam P, et al. MKK3 regulates mitochondrial biogenesis and mitophagy in sepsis-induced lung injury. Am J Physiol Lung Cell Mol Physiol. 2014;306:L604–19.
66. Ward PA. Immunosuppression in sepsis. JAMA. 2011;306:2618–9.

67. Faubert B, et al. AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. Cell Metab. 2013;17:113–24.

68. Treins C, Murdaca J, Van Obberghen E, Giorgetti-Peraldi S. AMPK activation inhibits the expression of HIF-1alpha induced by insulin and IGF-1. Biochem Biophys Res Commun. 2006;342:1197–202.

69. Metformin in Longevity Study (MILES) [Internet] 2016. [updated 2015 Dec 8; cited 2016 Jan 29]. Available from:

https://clinicaltrials.gov/ct2/show/record/NCT02432287 ClinicalTrials.gov identifier: NCT02432287.

Figures

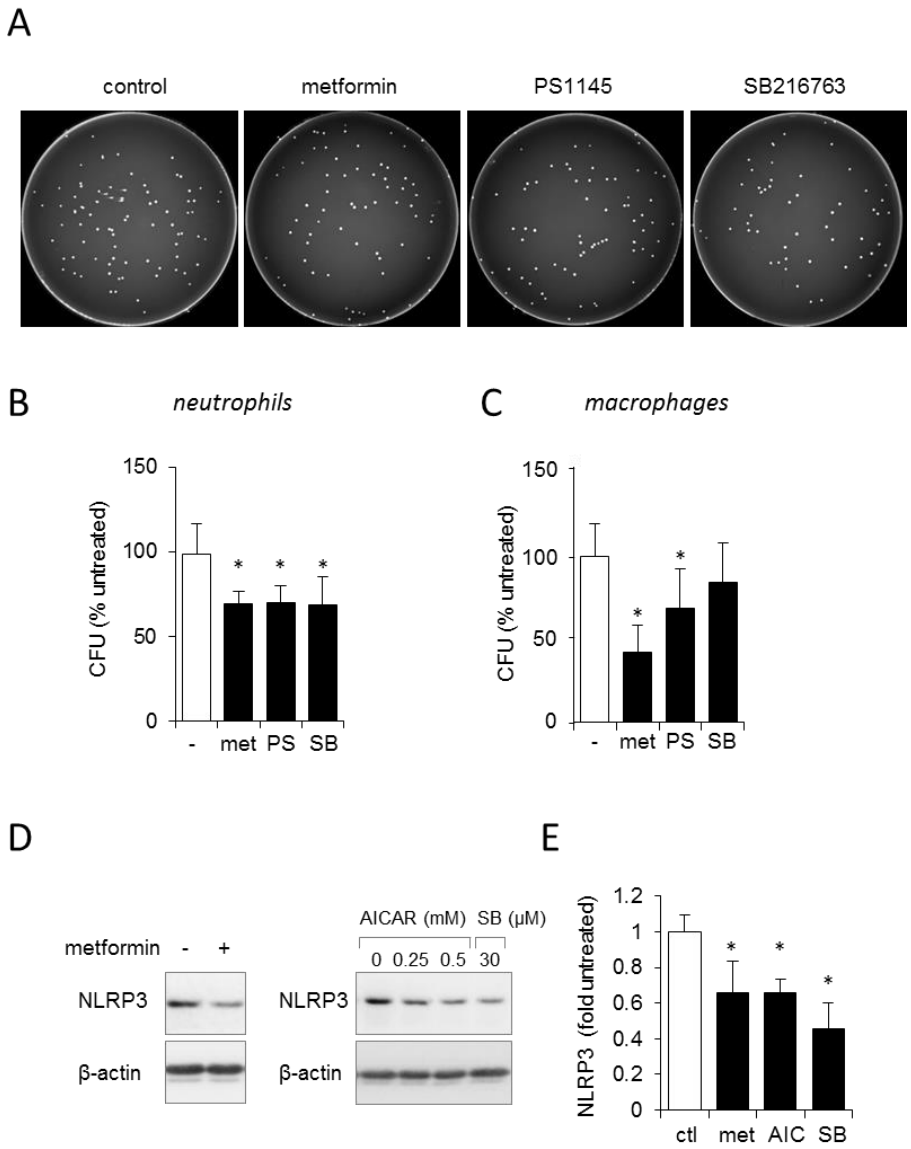


Figure 1. Metformin or IKK1/2 or GSK3β inhibitors affect bacterial killing by neutrophils or macrophages. (A,B,C) Bone marrow neutrophils or peritoneal macrophages were treated with metformin (0 or 1 mmol/L), PS1145 (0 or 10 μmol/L) or SB216763 (0 or 30 μmol/L) and then bacterial killing determined after exposure to P. aeruginosa. (A) Images show agar plates with bacterial colonies, an indicator of amounts surviving bacteria after coincubation of neutrophils with P. aeruginosa. (B and C) CFUs were calculated after treatment with (B) neutrophils or (C) macrophages with or without metformin, PS1145 or SB216763 followed by exposure to P. aeruginosa. Means ± SD (n = 3), *p < 0.05. (D,E,F) Western blot analysis shows the extent of NLRP3 degradation in peritoneal macrophages treated with (D) metformin (0 or 500 μmol/L), (E) AICAR (0, 0.25 or 0.5 mmol/L) or (F) SB216763 (0 or 30 μmol/L) for 8 h. Means ± SD (n = 3), *p < 0.05.

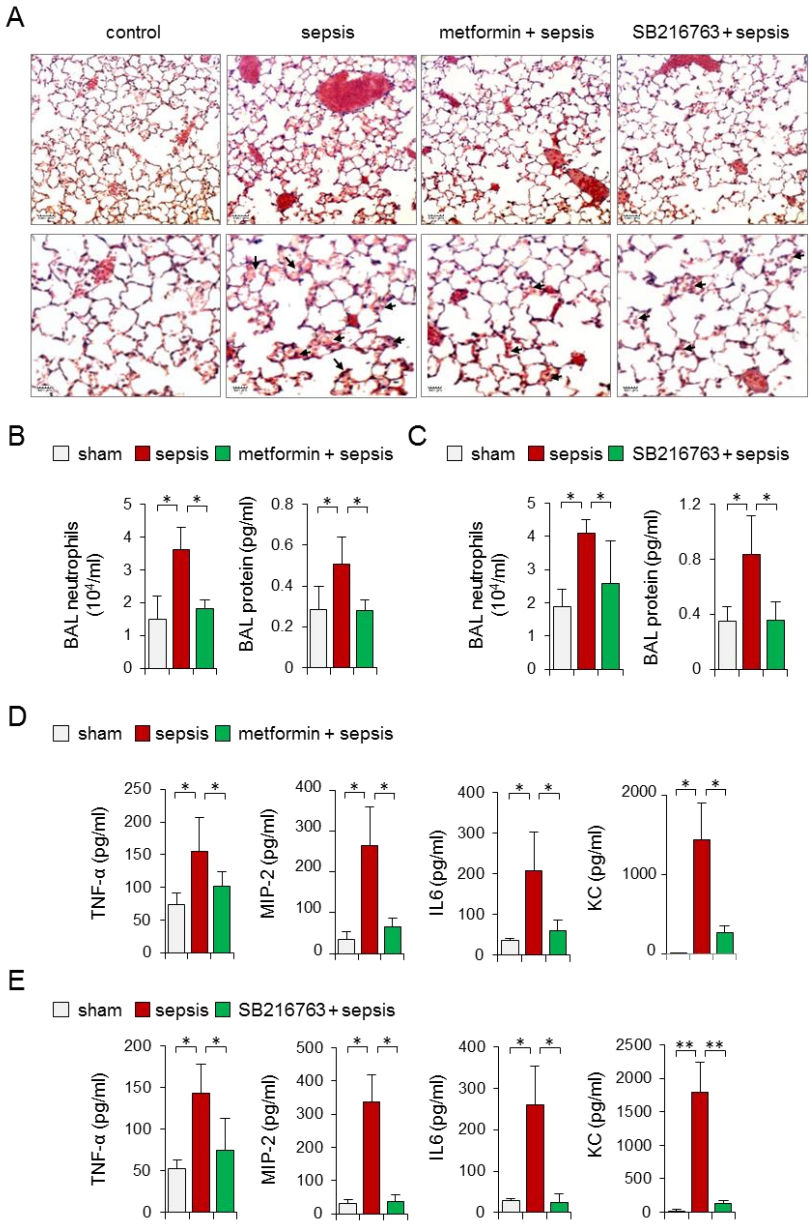


Figure 2. The effects of AMPK activator metformin or GSK3β inhibitor SB216763 on the severity of lung inflammation in mice subjected to CLP-induced sepsis. (A) Images show lung sections obtained of control or mice treated with metformin or GSK3β inhibitor SB216763 following CLP. Bars: 1,000 μm (upper panel) or 100 μm (lower panel). Arrows indicate spaces filled with a mixed mononuclear/neutrophilic infiltrate, cellular debris and proteinaceous material. Alveolar walls are also thickened, and the septa are edematous. (B) and (C) show the ability of metformin or SB216763 to diminish the amount of BAL neutrophils and to reduce lung permeability (BAL proteins). (D and E) Metformin lowers BAL pro-inflammatory cytokines content, including TNF-α, MIP-2, KC and IL-6 in mice subjected to CLP and metformin or CLP and SB216763, compared with control (sham) or mice with CLP alone (means ± SD, n = 6 mice/group, *$p < 0.05$, **$p < 0.01$).

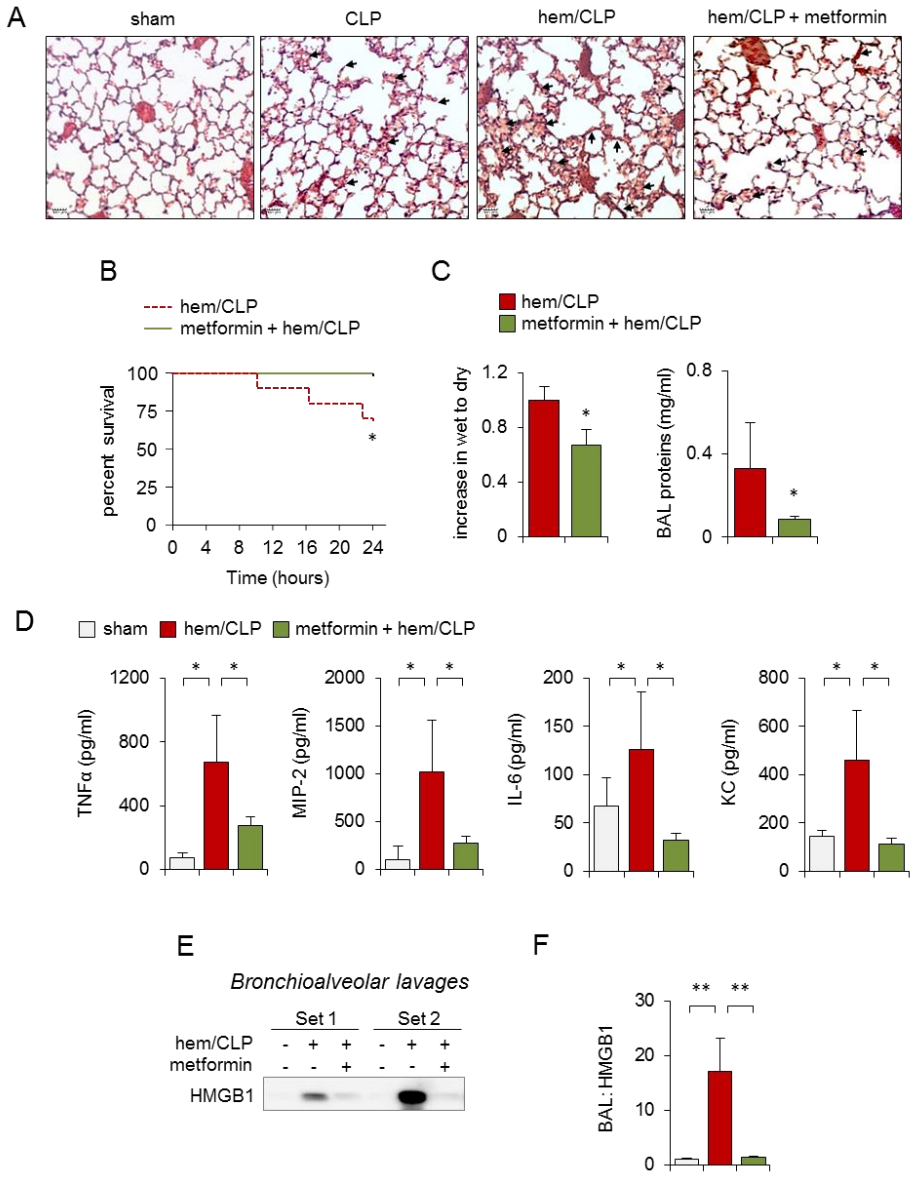


Figure 3. Metformin diminishes the severity of ALI in mice subjected to hemorrhage and CLP-induced sepsis. (A) Images show lung sections of control (sham), CLP- or hemorrhage/CLP- or metformin and hemorrhage/CLP treated mice. Bars: 100 μm. Arrows indicate spaces filled with a mixed mononuclear/neutrophilic infiltrate, cellular debris and proteinaceous material. Alveolar walls are also thickened, and the septa are edematous. (B) Metformin increased survival rates following hemorrhage and CLP. (C) Decrease of pulmonary edema and lung permeability was evidenced by measurement of lung wet-to-dry ratios and BAL proteins in mice treated with metformin and hemorrhage/CLP, as compared with hemorrhage/CLP group. Panels (D), (E) and (F) show amounts of BAL TNF-α, MIP-2, KC, IL-6 or HMGB1 in sham, hemorrhage/CLP- or metformin and hemorrhage/CLP treated mice (means ± SD (n = 5 mice/group), *p < 0.05, **p < 0.01).

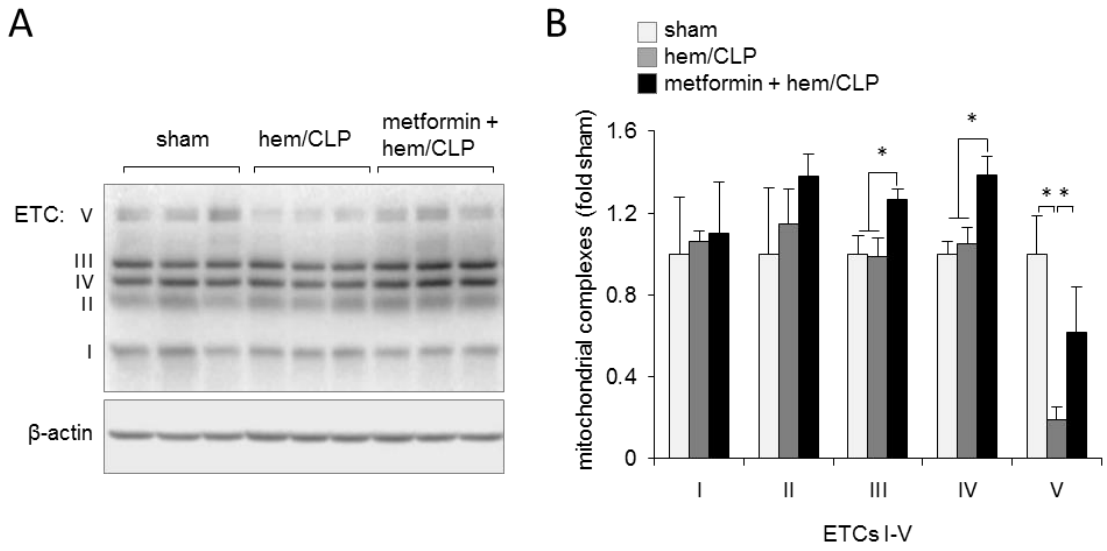


Figure 4. Metformin prevents the loss of mitochondrial ETC complex V and increased ETC complexes III and IV in lungs of mice subjected to sepsis-induced ALI. (A) Representative Western blots and (B) quantitative data show the amount of ETC complexes I–V in control (sham), hemorrhage/CLP- or metformin and hemorrhage/CLP treated mice (means ± SD, n = 3, *p < 0.05). Comp. I: NDUFB8; Comp. II: 30 kDa FeS complex; Comp. III: Core protein 2; Comp. IV: subunit I; Comp. V: α subunit.

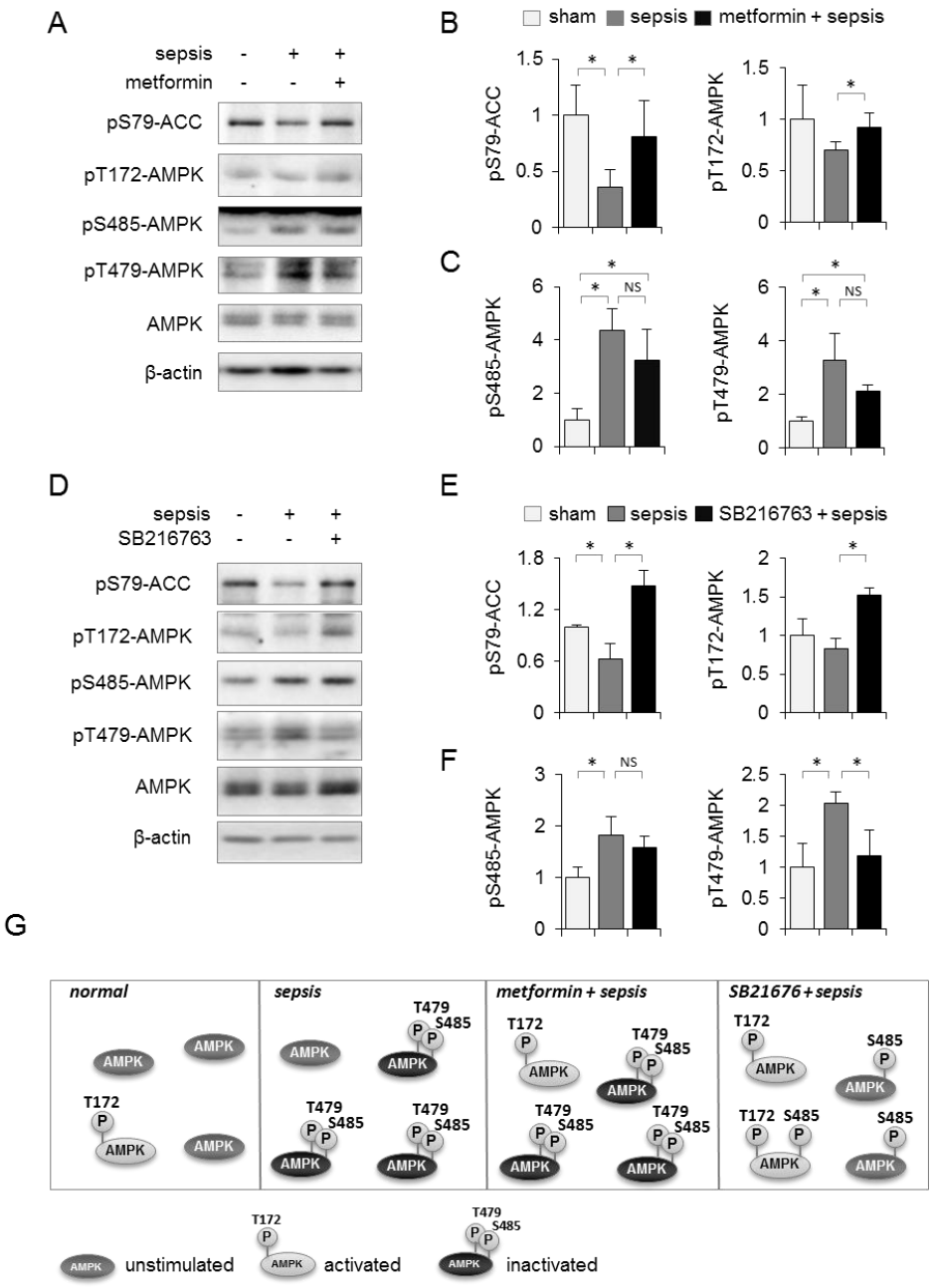


Figure 5. Metformin and GSK3β inhibitor SB216763 affect T172-AMPK phosphorylation in lungs of mice subjected to sepsis. Representative Western blots and quantitative data show the extent of pT172-AMPK, pS485-AMPK, pT479-AMPK and AMPK downstream target pS79-ACC in lung homogenates obtained from mice treated with (A,B,C) metformin or (C,D,E) GSK3β inhibitor SB216763 and then subjected to CLP-induced sepsis (means ± SD, n = 3–5, *p < 0.05, NS = not significant). (G) Cross-talk between metformin-stimulated pT172-AMPK and inhibitory IKKβ/GSK3β-induced S485/T479-AMPK phosphorylation modulates AMPK activity in lung of mice subjected to abdominal sepsis.

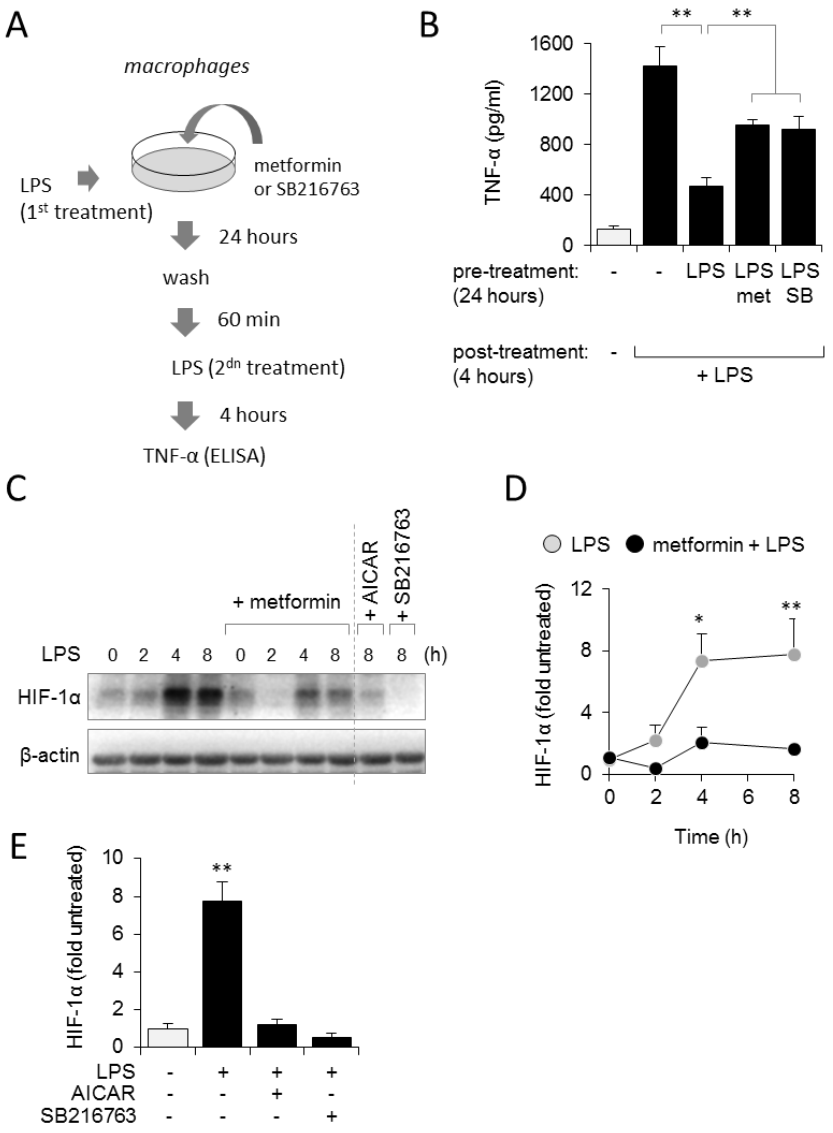


Figure 6. AMPK activation or inhibition of GSK3β diminishes development of endotox-in tolerance in LPS treated macrophage. (A,B) Macrophages were first incubated with LPS (0 or 10 ng/mL) for 24 h. Next, cells were washed and treated with a second dose of LPS (0 or 10 ng/mL) for 4 h followed by measurement of TNF-α in culture media (ELI-SA). As indicated, cells were also treated with AMPK activators metformin (0 or 500 μmol/L) or GSK3β inhibitor SB216763 (0 or 30 μmol/L) for 60 min followed by inclu-sion of LPS (first dose). (C,D,E) AMPK activators or GSK3β inhibition prevented HIF-1α accumulation in LPS treated macrophages. Peritoneal macrophages were treated with metformin (0 or 1 mmol/L) and LPS (30 ng/mL) for 0, 2, 4, 8 h followed by Western blot analysis of HIF-1α and β-actin. Cells were also treated with AICAR (0 or 300 μmol/L) or SB216763 (0 or 30 μmol/L) and then cultured with LPS for an additional 8 h (means ± SD, n = 3, *$p < 0.05$, **$p < 0.01$).

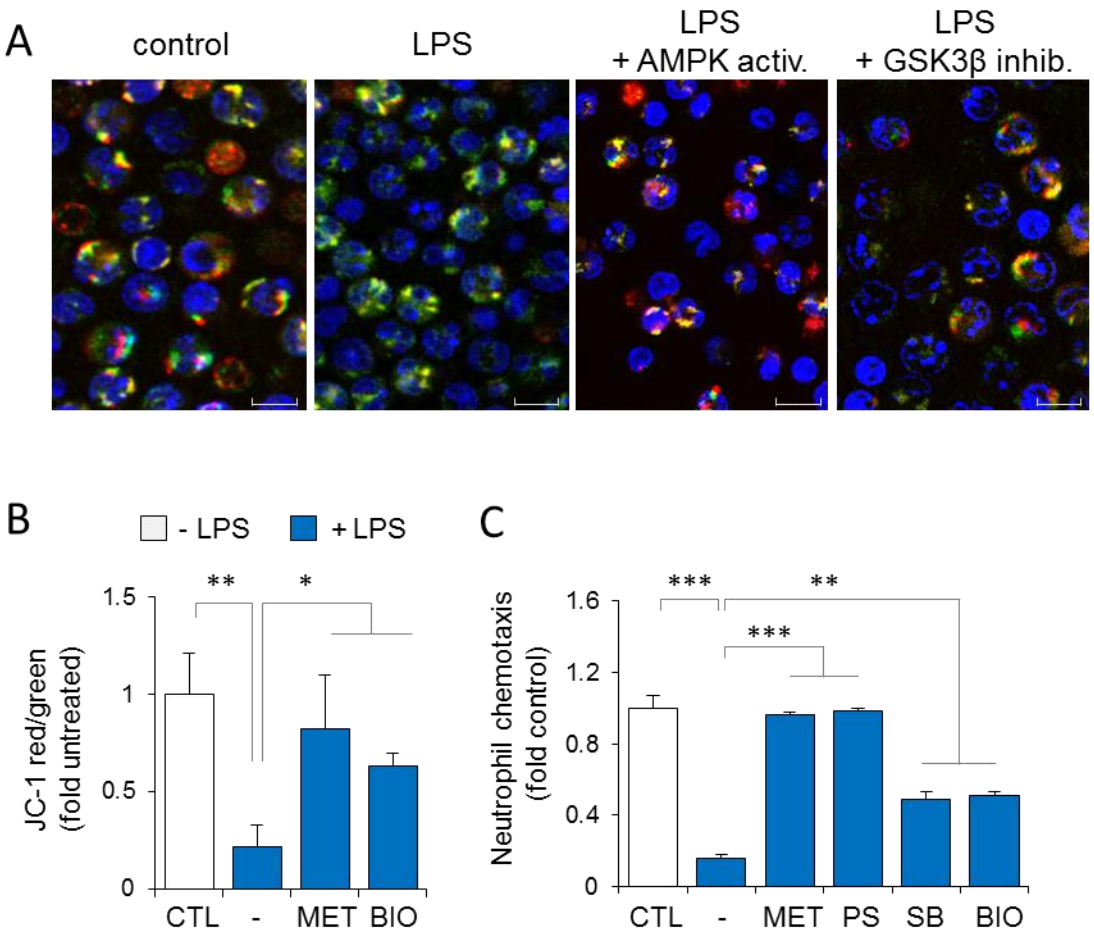


Figure 7. GSK3β inhibition and AMPK activation prevented mitochondrial membrane depolarization and chemotaxis dysfunction in LPS treated neutrophils. Bone marrow neutrophils were pretreated with metformin (0 or 500 μmol/L) or GSK3β inhibitor (BIO; 0 or 20 μmol/L) for 60 min prior to inclusion of LPS (300 mg/mL) for an additional 60 min. (A) Representative images show JC-1 and nuclei fluorescence in control and treated neutrophils. Red-high Δψ; green-low Δψ; blue-nuclei (Bars; 2 μm). (B) Means ± SD red/green pixel intensity, n = 5, *p < 0.05, **p < 0.01. (C) Neutrophils were pretreated with AMPK activator metformin (0 or 500 μmol/L), IKK1/2 inhibitor PS1145 (0 or 10 μmol/L), or GSK3β inhibitors SB216763 (0 or 30 μmol/L) for 120 min followed by incubation with LPS (0 or 300 ng/mL) for an additional 60 min. Quantitative data show the amount of neutrophils that migrated from upper to lower reservoir of transmigrating chamber (B) Means ± SD (n = 3), **p < 0.01, ***p < 0.001.

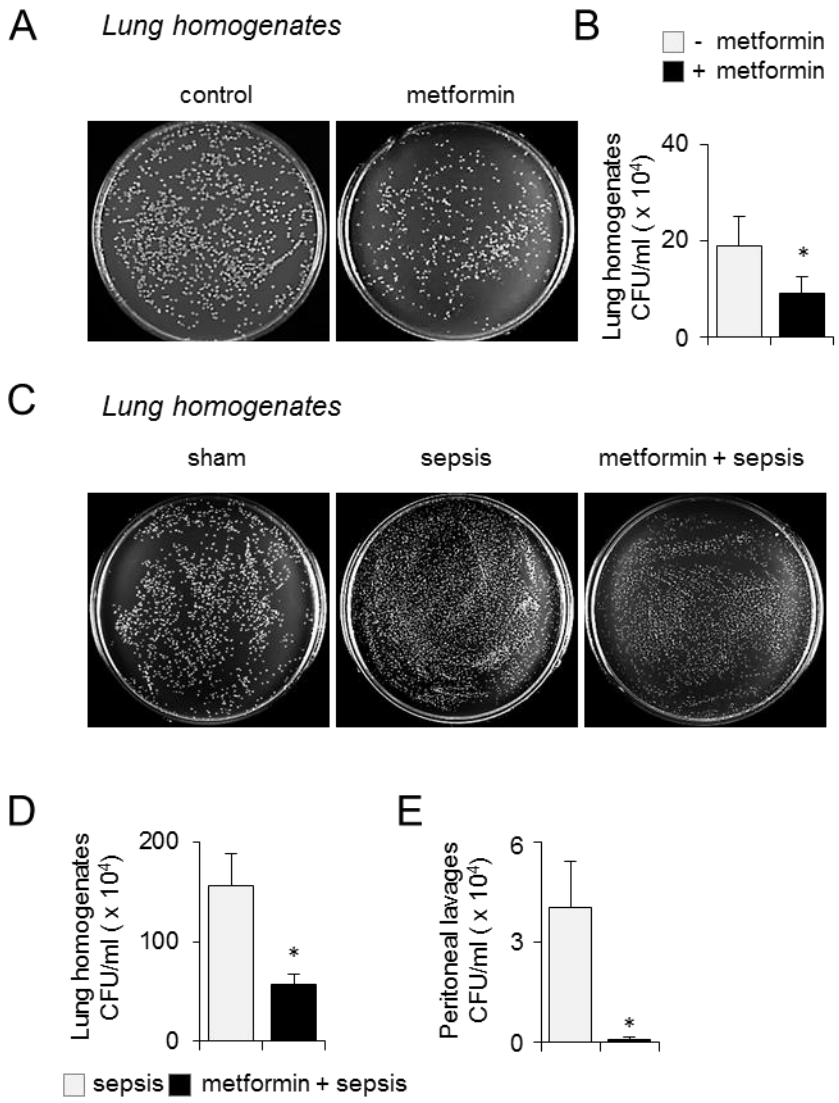
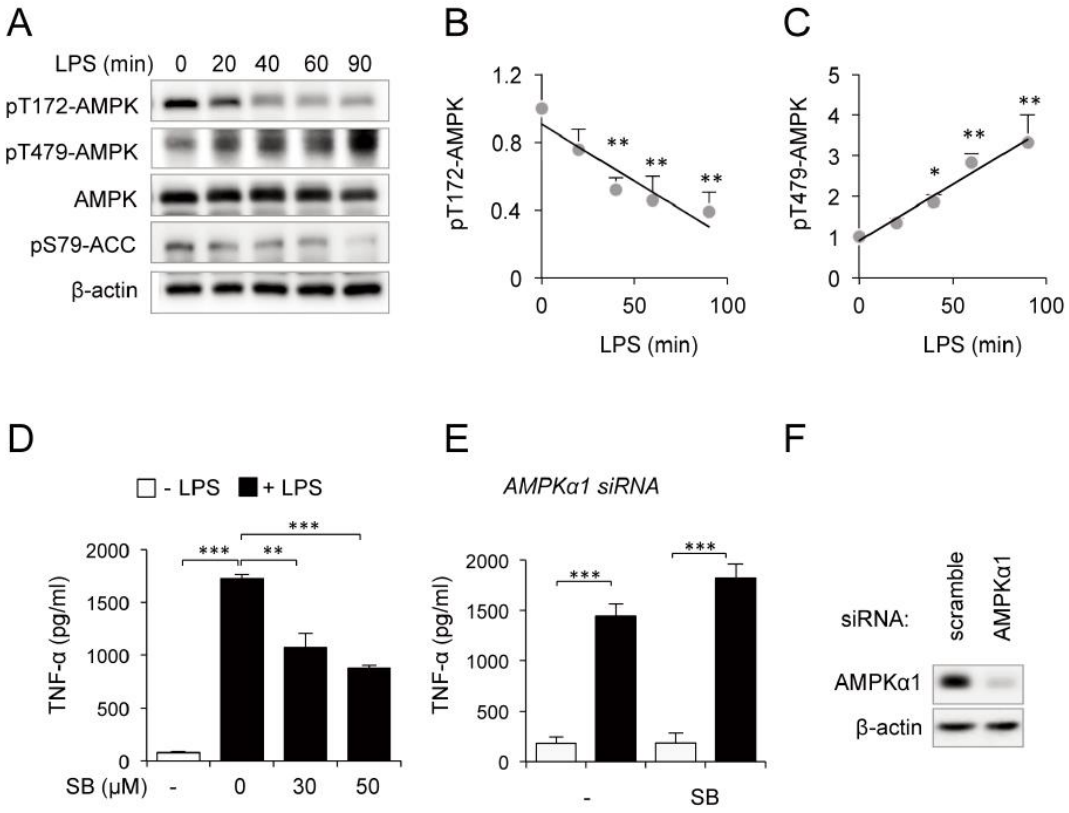


Figure 8. Metformin diminishes sepsis-mediated immunosuppression. Mice were treated with saline, metformin, CLP or metformin and CLP for 24 h followed by i.t. instillation of *P. aeruginosa* (PAK, 2.5×10^7/mouse) for an additional 4 h. Next, amounts of surviving bacteria were determined in lung homogenates using CFU assay. (A,C) Representative images show the amounts of bacterial colonies grown on agar plates, whereas (B,D) show CFU data obtained from lung homogenates of control, metformin, CLP, or metformin and CLP mice. Panel (E) shows the amounts of bacteria in peritoneal lavages of mice treated with CLP or a combination of metformin and CLP for 7 d (means ± SD n = 5, *$p < 0.05$).



Supplementary Figure S1. LPS diminished T172-AMPK and increased phosphorylation of T479-AMPK. (A, B and C) Peritoneal macrophages were incubated with LPS (300 ng/ml) for 0, 20, 40, 60 or 90 min followed by (A) Western Blot analysis of T172-AMPK, T479-AMPK and S79-ACC phosphorylation, and total amounts of AMPK and β-actin. (B and C) Quantitative data show an inverse correlation between T172 and T479 phosphorylation of AMPK (means ± SD (n = 3), * $p < 0.05$, ** $p < 0.01$, as compared to untreated. (D and E) GSK3β-dependent inhibition of AMPK promoted macrophage pro-inflammatory activation. (D) Peritoneal macrophages were pretreated with GSK3β inhibitor SB216763 (0, 30, or 50 μM) for 2 h followed by exposure to LPS (0 or 3 ng/ml) for an additional 4.5 h. The amount of TNF-α in the culture media was measured using ELISA. (E) Unaltered (control) or peritoneal macrophages with siRNA-mediated knockdown of AMPKα1 were treated with SB216763 (o or 50 μM) for 2 h. LPS (0 or 3 ng/ml) was added to the culture media for 4.5 h. The amount of TNF-α was measured in the culture media. Means ± SD, n = 3, ** $p < 0.01$, *** $p < 0.001$. (F) Representative Western blots show the amounts of total AMPK and β-actin in the peritoneal macrophages treated with scramble (control) or siRNA to AMPKα1.

THE AMPK-AUTOPHAGY AXIS IS CRUCIAL FOR DIMINISHING SEVERITY OF SEPSIS

Autophagy is an evolutionarily conserved cellular process that promotes maintenance of cellular homeostasis. Autophagy was observed for the first time in the mid-twentieth century and was followed by the major discovery of autophagy-related genes in yeast in the 1990s. The 2016 Nobel Prize in Physiology or Medicine was awarded to Yoshinori Ohsumi who was instrumental to these discoveries. These studies opened doors for more in-depth research into the functions of autophagy in disease[141-143], including nutrient, energetic, and stress sensing mechanisms that regulate autophagy activity. Autophagy was initially recognized as a protective mechanism against starvation or stress through non-selective degradation of cellular components, including misfolded proteins and dysfunctional organelles. The major mechanism of degradation is supported by formation of specialized phagosomes followed by fusion with the lysosome[144].

Autophagy encompasses three degradative mechanisms described as macroautophagy, microautophagy, and chaperone-mediated autophagy[144]. Macroautophagy involves engulfment of cytoplasmic targets, including protein and organelles. Autophagosomes then undergo fusion with the lysosome initiating cargo degradation. Macroautophagy includes both nonselective bulk degradation of multiple substrates and selective degradation of specific cellular components such as mitochondria. Autophagy can also occur through microautophagy where cellular components directly enter the lysosome through

contact and encapsulation by the lysosomal membrane. Another form autophagy is Chaperone-mediated autophagy; protein degradation mediated by a HSC70-dependent targeting mechanism. In this setting, the HSC70 containing complex directly delivers its cargo to the lysosome followed by transfer of unfolded proteins across the lysosome membrane[144, 145].

In this dissertation, our studies mainly focus on macroautophagy (referred to from now on as autophagy). Autophagy is essential to maintain cellular homeostasis; however autophagic flux is known to increase under stress conditions. Autophagy begins with the formation of the isolation membrane, or phagophore by the beclin-1/VPS34 complex. This membrane continues to elongate until the cargo is engulfed through activation of Atg3 and LC3. The completed autophagosome then migrates to the lysosome where fusion takes place leading to degradation of the contents by acidic lysosomal hydrolases. These degradation products are then able to be recycled by metabolic and biosynthetic pathways[144].

Autophagy dysfunction has been implicated in many pathophysiological conditions including pulmonary and cardiovascular diseases, cancer, and neurological disorders[146-148]. Increased autophagy has been associated with chronic obstructive pulmonary disease where increased LC3B-II and autophagosome formation was observed in patient lung tissue [149]. Dysfunctional autophagy of protein aggregates is evidenced in airway epithelial cells and nasal biopsy specimen from cystic fibrosis patients[150]. Defective autophagy has also been noted in idiopathic pulmonary fibrosis[151].

Mitochondria provide most of the energy production for the cells and is involved in multiple other cellular processes, including pro-inflammatory activation, bacterial killing, and removal of dying cells, necessitating regulation of mitochondrial quality[152]. Mitophagy is a form of selective autophagy of the mitochondria which can occur through basal constitutive turnover, starvation-induced turnover, and enhanced damaged mitochondria degradation. One of the best characterized pathways of mitophagy involves PINK1 and Parkin. PINK1 is constitutively imported into the mitochondrial membranes via the TOM and TIM complexes which involves protein cleavage followed by degradation of PINK1 by mitochondrial proteases, keeping concentrations of PINK1 low in the mitochondria. In turn, PINK1 is stabilized in depolarized mitochondria followed by recruitment of the E3 ubiquitin ligase Parkin that ubiquitinates outer mitochondrial membrane proteins, including mitofusin-1 and 2 and VDAC[153-155]. It is important to note that mitophagy can occur through a Parkin-independent pathway but a beclin-1-dependent mechanism and *vice versa*[156, 157]. In addition to mitophagy, Parkin is also involved in xenophagy, including degradation of bacteria, viruses, fungi, and aggrephagy-related neutralization of protein aggregates[158, 159]. Besides Parkinson's disease, mutations in Parkin are associated with development of lung, colon, and brain cancer[160, 161]. Parkin dysfunction and subsequent reduction in mitophagy are linked to accumulation of dysfunctional mitochondria along with appearance of protein aggregates. Importantly, Parkinson's disease patients show immune dysfunction and worse symptoms during infection[162]. Notably, more than 60% of idiopathic Parkinson's disease individuals die due to pneumonia[163].

Recent studies have revealed that autophagy is involved in the host immune response and intracellular pathogen elimination, antigen presentation, and turnover of inflammatory mediators[164]. Severe sepsis is associated with autophagy dysfunction caused by prolonged inflammation. Moreover, loss of autophagy will also lead to accumulation of dysfunctional mitochondria, bioenergetic reprogramming, and altered redox homeostasis. Given the importance of innate immunity in neutralization of microbial infection and the crucial impact of mitochondria on bioenergetic and redox homeostasis, our studies explore the influence of Parkin deficiency in sepsis and experimental ALI.

AMP-ACTIVATED PROTEIN KINASE ACTIVATES AUTOPHAGY AND DIMINISHES ENDOTOXIN-INDUCED LUNG INJURY THROUGH A PARKIN INDEPENDENT PATHWAY

by

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Abstract

Successful injury and infection resolution by innate immunity is ultimately controlled by autophagy and bioenergetics. Parkin is an ubiquitin ligase implicated in autophagy related processes including mitophagy and microbial eradication. We found loss of Parkin protein occurs several hours after pro-inflammatory engagement in macrophages and lungs of mice subjected to endotoxin-induced acute lung injury (ALI). We examined if AMPK activation recovers Parkin and thus reduce severity of ALI. Although AMPK activators did not recover Parkin, AMPK activation promoted autophagy and improved bacterial clearance in Parkin deficient macrophages. *In vivo,* the importance of AMPK activation was determined in a mouse model of endotoxin-induced ALI. Metformin application prior to LPS instillation partially prevented development of inflammation and decreased the severity of ALI. In summary, these results suggest that Parkin deficiency leads to diminished autophagy along with increased macrophage pro-inflammatory activation and severity of ALI which can be circumvented through AMPK-dependent autophagy activation.

Background

Acute respiratory distress syndrome (ARDS) is a form of respiratory failure resulting from infection or sterile injury induced inflammation. ARDS is a highly prevalent form of lung injury affecting about 250,000 people in the United States annually. In spite of improvements in hospital care, morbidity and mortality from this condition remain high (1, 2). Only supportive care is currently accessible for critically ill patients, and new effective therapeutics are crucially needed (3, 4). Besides pro-inflammatory components, loss of bioenergetic homeostasis, autophagy, and metabolic dysregulation of immune and parenchyma cells are important issues in sepsis and ARDS (5, 6).

Autophagy is an evolutionarily conserved lysosome-dependent degradation system involved in protein and organelle turnover (7). Autophagy preserves cellular homeostasis and appears to be indispensable for the adaptive response to stress, such as nutrient deprivation or oxidative stress (8). Along with organelle turnover, a major impact of autophagy is also relevant in host immune defense, including intracellular neutralization of *Mycobacterium*, *Salmonella*, and viruses (9). It is suggested that preservation of a "healthy" mitochondria pool affects bioenergetics and anti-bacterial capacity of immune cells (10, 11). Moreover, preservation of mitochondrial function is closely associated with reduced organ injury and recovery from trauma and inflammatory conditions (12). Impaired autophagy is associated with development of more severe lung, heart, and kidney injury in murine models (13-15). Loss of autophagy also has a significant impact in the clinical setting, including neurodegenerative disorders, cardiovascular complications, and exaggerated organ injury due to infections, including development of ARDS (16-20).

Neutralization of intracellular bacteria and viruses is a multistep process involving intricate signaling that promotes tagging of selective substrates, assembly of functional phagosomes, and phagosome-lysosome fusion for substrate degradation. In particular, specialized E3 ubiquitin ligases are responsible for tagging of microbial surface proteins with ubiquitin moieties followed by subsequent entrapment by phagosomes (21). Parkin is among the major ubiquitin ligases that promote ubiquitination of microbial proteins and ensuing phagosome-lysosome dependent degradation (22, 23). Similarly, ubiquitination of outer mitochondrial membrane proteins targets dysfunctional/depolarized mitochondria for autophagy-dependent degradation (24, 25). Mutations that impair Parkin function are linked to mitochondrial dysfunction and protein aggregation which have been observed in Parkinson's disease (PD) (26, 27). In addition to neurological complications, leukocytes from PD patients show many abnormalities and patients often do not recover from worsened symptoms after systemic infections (28, 29). Interestingly, Parkin deficient (*PARK2*$^{-/-}$) mice exhibit poor activation of cardiac mitophagy and limited recovery after acute peritonitis (30). Although PD is associated with reduced recovery from pneumonia, little is known if Parkin affects development of ARDS. Furthermore, it has not been determined if Parkin dysfunction could be circumvented by alternative autophagy mechanism(s) that reduce immune dysfunction and thereby improve lung tissue homeostasis.

AMP-activated protein kinase (AMPK) is a key sensor and modulator of cellular bioenergetics, including key components involved in the autophagy network (31, 32). When blood flow is restricted and oxygen and nutrient bioavailability is limited, AMPK activation reduces energy expenditure by limiting anabolic processes while promoting

energy production via catabolic pathways (33, 34), including ATP production by mitochondria (35). Bioenergetic homeostasis is also supported by AMPK-dependent regulation of autophagy (33). AMPK stimulates autophagy indirectly via suppression of mTOR signaling, and/or directly through autophagic switches, including phosphorylation (activation) of beclin-1 and ULK1 (36, 37). Recent studies indicate that diminished AMPK activity has been associated with increased severity of organ injury (38, 39). Although AMPK is known to elicit anti-inflammatory effects, the exact role of AMPK-autophagy axis is not well understood in lung disorders.

Our initial results indicate that Parkin dysfunction is associated with LPS treated macrophages, injury of epithelial cells, and in the lung tissue of mice subjected to endotoxin-induced ALI. We have previously shown that these conditions are associated with AMPK inactivation. We hypothesize that recovery of AMPK function is essential for autophagy activation, potentially via restoring Parkin, and thereby diminishing the severity of ALI.

Results

Parkin Depletion Is a Result of Macrophage Pro-Inflammatory Activation and Epithelial Injury in Lungs of Mice Subjected To LPS-Induced ALI

In the first set of experiments, we examined the possibility of Parkin protein turnover associated with macrophage pro-inflammatory activation, alveolar epithelial injury, and in vivo in lungs of mice subjected to endotoxin-induced ALI. Western blot analysis revealed that exposure to LPS resulted in a time-dependent decrease in the Parkin levels of stimulated macrophages (Figure 1a). Similarly, the amounts of Parkin were diminished in alveolar lung epithelial cells that were treated with tunicamycin-induced ER stress. Notably, ER dysfunction is a relevant situation associated with epithelial injury in experimental models of ALI or patients with ARDS (40, 41). Consistent with results obtained from macrophages and lung epithelial cells, significant reduction of Parkin protein was also observed in lung homogenates of mice subjected to intratracheal LPS administration (Figure 1c). To further explore a possible link between inflammatory conditions implicated in ALI and Parkin dissipation, alveolar epithelial cells were cultured with control (unstimulated) or media obtained from activated macrophages. We found that Parkin protein was significantly diminished after treatment with conditioned media, but not after inclusion of control media (Figure 1d). These results indicate that inflammatory conditions are leading to Parkin dissipation; in particular this occurred several hours after TLR4-LPS engagement in macrophages and in lung tissue 24 hours after endotoxin-induced ALI.

Parkin Deficiency Augments Macrophage Pro-Inflammatory Activation

To determine the impact of Parkin on immune function, we first measured the pro-inflammatory response in peritoneal macrophages isolated from wild type (*PARK2*$^{+/+}$) and Parkin knockout (*PARK2*$^{-/-}$) mice. In particular, cells were treated with or without LPS followed by detection of pro-inflammatory mediators in culture media and NLRP3 accumulation in lysates. As compared to their wild type counterparts, Parkin deficient macrophages had a more robust pro-inflammatory response, including LPS-induced NLRP3 activation (Figure 1e) and enhanced production of TNF-α and MIP-2 (Figure 1f). These results indicate that Parkin deficiency is associated with enhanced macrophage pro-inflammatory response.

Parkin Deficient Macrophages Exhibit Impaired Autophagy/Mitophagy

Given that Parkin is involved in mitophagy, we hypothesized that impaired mitochondrial redox homeostasis is priming macrophages for a robust pro-inflammatory response in Parkin deficient macrophages. This possibility was explored using the fluorogenic probe mitoSOX, a mitochondria targeted fluorogenic probe that is specific toward superoxide detection. Confocal images and quantitative analysis have shown a nearly 25% increase in ROS formation occurred in *PARK2*$^{-/-}$ *versus PARK2*$^{+/+}$ macrophages (Figures 2a and b). Further analysis revealed ROS formation in *PARK2*$^{-/-}$ macrophages also exhibited mitochondrial fragmentation, as compared to *PARK2*$^{+/+}$ (Figure 2c). Of note, accumulation of impaired mitochondria was likely a result of deficient mitophagy in *PARK2*$^{-/-}$ macrophages. Indeed, results shown in Figures 2d and e indicate that dissipa-

tion of mitochondrial membrane potential (mΔΨ) induced autophagy in *PARK2*$^{+/+}$ but not in *PARK2*$^{-/-}$ macrophages. This was evidenced by Western blot analysis of adaptor protein and autophagy marker LC3B in macrophages treated with mitochondrial membrane uncoupler FCCP. These findings suggest that loss of Parkin and a robust macrophage pro-inflammatory response were associated with accumulation of ROS-producing dysfunctional mitochondria.

AMPK Activation Rescues Autophagy in Parkin Deficient Macrophages

AMPK activation is a key signaling event associated with autophagy initiation. Therefore we examined if AMPK activation is capable of preventing Parkin dissipation in LPS treated macrophages. Our results show that despite AMPK activation, the significant loss of Parkin persisted in LPS treated macrophages (Figure 3a-e). Although AMPK did not prevent Parkin turnover, our next question was as to whether AMPK activators stimulate autophagy despite Parkin deficiency. We tested this possibility using AMPK activator AICAR (AMP mimetic) in *PARK2*$^{-/-}$ macrophages. Western blot analysis revealed that AMPK activation significantly increased the LC3B2/LC3B1 ratio in Parkin deficient cells, in particular within 8 to 24 hours after inclusion of AICAR (Figures 3f and g). To determine the signaling pathway by which AMPK activates autophagy, *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages were treated with AICAR for 24 hours followed by measuring phosphorylation of a potential AMPK target beclin-1. We found that AMPK increases Ser93-beclin-1 phosphorylation (Figures 3h and i), indicative of AMPK-mediated activation of autophagy, even in Parkin deficient macrophages.

Besides removal of defective organelles, the Parkin has been implicated in clearance of intracellular bacteria (9). Both *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages exhibited similar uptake of fluorescently tagged *E. coli*. However, further analysis confirmed that *PARK2*$^{-/-}$ macrophages had an impaired ability to kill bacteria (Figure 3k). Because AMPK activation promoted autophagy, even in the absence of Parkin (Figure 3f), we tested if AMPK stimulates bacterial killing in *PARK2*$^{-/-}$ macrophages. As shown in Figure 3k, pretreatment with AICAR resulted in a significant increase in *P. aeruginosa* clearance *ex vivo* by *PARK2*$^{-/-}$ macrophages. These results suggest that AMPK activation was able to bypass Parkin-related deficiency of autophagy and effectively increased bacterial killing.

AMPK Activation Increases Mitochondrial Quality Control and Mitochondrial Bioenergetics

Defective autophagy is likely affecting mitochondrial quality control in Parkin deficient cells. We found basal and ATP-link oxygen consumption rates were significantly decreased in *PARK2*$^{-/-}$ macrophages, as compared to *PARK2*$^{+/+}$ macrophages (Figures 4a-c). Notably, *PARK2*$^{-/-}$ macrophages also showed reduced non-mitochondrial OCR (Figure 4g), these results suggest an overall diminished bioenergetic capacity in *PARK2*$^{-/-}$ macrophages. Additional experiments indicate only a negligible impact of AICAR on basal and ATP-link OCR (Figures a-c). However, AMPK activation greatly improved the quality of mitochondria in Parkin deficient cells, as evidenced by increased maximal and reserve capacity (Figures 4a, e-f). Mitochondrial dynamics is also dependent on biogenesis. We

found, AMPK activation effectively stimulated expression of major components in electron transport chain complexes I-IV, and ATP-synthase alpha subunit (Complex V) (Figures 4h and i). These results suggest that despite Parkin deficiency, AMPK improved mitochondrial quality by activating autophagy and biogenesis.

Parkin Deficiency Increases the Severity of LPS-Induced Lung Injury

Given that Parkin deficiency is associated with mitochondrial dysfunction and exaggerated pro-inflammatory activation of immune cells, we hypothesized that *PARK2*$^{-/-}$ mice may development more severe ALI. To test this possibility, *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ mice were subjected to intratracheal instillation of saline (control) or LPS (2 mg/kg) for 24 hours. Both *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ mice show indices of lung injury after LPS administration. However, *PARK2*$^{-/-}$ mice exhibit more enhanced neutrophil flux, more extensive thickened septa, and accumulation of cellular debris in alveoli, compared to wild type mice (Figures 5a and e). *PARK2*$^{-/-}$ mice also exhibit significant increases in edema (wet-to-dry ratio) along with enhanced lung permeability (BAL fluid protein concentrations), as compared to wild type counterparts (Figures, b-d). Consistent with results obtained from LPS treated *PARK2*$^{-/-}$ peritoneal macrophages (Figure 1), intratracheal instillation of LPS also triggered more robust production of pro-inflammatory cytokines TNF-α, MIP-2, and IL-6 in lungs (BALs) of *PARK2*$^{-/-}$ *versus PARK2*$^{+/+}$ mice (Figures 5f-h). Notably, Parkin deficient mice exhibit a greater accumulation of damage associated molecular pattern proteins (DAMPs), including in HMGB1 and histone H3 in BAL fluids

(Figures 5i and j). These results indicate that Parkin deficient mice had increased susceptibility to endotoxin-induced ALI.

Metformin Reduces the Severity of LPS-Induced ALI in PARK2$^{-/-}$ Mice

We next examined whether AMPK activator metformin affects the severity of ALI in LPS treated *PARK2*$^{-/-}$ mice. Mice were treated to saline (control; i.p.) or metformin (90 mg/kg; i.p.) followed by intratracheal instillation of LPS (2 mg/kg) or saline. As shown in Figure 6, pretreatment with metformin significantly diminished LPS-induced lung injury, as evidenced by reduced permeability (BAL protein levels), a lower extent of neutrophil accumulation in the lungs, and reduced BAL fluid cytokines, including TNF-α and MIP-2, as compared to mice that received LPS alone. These findings indicate that AMPK activation prevented development of ALI despite Parkin deficiency.

Myeloid AMPKα1$^{-/-}$ Mice Exhibit Diminished Clearance of Bacteria in the Lungs

To further determine the importance of AMPK activation, we developed myeloid specific AMPKα1$^{-/-}$ mice. These mice are deficient in AMPKα1 in their monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, and platelets. We used these mice to investigate *in vivo* bacterial clearance in the lungs and found that the myeloid AMPKα1$^{-/-}$ mice had significantly more *P. aeruginosa* remaining in the lungs (Figure 7).

Discussion

In the present study, we found an interesting correlation between inflammatory conditions implicated in ALI and dissipation of Parkin, a major ubiquitin ligase involved in regulating autophagy. Besides a time dependent decrease of Parkin protein in LPS treated macrophages, reduced Parkin levels were associated with alveolar epithelial cell injury and was also observed in lungs of mice subjected to endotoxin-induced ALI. *PARK2*$^{-/-}$ mice were prone to develop a more robust macrophage pro-inflammatory response, while autophagy-dependent neutralization of intracellular bacteria was defective. Parkin has cellular substrates across multiple pathways (42), and our results suggest that mitochondrial dysfunction is priming Parkin deficient macrophages for an exaggerated pro-inflammatory response. In order to stimulate autophagy, we examined the effects of AMPK activators in *PARK2*$^{-/-}$ mice. Interestingly, AMPK activation did not prevent Parkin dissipation. However, AMPK stimulated autophagy-dependent neutralization of *P. aeruginosa* and improve mitochondrial function in *PARK2*$^{-/-}$ macrophages. Finally, AMPK activators effectively reduced the severity of LPS-induced ALI in *PARK2*$^{-/-}$ mice.

The Parkin-autophagy axis plays a crucial role in regulating innate immune responses, as autophagy is directly involved in intracellular removal of phagocytosed bacteria (43-45). Autophagy can also indirectly affect the immune response, in particular via mitochondrial quality control. Mitochondria are essential bioenergetic hubs, but also serve as a signaling platform for many cellular processes, including pro-inflammatory activation, bacterial killing, and phagocytic clearance of dying cells (46). Our results are consistent with previous studies that loss of autophagy and increased mitochondrial ROS formation are implicated in inflammasome activation. In turn, autophagy activation has

been shown to reduce inflammation due to downregulation of NLRP3-dependent mitochondrial ROS generation and mitochondrial DNA release (47-49). Preservation of a "healthy" mitochondria pool and biogenesis has recently been implicated in increased macrophage and neutrophil antimicrobial activity (50). Similarly, preservation or recovery of mitochondrial function was beneficial in models of sepsis and ALI (12, 39). These findings are clinically relevant, as mitochondrial dysfunction in lungs of critically ill patients is correlated with the severity of injury and mortality (5, 6, 51). In this setting, the increased inflammatory burden and inability to eradicate bacteria is likely related to Parkin dissipation followed by reduction in autophagy and accumulation of dysfunctional mitochondria.

Recent studies suggest that Parkin may affect immune function, including sensitivity to pro-inflammatory activation that was observed in monocytes isolated from PD patients (9, 52, 53). Defects in the Parkin pathway have also been implicated in lung cancer (54, 55), pulmonary fibrosis (56), COPD (57), and possibly sepsis-induced lung injury (58). By measuring Parkin in immune cells and lung homogenates, we provide evidence for enhanced turnover of Parkin that occurs during inflammatory conditions. It is possible that along with bacterial clearance and inflammatory lung injury, Parkin also contributes to macrophage dependent neutralization of apoptotic cells and cellular debris (19, 59, 60). In particular, enhanced efferocytosis has been associated with reduced severity of sterile inflammatory conditions and development of ALI. Overall, our results revealed that (i) Parkin deficiency is characterized by reduced autophagic bacterial killing, (ii) loss of Parkin-autophagy axis leads to accumulation of dysfunctional mitochondria and ROS –mediated priming for macrophage pro-inflammatory activation, and (iii) exag-

gerated pro-inflammatory activation of immune cells in Parkin deficient mice is associated with development of severe ALI. These findings establish a strong link between the Parkin-autophagy axis and immune bioenergetics, pro-inflammatory activation and bacterial killing.

Given that Parkin-dependent autophagy is a perishable signaling, we examined if activation AMPK can rescue autophagy even in Parkin deficient macrophages and lungs of mice subjected to ALI. We tested this possibility using AMPK activators metformin and AICAR, both well-established in cultured cells and *in vivo* models of ALI. First, we provided evidence that AMPK activation promoted autophagy and bacterial killing in Parkin deficient macrophages. Secondly, application of metformin diminished LPS-induced development of ALI in *PARK2*$^{-/-}$ mice. A possible mechanism by which AMPK promoted autophagy is linked to phosphorylation of ULK1, beclin-1, and inhibition of mTOR. Notably, beclin-1 has been previously shown to activate autophagy in Parkin independent fashion (61), and our results suggest that this is a relevant mechanism by which AMPK bypasses Parkin deficiency. Our findings are likely impactful when considering a few studies that suggest a benefit of AMPK activation in PD patients (62-64). Idiopathic PD is characterized by worsen symptoms due to infection, and a nearly 60% mortality rate is associated with aspiration pneumonia (65). Thus, AMPK activation is likely a relevant therapeutic target to reduce indices of lung injury.

Our findings suggest that AMPK activators, including metformin could be repurposed for treatment of ARDS and sepsis. Metformin is an FDA approved drug typically prescribed to type II diabetics. Recent clinical studies revealed that use of metformin prior to sepsis-related hospitalization had better outcomes (66). Taken together, our studies

suggest that AMPK activation has a substantial benefit due to reduced inflammatory conditions and enhanced bacterial killing. Moreover, AMPK also improves mitochondrial bioenergetics, thus it is expected that AMPK activation will improve immune cells and lung tissue homeostasis.

Materials and Methods

Mice

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *PARK2*$^{-/-}$ were originally purchased from The Jackson Laboratory and bred in facilities at the University of Alabama at Birmingham. Mice 10 to 12 weeks of age were used for experiments. Mice were given food and water ad libitum and kept on a 12-hours light-dark cycle. All experiments were conducted in accordance with approved protocols by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Antibodies

The following antibodies were used: Anti-HMGB1 (R&D Systems, MAB1690), phospho-Thr172-AMPK (Cell Signaling Technology, 2531), AMPKα1 (Cell Signaling Technology, 2532), phospho-Ser93-beclin-1 (Cell Signaling Technology, 14717) phospho-Ser79-Acetyl CoA Carboxylase (Cell Signaling Technology, 3661), LC3B (Cell Signaling Technology, 2775), Histone H3 (Santa Cruz Biotechnology, sc-8654-R), Parkin (Santa Cruz Biotechnology, sc-32282), and HRP-conjugated β-actin antibody (Santa Cruz Biotechnology, sc-47778).

Peritoneal Macrophage Isolation and Culture

Peritoneal macrophages were isolated as previously described (67). Macrophages were elicited in 10- to 12- week-old mice by intraperitoneal application of Brewer thioglycol-

late (Sigma-Aldrich, B2551). Cells were collected 4 days after thioglycollate injection. Peritoneal macrophages were cultured in RPMI 1640 media (GE Health, SH30027) supplemented with 8% FBS (Atlanta Biologicals, S11150) at 37°C. In select experiments macrophages were incubated with RPMI 1640 media supplemented with 0.5% FBS for 2 hours prior to LPS (Sigma-Aldrich, Cat # L2630) exposure, as indicated in Figure legends. For experiments where cytokines were measured, macrophages were left in culture for 3-4 days prior to experimentation.

Alveolar epithelial cell culture

L2, alveolar cell line, cells were cultured in F-12K (Gibco, 21127022) supplemented with 8% FBS (Atlanta Biologicals, S11150) at 37°C.

Paracrine signaling

Macrophages were stimulated with LPS (300 ng/ml) for 24 hours and conditioned culture media was collected. Alveolar epithelial cells were then incubated with macrophage conditioned media for 24 hours.

Bacterial Uptake and Killing

Bacterial uptake was determined by culturing macrophages with metformin (0 or 500 μM) for 2 hours followed by incubation with *E. coli* for 20 minutes. *PARK2*$^{+/+}$ and

PARK2$^{-/-}$ macrophages were cultured with AICAR (0 or 500 μM) for 1 hour followed by incubation with *P. aeruginosa* (10 fold) for an additional 1 hour. The surviving bacteria were cultured overnight on agar plates and CFUs counted for bacterial killing determination. Bacteria to macrophages was 10:1.

Measurement of Cellular Bioenergetics

The bioenergetics of macrophages was determined using the XF96 analyzer from Seahorse Bioscience, which measures O_2 consumption and proton production (pH) in intact cells. The O_2 consumption rate (OCR) is correlated with oxidative phosphorylation, and proton production (extracellular acidification rate) can be related to glycolysis. Measurements were performed using macrophages (1×10^5) that were plated on XF96 plates, after which they were treated with AICAR (150 μM) for 48 hours. The plate was then washed with XF assay buffer (DMEM, 5% FBS supplemented with 5.5 mm, D-glucose, 4 mm l-glutamine, and 1 mm pyruvate (pH 7.4)) and incubated in XF buffer for 30–60 min before the assay. After the assay, the cells were lysed with radioimmune precipitation assay (RIPA) buffer, and protein concentration was determined by Bradford assay. All results were corrected to protein levels in individual wells.

Cytokine ELISA

ELISA was used to measure cytokine levels in culture media and bronchoalveolar lavage (BAL) fluids as previously described (67). Levels of TNF-α (R&D Systems, DY410),

MIP-2 (R&D Systems, DY452), and IL-6 (R&D Systems, DY406) were determined using ELISA kits according to manufacturer's instructions.

Western Blot Analysis

Western Blot analysis was performed as described previously (67). Each experiment was carried out two or more times with cell populations obtained from separate groups of mice. In selected experiments, BAL fluids (30 μl) were mixed with Laemmli sample buffer (BostonBioProducts, BP-111NR) and boiled for 5 min followed by Western Blot analysis with anti-HMGB1 antibody.

A Mouse Model for Endotoxin-Induced Lung Injury

Lung injury was induced by intratracheal administration of LPS (2 mg/kg in 50 μl of PBS) (Sigma-Aldrich, L2630) as previously described (68, 69). Characterization of lung injury is by neutrophil infiltration in to the interstitium and airways of the lungs, interstitial edema development, and increased proinflammatory cytokine production. Briefly, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was gently extended and LPS or PBS solution deposited into the pharynx followed by aspiration into the lungs. Metformin (Sigma, D150959) (100 mg/kg) in 0.5 ml of saline or control vehicle (saline) was injected intraperitoneally for 3 consecutive days with the third dose immediately prior to LPS intratracheal instillation. Mice were euthanized 24 hours after LPS administration. Bronchoalveolar fluids

(BALs) were obtained by lavaging the lungs three times with 1 ml PBS. In experiments where metformin was utilized, mice received 100 mg/kg (i.p.) 24 hours and 30 minutes prior to LPS administration.

Statistical Analysis

Multigroup comparisons were performed using one-way ANOVA with Tukey's post hoc test. Values were normally distributed. Statistical significance was determined by the Student's t-test for comparisons between two groups. A value of $p < 0.05$ was considered significant. Analyses were performed on SPSS version 16.0 (IBM, Armonk, NY) for Windows (Microsoft Corp., Redmond, WA).

References

1 Hudson, L. D. & Steinberg, K. P. Epidemiology of acute lung injury and ARDS. *Chest* **116**, 74S-82S (1999).

2 Rubenfeld, G. D. *et al.* Incidence and outcomes of acute lung injury. *The New England journal of medicine* **353**, 1685-1693, doi:10.1056/NEJMoa050333 (2005).

3 Acute Respiratory Distress Syndrome, N. *et al.* Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* **342**, 1301-1308, doi:10.1056/NEJM200005043421801 (2000).

4 Guerin, C., Reignier, J. & Richard, J. C. Prone positioning in the acute respiratory distress syndrome. *N Engl J Med* **369**, 980-981, doi:10.1056/NEJMc1308895 (2013).

5 Singer, M. The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. *Virulence* **5**, 66-72, doi:10.4161/viru.26907 (2014).

6 Carre, J. E. *et al.* Survival in critical illness is associated with early activation of mitochondrial biogenesis. *American journal of respiratory and critical care medicine* **182**, 745-751, doi:10.1164/rccm.201003-0326OC (2010).

7 Klionsky, D. J. & Emr, S. D. Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717-1721 (2000).

8 Mihalache, C. C. & Simon, H. U. Autophagy regulation in macrophages and neutrophils. *Experimental cell research* **318**, 1187-1192, doi:10.1016/j.yexcr.2011.12.021 (2012).

9 Manzanillo, P. S. *et al.* The ubiquitin ligase parkin mediates resistance to intracellular pathogens. *Nature* **501**, 512-516, doi:10.1038/nature12566 (2013).

10 Garaude, J. *et al.* Mitochondrial respiratory-chain adaptations in macrophages contribute to antibacterial host defense. *Nature immunology* **17**, 1037-1045, doi:10.1038/ni.3509 (2016).

11 West, A. P. *et al.* TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* **472**, 476-480, doi:10.1038/nature09973 (2011).

12 Mannam, P. *et al.* MKK3 regulates mitochondrial biogenesis and mitophagy in sepsis-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* **306**, L604-619, doi:10.1152/ajplung.00272.2013 (2014).

13 Aguirre, A. *et al.* Defective autophagy impairs ATF3 activity and worsens lung injury during endotoxemia. *Journal of molecular medicine* **92**, 665-676, doi:10.1007/s00109-014-1132-7 (2014).

14 Zhao, W. *et al.* Atg5 deficiency-mediated mitophagy aggravates cardiac inflammation and injury in response to angiotensin II. *Free radical biology & medicine* **69**, 108-115, doi:10.1016/j.freeradbiomed.2014.01.002 (2014).

15 Liu, S. *et al.* Autophagy plays a critical role in kidney tubule maintenance, aging and ischemia-reperfusion injury. *Autophagy* **8**, 826-837, doi:10.4161/auto.19419 (2012).

16 Wong, E. & Cuervo, A. M. Autophagy gone awry in neurodegenerative diseases. *Nature neuroscience* **13**, 805-811, doi:10.1038/nn.2575 (2010).

17 Nishino, I. *et al.* Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature* **406**, 906-910, doi:10.1038/35022604 (2000).

18 Verheye, S. *et al.* Selective clearance of macrophages in atherosclerotic plaques by autophagy. *Journal of the American College of Cardiology* **49**, 706-715, doi:10.1016/j.jacc.2006.09.047 (2007).

19 Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. *Nature* **469**, 323-335, doi:10.1038/nature09782 (2011).

20 Patel, A. S. *et al.* Autophagy in idiopathic pulmonary fibrosis. *PloS one* **7**, e41394, doi:10.1371/journal.pone.0041394 (2012).

21 Rogov, V., Dotsch, V., Johansen, T. & Kirkin, V. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Molecular cell* **53**, 167-178, doi:10.1016/j.molcel.2013.12.014 (2014).

22 Narendra, D., Tanaka, A., Suen, D. F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of cell biology* **183**, 795-803, doi:10.1083/jcb.200809125 (2008).

23 Zhang, Y. *et al.* Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13354-13359, doi:10.1073/pnas.240347797 (2000).

24 Chan, N. C. *et al.* Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Human molecular genetics* **20**, 1726-1737, doi:10.1093/hmg/ddr048 (2011).

25 Bertolin, G. *et al.* The TOMM machinery is a molecular switch in PINK1 and PARK2/PARKIN-dependent mitochondrial clearance. *Autophagy* **9**, 1801-1817, doi:10.4161/auto.25884 (2013).

26 Henn, I. H., Gostner, J. M., Lackner, P., Tatzelt, J. & Winklhofer, K. F. Pathogenic mutations inactivate parkin by distinct mechanisms. *Journal of neurochemistry* **92**, 114-122, doi:10.1111/j.1471-4159.2004.02854.x (2005).

27 Sriram, S. R. *et al.* Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin. *Human molecular genetics* **14**, 2571-2586, doi:10.1093/hmg/ddi292 (2005).

28 Harris, M. A., Tsui, J. K., Marion, S. A., Shen, H. & Teschke, K. Association of Parkinson's disease with infections and occupational exposure to possible vectors. *Movement disorders : official journal of the Movement Disorder Society* **27**, 1111-1117, doi:10.1002/mds.25077 (2012).

29 Bower, J. H., Maraganore, D. M., Peterson, B. J., Ahlskog, J. E. & Rocca, W. A. Immunologic diseases, anti-inflammatory drugs, and Parkinson disease: a case-control study. *Neurology* **67**, 494-496, doi:10.1212/01.wnl.0000227906.99570.cc (2006).

30 Piquereau, J. *et al.* Protective role of PARK2/Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction. *Autophagy* **9**, 1837-1851, doi:10.4161/auto.26502 (2013).

31 O'Neill, L. A. & Hardie, D. G. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* **493**, 346-355, doi:10.1038/nature11862 (2013).

32 Wu, S. B., Wu, Y. T., Wu, T. P. & Wei, Y. H. Role of AMPK-mediated adaptive responses in human cells with mitochondrial dysfunction to oxidative stress. *Biochimica et biophysica acta* **1840**, 1331-1344, doi:10.1016/j.bbagen.2013.10.034 (2014).

33 Hardie, D. G., Ross, F. A. & Hawley, S. A. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* **13**, 251-262, doi:10.1038/nrm3311 (2012).

34 Assifi, M. M. *et al.* AMP-activated protein kinase and coordination of hepatic fatty acid metabolism of starved/carbohydrate-refed rats. *American journal of physiology. Endocrinology and metabolism* **289**, E794-800, doi:10.1152/ajpendo.00144.2005 (2005).

35 Carling, D. AMPK signalling in health and disease. *Current opinion in cell biology* **45**, 31-37, doi:10.1016/j.ceb.2017.01.005 (2017).

36 Kim, J., Kundu, M., Viollet, B. & Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature cell biology* **13**, 132-141, doi:10.1038/ncb2152 (2011).

37 Zhang, D. *et al.* AMPK regulates autophagy by phosphorylating BECN1 at threonine 388. *Autophagy* **12**, 1447-1459, doi:10.1080/15548627.2016.1185576 (2016).

38 Escobar, D. A. *et al.* Adenosine monophosphate-activated protein kinase activation protects against sepsis-induced organ injury and inflammation. *The Journal of surgical research* **194**, 262-272, doi:10.1016/j.jss.2014.10.009 (2015).

39 Liu, Z. *et al.* AMP-activated protein kinase and Glycogen Synthase Kinase 3beta modulate the severity of sepsis-induced lung injury. *Mol Med*, doi:10.2119/molmed.2015.00198 (2015).

40 Zeng, M. *et al.* 4-PBA inhibits LPS-induced inflammation through regulating ER stress and autophagy in acute lung injury models. *Toxicology letters* **271**, 26-37, doi:10.1016/j.toxlet.2017.02.023 (2017).

41 Matthay, M. A. & Zimmerman, G. A. Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. *American journal of respiratory cell and molecular biology* **33**, 319-327, doi:10.1165/rcmb.F305 (2005).

42 Sandebring, A. & Cedazo-Minguez, A. Parkin- An E3 Ubiquitin Ligase with Multiple Substrates. *Alzheimer's Disease & Parkinsonism*, doi:10.4172/2161-0460.S10-002 (2012).

43 Gutierrez, M. G. *et al.* Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766, doi:10.1016/j.cell.2004.11.038 (2004).

44 Birmingham, C. L., Smith, A. C., Bakowski, M. A., Yoshimori, T. & Brumell, J. H. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *The Journal of biological chemistry* **281**, 11374-11383, doi:10.1074/jbc.M509157200 (2006).

45 Paludan, C. *et al.* Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* **307**, 593-596, doi:10.1126/science.1104904 (2005).

46 West, A. P., Shadel, G. S. & Ghosh, S. Mitochondria in innate immune responses. *Nature reviews. Immunology* **11**, 389-402, doi:10.1038/nri2975 (2011).

47 Heid, M. E. *et al.* Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *Journal of immunology* **191**, 5230-5238, doi:10.4049/jimmunol.1301490 (2013).

48 Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221-225, doi:10.1038/nature09663 (2011).

49 Nakahira, K. *et al.* Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nature immunology* **12**, 222-230, doi:10.1038/ni.1980 (2011).

50 Yang, C. S. *et al.* The AMPK-PPARGC1A pathway is required for antimicrobial host defense through activation of autophagy. *Autophagy* **10**, 785-802, doi:10.4161/auto.28072 (2014).

51 Brealey, D. *et al.* Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. *American journal of physiology. Regulatory, integrative and comparative physiology* **286**, R491-497, doi:10.1152/ajpregu.00432.2003 (2004).

52 Watson, R. O., Manzanillo, P. S. & Cox, J. S. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* **150**, 803-815, doi:10.1016/j.cell.2012.06.040 (2012).

53 Grozdanov, V. *et al.* Inflammatory dysregulation of blood monocytes in Parkinson's disease patients. *Acta neuropathologica* **128**, 651-663, doi:10.1007/s00401-014-1345-4 (2014).

54 Lee, S. *et al.* Multiple-level validation identifies PARK2 in the development of lung cancer and chronic obstructive pulmonary disease. *Oncotarget* **7**, 44211-44223, doi:10.18632/oncotarget.9954 (2016).

55 D'Amico, A. G. *et al.* Expression pattern of parkin isoforms in lung adenocarcinomas. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **36**, 5133-5141, doi:10.1007/s13277-015-3166-z (2015).

56 Bueno, M. *et al.* PINK1 deficiency impairs mitochondrial homeostasis and promotes lung fibrosis. *The Journal of clinical investigation* **125**, 521-538, doi:10.1172/JCI74942 (2015).

57 Ito, S. *et al.* PARK2-mediated mitophagy is involved in regulation of HBEC senescence in COPD pathogenesis. *Autophagy* **11**, 547-559, doi:10.1080/15548627.2015.1017190 (2015).

58 Kang, R. *et al.* A novel PINK1- and PARK2-dependent protective neuroimmune pathway in lethal sepsis. *Autophagy* **12**, 2374-2385, doi:10.1080/15548627.2016.1239678 (2016).

59 Jiang, S. *et al.* Mitochondria and AMP-activated protein kinase-dependent mechanism of efferocytosis. *J Biol Chem* **288**, 26013-26026, doi:10.1074/jbc.M113.489468 (2013).

60 Bae, H. B. *et al.* AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. *Faseb J* **25**, 4358-4368, doi:10.1096/fj.11-190587 (2011).

61 Strappazzon, F. *et al.* AMBRA1 is able to induce mitophagy via LC3 binding, regardless of PARKIN and p62/SQSTM1. *Cell death and differentiation* **22**, 517, doi:10.1038/cdd.2014.190 (2015).

62 Wu, Y. *et al.* Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease. *Neuro-Signals* **19**, 163-174, doi:10.1159/000328516 (2011).

63 Wahlqvist, M. L. *et al.* Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson's disease occurring with Type 2 diabetes in a Taiwanese population cohort. *Parkinsonism & related disorders* **18**, 753-758, doi:10.1016/j.parkreldis.2012.03.010 (2012).

64 Ng, C. H. *et al.* AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in Drosophila models of Parkinson's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 14311-14317, doi:10.1523/JNEUROSCI.0499-12.2012 (2012).

65 Mehanna, R. & Jankovic, J. Respiratory problems in neurologic movement disorders. *Parkinsonism & related disorders* **16**, 628-638, doi:10.1016/j.parkreldis.2010.07.004 (2010).

66 Doenyas-Barak, K., Beberashvili, I., Marcus, R. & Efrati, S. Lactic acidosis and severe septic shock in metformin users: a cohort study. *Critical care* **20**, 10, doi:10.1186/s13054-015-1180-6 (2016).

67 Park, D. W. *et al.* GSK3beta-dependent inhibition of AMPK potentiates activation of neutrophils and macrophages and enhances severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* **307**, L735-745, doi:10.1152/ajplung.00165.2014 (2014).

68 Zhao, X. *et al.* Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* **295**, L497-504 (2008).

69 Zmijewski, J. W. *et al.* Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. *Am J Respir Crit Care Med* **178**, 168-179 (2008).

Figures

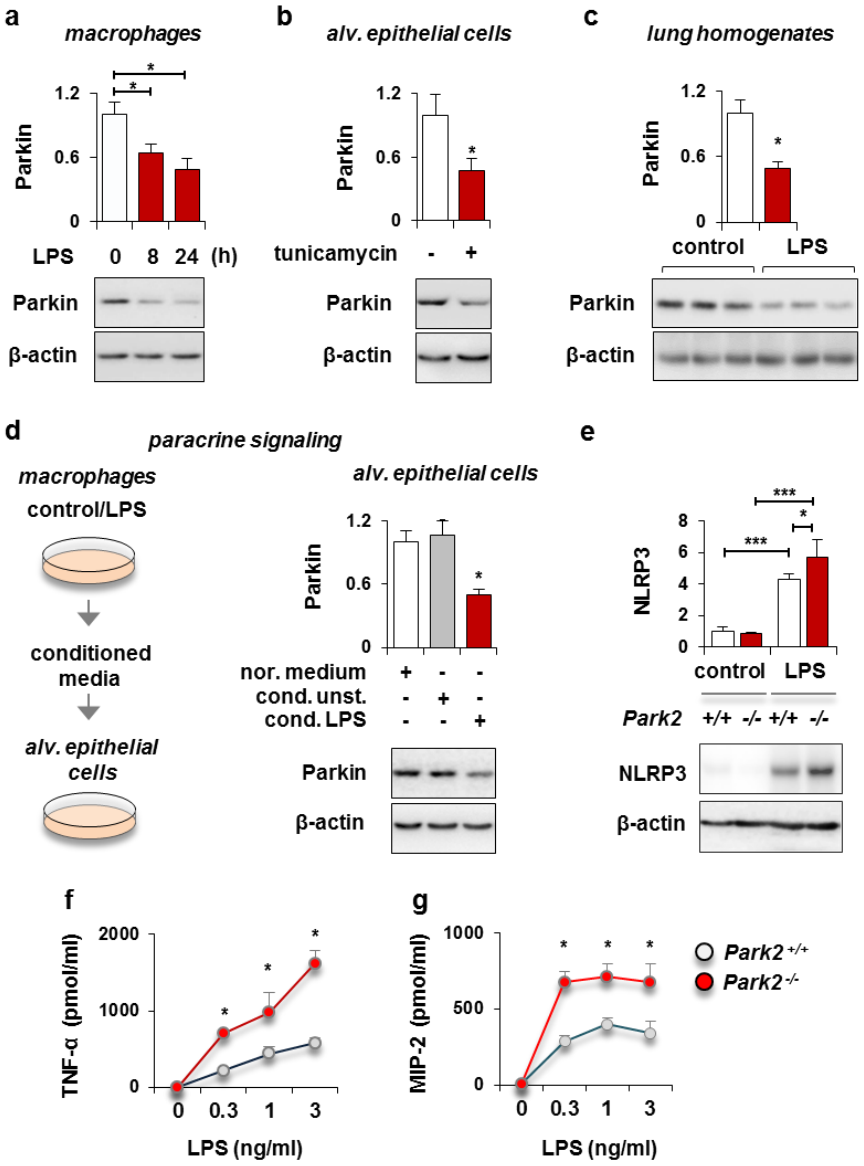

Figure 1. Inflammatory conditions are associated with dissipation of Parkin protein. Western blot analysis of Parkin protein in lysates of (**a**) LPS treated (1 μM) macrophages, (**b**) tunicamycin (10 μg/ml, 24 h) treated epithelial cells, and (**c**) in lung homogenates obtained from mice subjected to LPS-induced ALI (n = 3). (**d**) Conditioned media obtained from activated macrophages caused Parkin dissipation in alveolar epithelial cells (n = 3). (**e**) *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages were cultured with LPS (0 or 300 ng/ml) for 24 hours followed by Western blot analysis of NLRP3 and β-actin (n = 3). (**f-g**) The amounts of TNF-α and MIP-2 cytokines were measured in culture media of *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages. Cells were treated with LPS for 4.5 hours (n = 4). Means ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

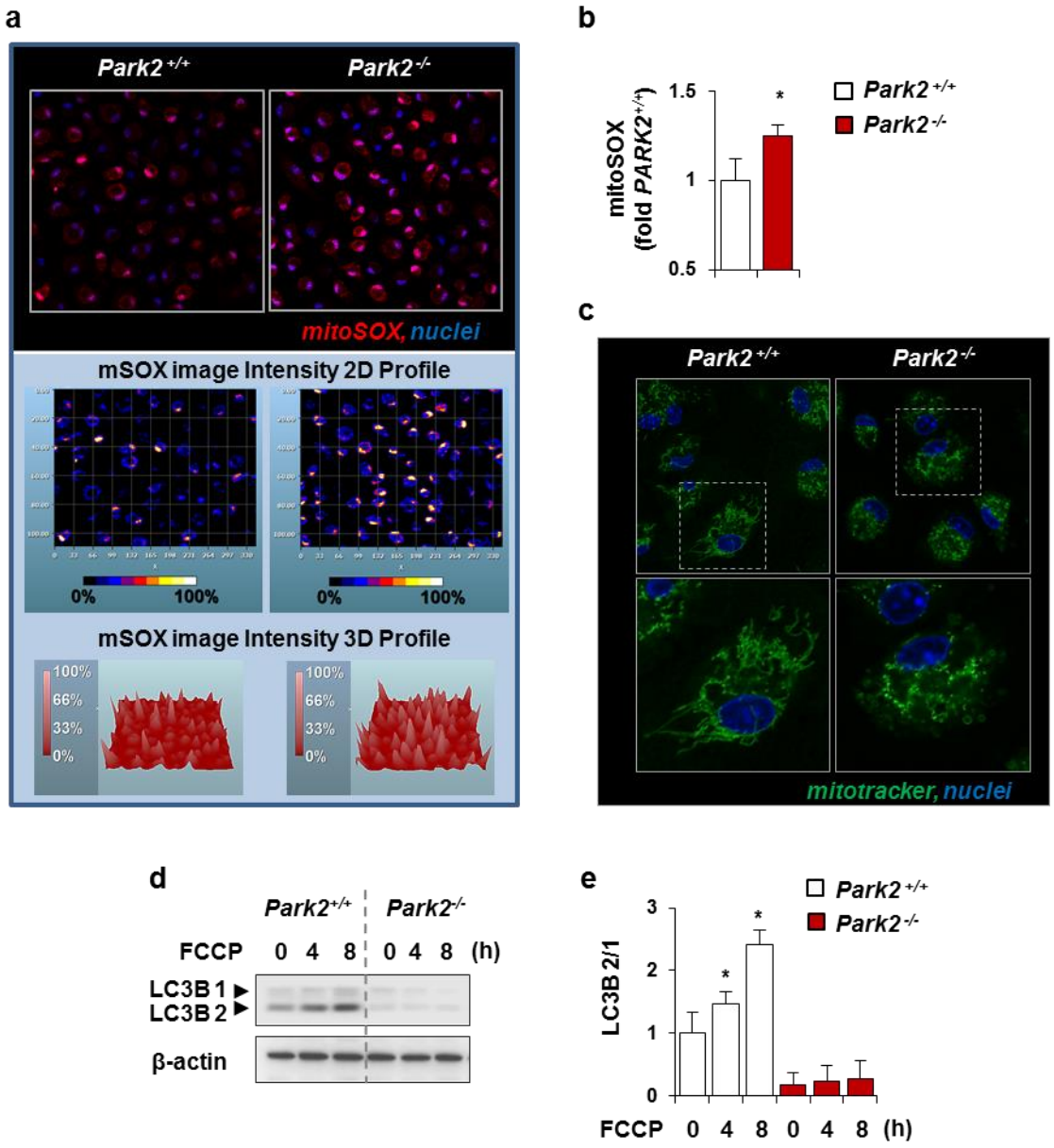

Figure 2. Mitophagy impairment and mitochondrial ROS formation are associated with Parkin deficiency. (**a**) Representative images, including 2-D and 3-D image profiles show the extent of mitoSOX fluorescence in *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages. Panel (**b**) shows mitoSOX fluorescent intensity (fold untreated *PARK2*$^{+/+}$). Means ± SD, $n = 4$, * $p < 0.05$). (**c**) Mitochondrial network in *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages was visualized using mito-Tracker Green dye. (**d-e**) Mitophagy activation was examined in *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages treated with uncoupler FCCP (500 nM) followed by WB analysis of LC3B2/LC3B1. Means ± SD, $n = 3$, * $p < 0.05$.

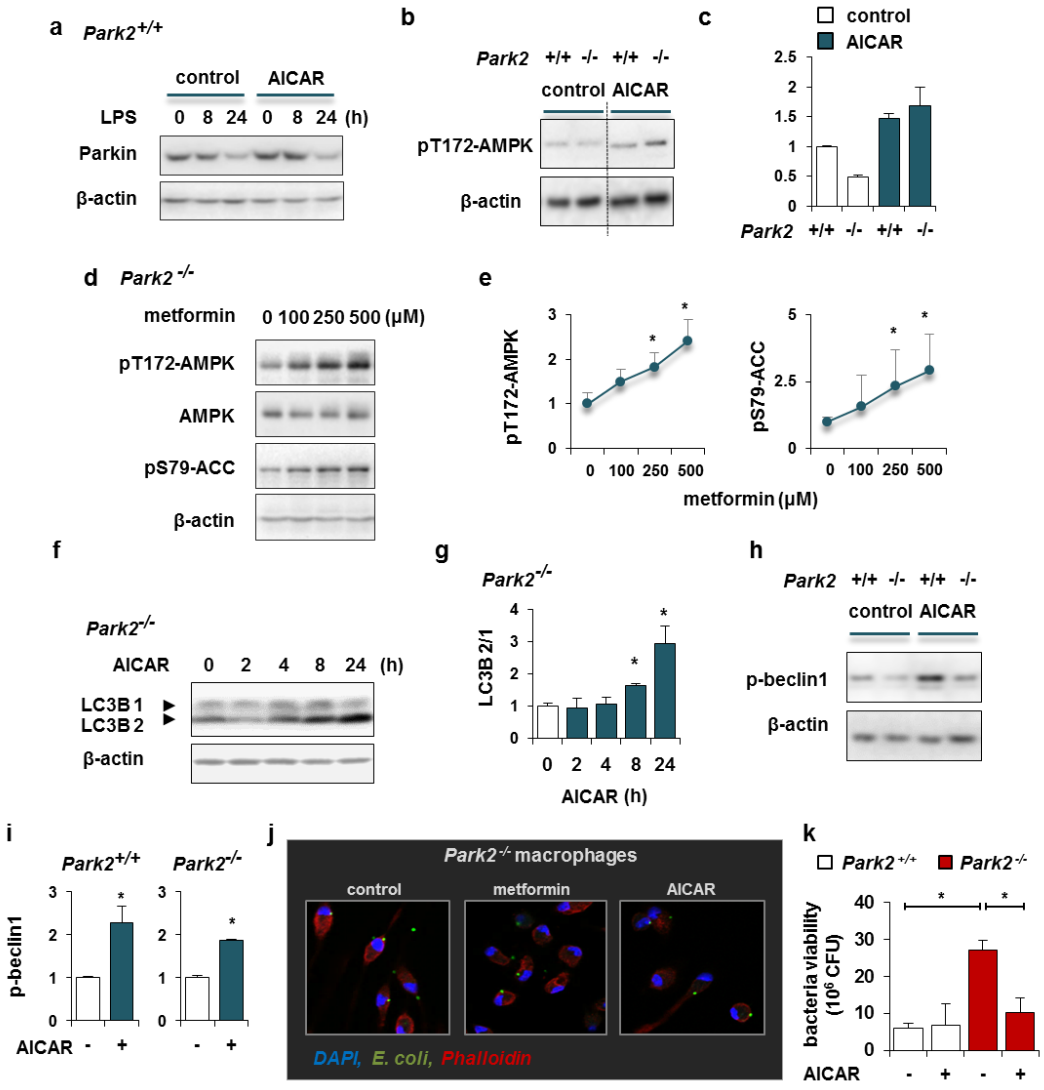

Figure 3. AMPK activation promotes autophagy despite Parkin deficiency. (**a**) Wild type macrophages were treated with AICAR for 2 hours prior to LPS exposure. The amounts of Parkin and β-actin is shown (n = 3). (**b-c**) *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages were treated with AICAR for 2 hours and Western blot analysis of pT172-AMPK and β-actin was performed (n = 3). (**d-e**) Western blot analysis of pT172-AMPK, AMPK, pS79-ACC, and β-actin in *PARK2*$^{-/-}$ macrophages treated with metformin (0-500 μM) for 4 hours (n = 3). (**f-g**) LC3B2/1 ratio (activation) in *PARK2*$^{-/-}$ macrophages treated with or without AICAR for indicated time (n = 3). (**h-i**) *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages were cultured with AICAR (0 or 500 μM) for 24 hours and then phospho-S93-beclin-1 determined in cell lysates (n = 3). (**j**) Representative images show bacterial uptake (fluorescein-tagged *E. coli*) in control and metformin treated *PARK2*$^{-/-}$ macrophages. Blue -nuclei; green -*E. coli*; red -Phalloidin. (**k**). *P. aeruginosa* viability (CFU) was determined in *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages pre-treated with AICAR (0 or 500 μM). Means ± SD, n = 3, * $p < 0.05$.

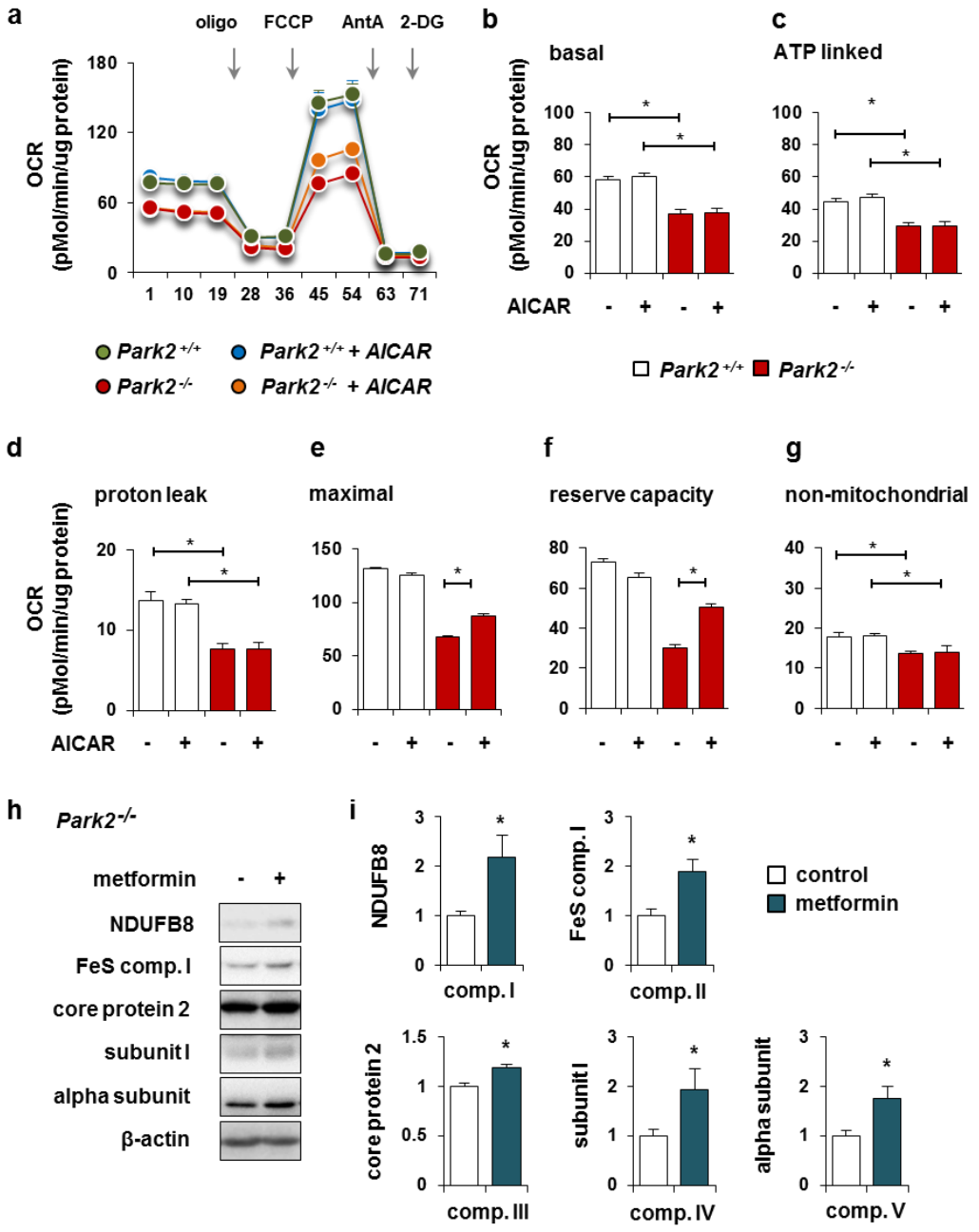

Figure 4. AMPK activation increased mitochondrial maximal OCR, reserve capacity and promoted biogenesis in Parkin deficient macrophages. (**a**-**g**) *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ macrophages were incubated with or without AICAR for 24 hours followed by measure of oxygen consumption rate (n = 5). Representative OCR profile and indices of mitochondrial functions are shown. (**h-i**) *PARK2*$^{-/-}$ macrophages were cultured with AICAR (0 or 500 μM) for 72 hours followed by analysis of major components of mitochondrial electron transport chain subunits: complex I: NDUFB8, complex II: FeS comp. l, complex III: core protein 2, complex IV: subunit I, and complex V: alpha subunit. Means ± SD, n = 4, * $p < 0.05$.

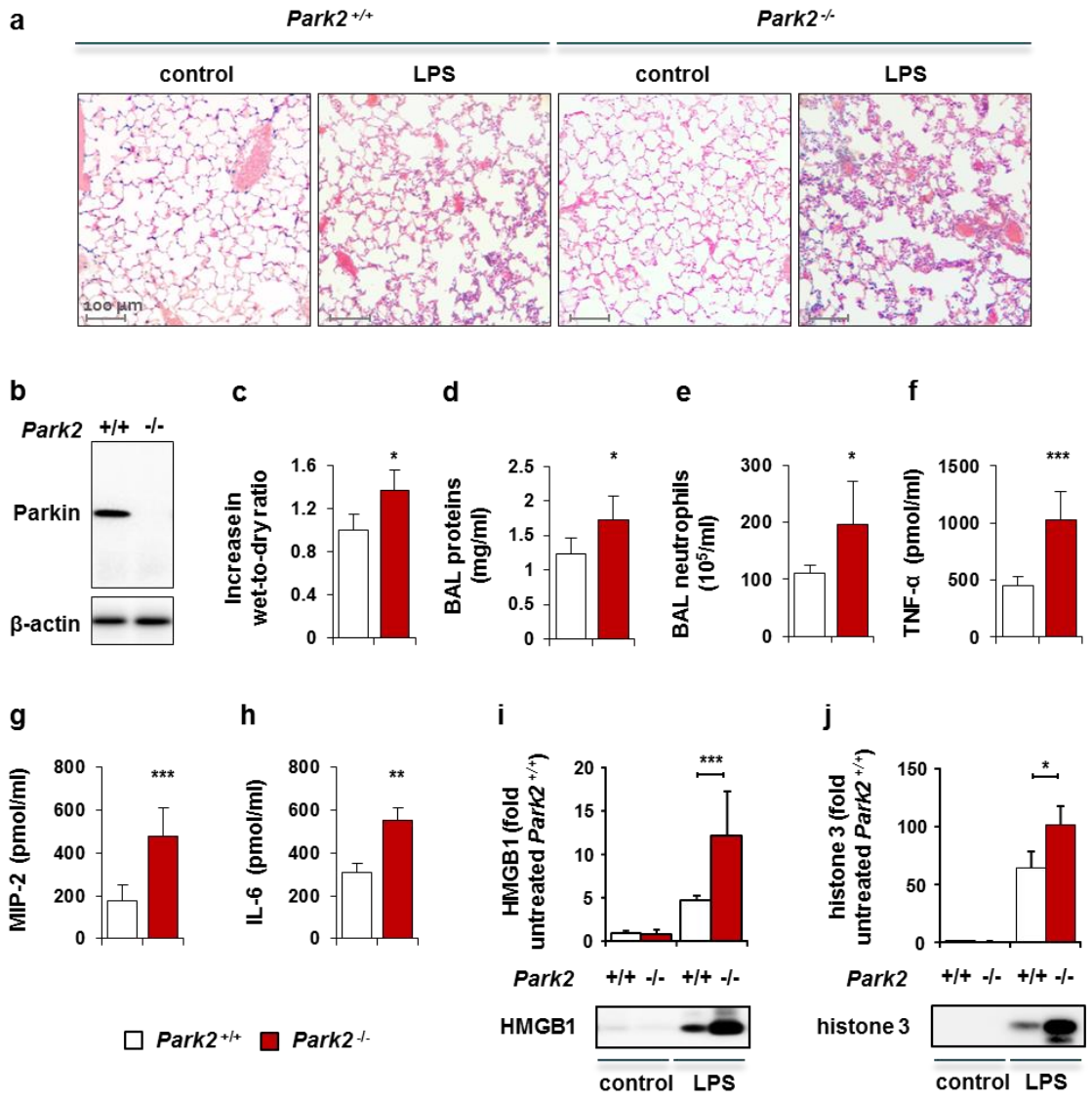

Figure 5. Parkin deficient mice have increased severity of LPS-induced acute lung injury. *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ mice were subjected with intratracheal instillation of saline (control, i.t.) or LPS (2 mg/kg, i.t.) for 24 h. Panel (**a**) shows representative images (10 x) of lung sections (H&E staining) obtained from control or mice treated with LPS. Bar 100 µM. (**b**) Western blot shows the extent of Parkin protein in *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ mouse lungs. (**c**) Increase in lung wet-to-dry ratios, (**d**) BAL proteins, and (**e**) number of lung neutrophils in BAL fluids were obtained 24 hours after exposure to LPS (n = 5). (**f**) TNF-α, (**g**) MIP-2, and (**h**) IL-6 was measured in BAL fluids of control and LPS treated *Park*$^{+/+}$ or *PARK2*$^{-/-}$ mice (n = 5). Representative Western blots and quantitative data show the amount of inflammatory mediators (**i**) HMGB1 and (**j**) histone H3 in BALs of *PARK2*$^{+/+}$ and *PARK2*$^{-/-}$ mice. BALs were collected 24 hours after exposure to saline or LPS (n = 3). Means ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to controls.

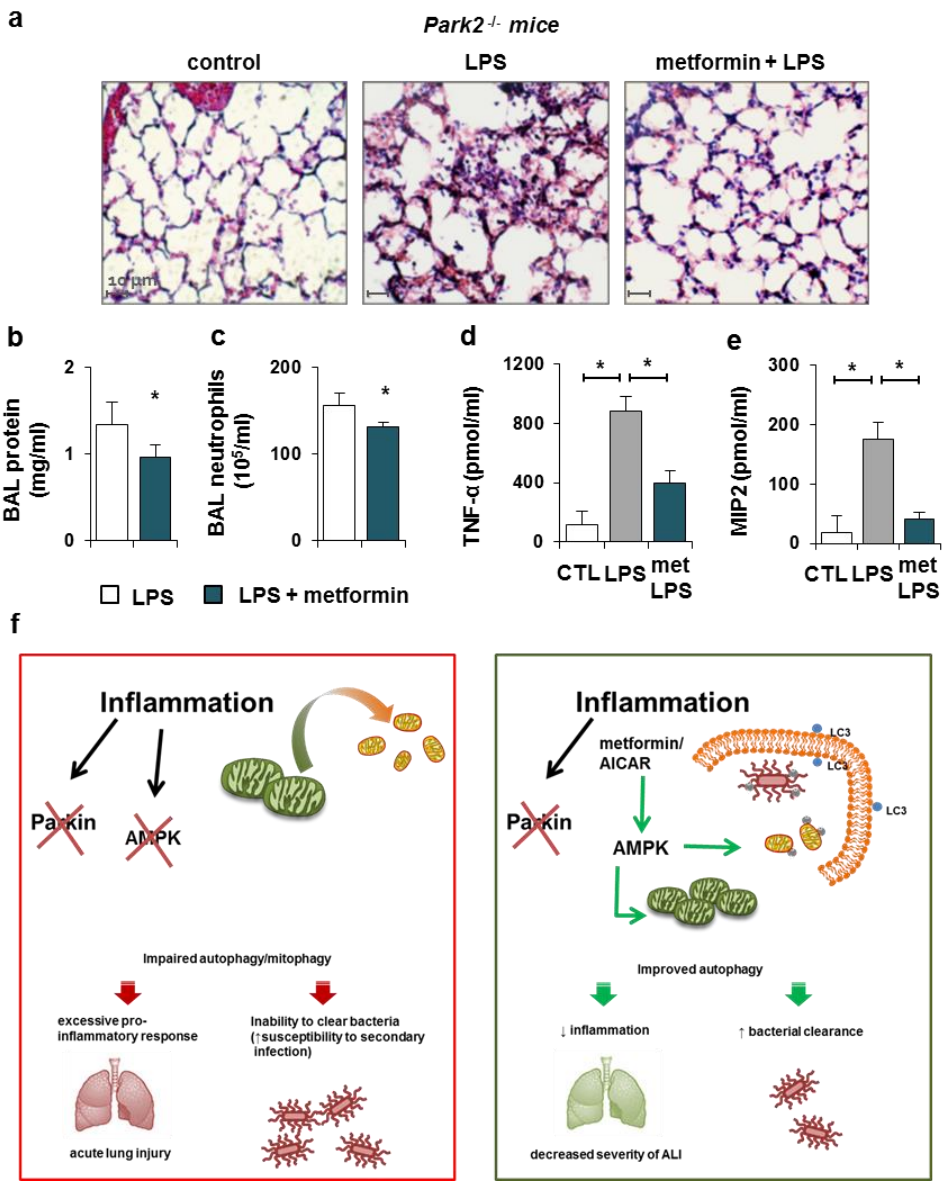

Figure 6. Metformin reduced severity of acute lung injury *PARK2*$^{-/-}$ mice. (**a**) Representative images (40x) show lung sections (H&E staining). Arrows indicate spaces filled with a mixed mononuclear/neutrophilic infiltrate, cellular debris and proteinaceous material. Alveolar walls are also thickened, and the septa are edematous. Quantitative data show (**b**) protein concentration, (**c**) number of BAL neutrophils, and the extent of BAL (**d**) TNF-α and (**e**) MIP-2, in *PARK2*$^{-/-}$ mice 24 hours after saline or LPS administration (n = 3). Means ± SD, * $p < 0.05$ compared to controls. (**f**) Inflammation leads to decreased levels of Parkin protein. The deficiency of Parkin protein is associated with bioenergetic dysfunction, impaired autophagy and bacterial killing, oxidative stress, and worse organ injury. Activation of AMPK by metformin or AICAR improves mitochondrial bioenergetics, promotes autophagy and bacterial clearance, and diminishes organ injury.

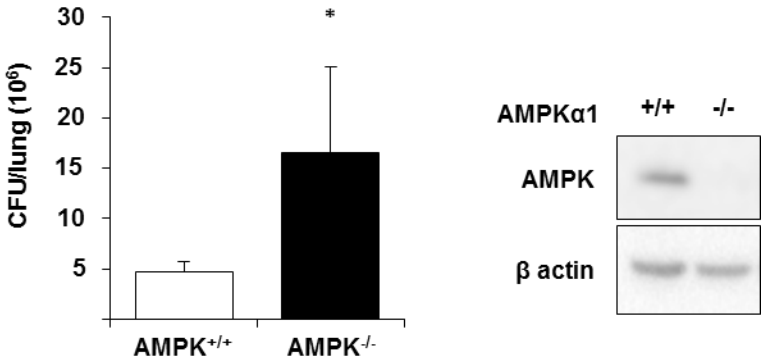

Figure 7. AMPK deficiency in myeloid cells results in impaired bacterial clearance. Wild type ($AMPK^{+/+}$) and myeloid specific AMPK deficient ($AMPK^{-/-}$) mice treated with i.t. instillation of P. aeruginosa (PAK, 2.5×10^7/mouse) for 4 hours. Next, amounts of surviving bacteria were determined in lung homogenates using CFU assay ($n = 5$). Means ± SD, * $p < 0.05$.

CONCLUSION

Sepsis is a multifactorial condition that has an enormous impact on the immune system and organ tissue homeostasis. Monocytes, macrophages, and neutrophils are essential components of the innate response to infection, but are also activated during injury. Immune activation is specifically designed to neutralize pathogens and apoptotic cells that allows for subsequent resolution and tissue repair. However, severe sepsis is linked to macrophage and monocyte dysfunction, uncontrolled microbial growth and dissemination, and ultimately multi-organ failure. In particular, macrophages exhibit sepsis-related dysfunction of autophagy, as evidenced by a reduced ability to clear intracellular bacteria. This deficiency also affects bioenergetic recovery due to accumulation of dysfunctional mitochondria. Recent studies suggest that autophagy is implicated in morbidity and mortality among critically ill patients[165]. Another previous observation has shown that loss of mitochondrial oxidative phosphorylation in immune and lung parenchyma cells is also correlated with mortality in sepsis and ARDS. In particular, pioneer studies conducted by Dr. Mervyn Singer have shown a significant correlation between the extent of mitochondrial dysfunction in lungs and mortality due to sepsis[87, 88]. For these reasons, autophagy (mitophagy), mitochondrial bioenergetics, and metabolic switches emerge as crucial therapeutic targets for sepsis and ARDS. It is important to note that anti-inflammatory approaches alone appear to have negligible effects in many clinical trials. Thus, we investigated the potential mechanism(s) by which

AMPK activation affects immune and parenchymal cell metabolism/bioenergetics to potentially diminish the severity of sepsis-induced lung injury.

Summary of studies

Study #1

It has been recently suggested that the central and peripheral nervous system, in particular activation of dopaminergic signaling, may provide a substantial benefit in experimental sepsis[26]. Because relatively little is known about how catecholamines affect bioenergetics, we examined a possible impact of dopaminergic signaling on AMPK activation and related mitochondrial function in immune and lung parenchyma cells during endotoxin-induced ALI. Our results showed:

- *Both dopamine and norepinephrine produce anti-inflammatory effects, however dopamine, specifically D1R signaling, may also be advantageous due to modulation of metabolic regulator AMPK.*
- *D1R mediated increase of AMPK activity prevented LPS-induced pT172-AMPK dephosphorylation (inactivation) in macrophages.*
- *The D1R-AMPK axis controls epithelial pro-inflammatory paracrine signaling influencing macrophage activation.*
- *The D1R- AMPK signaling axis diminished development of lung injury in a mouse model of endotoxin-induced ALI.*

Study #2

In order to better understand how preservation of bioenergetics affects sepsis-induced lung injury, we investigated the role of AMPK regulation during polymicrobial sepsis. Our therapeutic strategy included (i) AMPK activation by pharmacological stimuli with metformin and (ii) AMPK activation due to blocking inhibitory phosphorylation by GSK3β. Our studies show:

- *AMPK activation in macrophages that acquire immunotolerance preserves the ability of these cells to respond to secondary stimulation with PAMPs.*
- *AMPK activation diminished mortality in a murine model of polymicrobial sepsis.*
- *Metformin or inhibition of GSK3β increased the ability of neutrophils and macrophages to eradicate bacteria both ex vivo and in vivo.*
- *Stimulation of AMPK by metformin or prevention of GSK3β dependent inhibition of AMPK effectively diminished development of lung injury in a mouse model of intra-abdominal microbial sepsis.*

Study #3.

Mitochondrial dysfunction, pro-inflammatory cytokine production, and impaired bacterial clearance are major issues in sepsis. Autophagy activation is an essential degradative mechanism that may diminish many of these indices. Our initial study shows that Parkin, a major E3 ubiquitin ligase involved in autophagy, is diminished in immune cells and lung tissue in experimental models of ALI. We hypothesized that AMPK activation will promote recovery of Parkin and improve autophagy. We have shown that:

- *Parkin deficiency is characterized by increased inflammatory cytokine production and decreased macrophage autophagy.*

- *AMPK activation does not rescue Parkin protein levels.*
- *AMPK activation promotes autophagy in the absence of Parkin through a beclin-1 related pathway to reduce hyper pro-inflammatory cytokine release, remove dysfunctional mitochondria, and enhance bacterial clearance while diminishing lung injury.*

A graphical summary of our findings is provided in Figure 1.

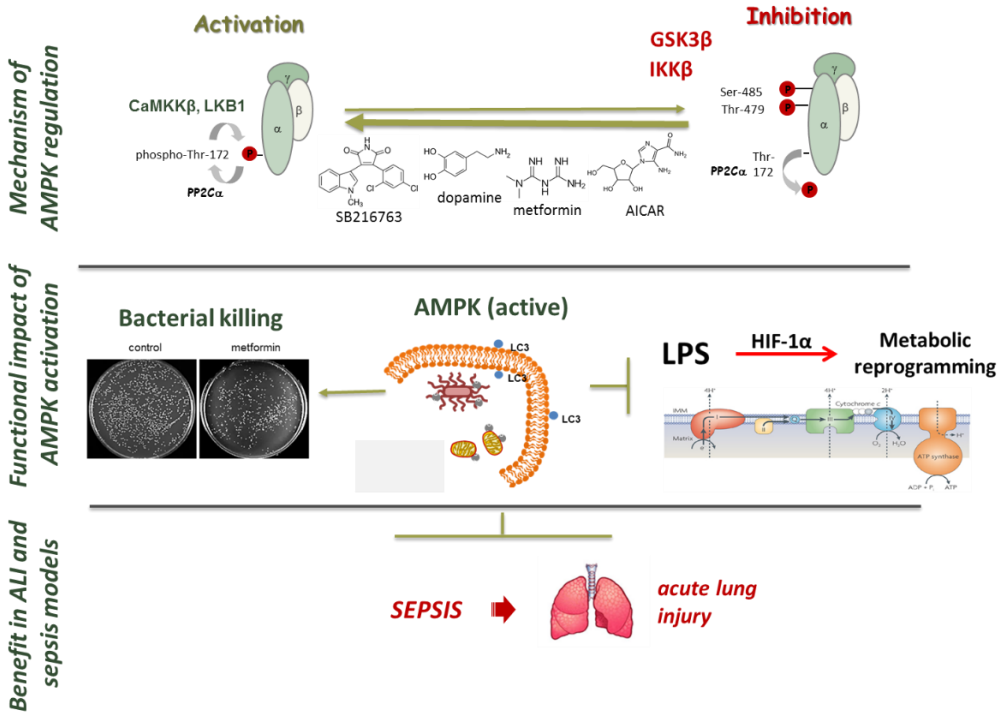

Figure 1. AMPK activation is a crucial target in sepsis and ALI. Selective pharmacological agents were identified to activate AMPK, including fenoldopam, metformin, SB216737, and AICAR. AMPK activation improved autophagy related to bacterial clearance, removal of defective mitochondrial (mitophagy), and decreased pro-inflammatory cytokine release. AMPK activation also reduced metabolic reprogramming associated with HIF-1α accumulation and glycolytic flux. These events diminished the severity of sepsis and ALI.

In summary, activation of AMPK by pharmacological means during sepsis may provide a useful treatment to reduce sepsis severity and development of ALI.

AMPK in the context of bacterial killing and development of ARDS

Despite many clinical trials having occurred over the years, effective therapeutic interventions are not available for sepsis, ARDS, and post-injury complications. Most potential therapeutic approaches target various inflammatory mediators, such as IL-1β or TNFα, but have been largely unsuccessful. We are first to instead target the bioenergetic pathway through AMPK activation. Severe sepsis and shock are frequently associated with dysfunction in immune and parenchyma cells. We have shown that AMPK activation is an important target in sepsis because AMPK activation (1) diminishes pro-inflammatory mediators in both macrophages and animal models; (2) increases clearance of bacteria in the lungs of septic mice and increased bacterial killing through alternative activation of xenophagy, which is defective in Parkin deficient mice; (3) reduces the severity of sepsis-induced ALI, including Parkin deficient mice subjected to LPS-induced ALI; and (4) preserves mitochondrial oxidative phosphorylation through reduction of macrophage metabolic reprogramming and preservation of mitochondrial electron transport chain complexes.

From a translational standpoint, activation of AMPK by pharmacological agents in sepsis patients may likely improve immune cell dependent autophagic clearance of bacteria and lessen inflammation and subsequent lung injury. FDA approved drugs which can activate AMPK already exist, including metformin, used to improve insulin sensitivity in Type II diabetics, and fenoldopam, used to diminish acute kidney injury. These drugs could potentially be repurposed and utilized in humans as a therapeutic to increase

bacterial killing and diminish inflammatory mediators during sepsis and thereby reduce lung injury. If either of these drugs is successful in clinical trials, they may pave the way for development of new therapeutics for sepsis.

Limitations

The studies included within this dissertation consist primarily of prophylactic activation of AMPK to reduce the severity of ALI and sepsis. This approach provides useful insight into mechanisms of AMPK regulation and development of ALI/sepsis. A major limitation is that many patients are admitted to ICUs with existing infections and injury. To further establish the therapeutic potential of AMPK activators, it is necessary to determine if AMPK activation is beneficial during establish sepsis and ARDS, as well as potentially reducing post-sepsis complications.

A substantial limitation of our studies is use of mouse models. Many studies criticize this approach and point out differences in receptor bioavailability and protein expression that affect the severity of immune cell pro-inflammatory activation, as compared to critically ill patients. Furthermore, many clinical trials fail even though pre-clinical models indicate there to be a benefit.

Concerns have arisen about the use of AMPK activator metformin due to a presumed elevation of serum lactate levels in sepsis and ARDS[166-168]. This notion is supported by studies that linked use of metformin to inhibition of ETC complex 1. This mechanism was also suggested to induce bioenergetic imbalance and thus AMPK activation[169, 170]. Because of potential mitochondrial function inhibition which will affect

lactate flux, metformin is currently not use in clinical sepsis. However, more recent studies revealed that the mechanism of AMPK activation by metformin is dependent on activation of a lysosomal signaling cascade, and only high non physiological concentrations of metformin are inhibiting activity of ETC complex I[171, 172]. Notably, two recent clinical studies have shown that lowering lactate had negligible therapeutic impact[17, 173]. In turn, a cohort study published in 2016 has found that metformin use prior to sepsis provides ~30% reduction in mortality rates[174].

Another issue is related to a relatively limited pool of AMPK available for activation during sepsis. In particular, we found that a significant amount of AMPK is inactivated due to phosphorylation of AMPKα subunit by GSK3β[175]. It is crucial to determine if efficacy of AMPK activators, such as metformin, are increased when used in combination of GSK3β inhibitors.

GENERAL LIST OF REFERENCES

[1] Angus DC, van der Poll T: Severe sepsis and septic shock. N Engl J Med 2013, 369:840-51.

[2] Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC: The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 2016, 315:801-10.

[3] Angus DC, van der Poll T: Severe sepsis and septic shock. N Engl J Med 2013, 369:2063.

[4] Vincent JL, Opal SM, Marshall JC, Tracey KJ: Sepsis definitions: time for change. Lancet 2013, 381:774-5.

[5] Deutschman CS, Tracey KJ: Sepsis: current dogma and new perspectives. Immunity 2014, 40:463-75.

[6] Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 2001, 29:1303-10.

[7] McPherson D, Griffiths C, Williams M, Baker A, Klodawski E, Jacobson B, Donaldson L: Sepsis-associated mortality in England: an analysis of multiple cause of death data from 2001 to 2010. BMJ Open 2013, 3.

[8] Melamed A, Sorvillo FJ: The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data. Crit Care 2009, 13:R28.

[9] Koh GC, Peacock SJ, van der Poll T, Wiersinga WJ: The impact of diabetes on the pathogenesis of sepsis. Eur J Clin Microbiol Infect Dis 2012, 31:379-88.

[10] Angele MK, Pratschke S, Hubbard WJ, Chaudry IH: Gender differences in sepsis: cardiovascular and immunological aspects. Virulence 2014, 5:12-9.

[11] Torio CM, Andrews RM: National Inpatient Hospital Costs: The Most Expensive Conditions by Payer, 2011: Statistical Brief #160. Healthcare Cost and Utilization Project (HCUP) Statistical Briefs. Rockville (MD), 2006.

[12] Iwashyna TJ, Cooke CR, Wunsch H, Kahn JM: Population burden of long-term survivorship after severe sepsis in older Americans. Journal of the American Geriatrics Society 2012, 60:1070-7.

[13] Gaieski DF, Edwards JM, Kallan MJ, Carr BG: Benchmarking the incidence and mortality of severe sepsis in the United States. Critical care medicine 2013, 41:1167-74.

[14] Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G, International Sepsis Definitions C: 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Intensive care medicine 2003, 29:530-8.

[15] Shetty AL, Brown T, Booth T, Van KL, Dor-Shiffer DE, Vaghasiya MR, Eccleston CE, Iredell J: Systemic inflammatory response syndrome-based severe sepsis screening algorithms in emergency department patients with suspected sepsis. Emergency medicine Australasia : EMA 2016, 28:287-94.

[16] Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ: Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 1992, 101:1644-55.

[17] Lokhandwala S, Andersen LW, Nair S, Patel P, Cocchi MN, Donnino MW: Absolute lactate value vs relative reduction as a predictor of mortality in severe sepsis and septic shock. Journal of critical care 2017, 37:179-84.

[18] Varis E, Pettila V, Poukkanen M, Jakob SM, Karlsson S, Perner A, Takala J, Wilkman E, group Fs: Evolution of Blood Lactate and 90-Day Mortality in Septic Shock. A Post Hoc Analysis of the Finnaki Study. Shock 2016.

[19] Puskarich MA, Shapiro NI, Massey MJ, Kline JA, Jones AE: Lactate Clearance in Septic Shock Is Not a Surrogate for Improved Microcirculatory Flow. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine 2016, 23:690-3.

[20] Rittirsch D, Flierl MA, Ward PA: Harmful molecular mechanisms in sepsis. Nat Rev Immunol 2008, 8:776-87.

[21] Myburgh JA, Higgins A, Jovanovska A, Lipman J, Ramakrishnan N, Santamaria J, investigators CATS: A comparison of epinephrine and norepinephrine in critically ill patients. Intensive care medicine 2008, 34:2226-34.

[22] Avni T, Lador A, Lev S, Leibovici L, Paul M, Grossman A: Vasopressors for the Treatment of Septic Shock: Systematic Review and Meta-Analysis. PloS one 2015, 10:e0129305.

[23] Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, Kumar A, Sevransky JE, Sprung CL, Nunnally ME, Rochwerg B, Rubenfeld GD, Angus DC, Annane D, Beale RJ, Bellinghan GJ, Bernard GR, Chiche JD, Coopersmith C, De Backer DP, French CJ, Fujishima S, Gerlach H, Hidalgo JL, Hollenberg SM, Jones AE, Karnad DR, Kleinpell RM, Koh Y, Lisboa TC, Machado FR, Marini JJ, Marshall JC, Mazuski JE, McIntyre LA, McLean AS, Mehta S, Moreno RP, Myburgh J, Navalesi P, Nishida O, Osborn TM, Perner A, Plunkett CM, Ranieri M, Schorr CA, Seckel MA, Seymour CW, Shieh L, Shukri KA, Simpson SQ, Singer M, Thompson BT, Townsend SR, Van der Poll T, Vincent JL, Wiersinga WJ, Zimmerman JL, Dellinger RP: Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Critical care medicine 2017, 45:486-552.

[24] Regnier B, Rapin M, Gory G, Lemaire F, Teisseire B, Harari A: Haemodynamic effects of dopamine in septic shock. Intensive care medicine 1977, 3:47-53.

[25] Beck G, Brinkkoetter P, Hanusch C, Schulte J, van Ackern K, van der Woude FJ, Yard BA: Clinical review: immunomodulatory effects of dopamine in general inflammation. Critical care 2004, 8:485-91.

[26] Torres-Rosas R, Yehia G, Pena G, Mishra P, del Rocio Thompson-Bonilla M, Moreno-Eutimio MA, Arriaga-Pizano LA, Isibasi A, Ulloa L: Dopamine mediates vagal modulation of the immune system by electroacupuncture. Nat Med 2014, 20:291-5.

[27] Stanojevic S, Dimitrijevic M, Kustrimovic N, Mitic K, Vujic V, Leposavic G: Adrenal hormone deprivation affects macrophage catecholamine metabolism and beta2-adrenoceptor density, but not propranolol stimulation of tumour necrosis factor-alpha production. Experimental physiology 2013, 98:665-78.

[28] Bergmann M, Sautner T: Immunomodulatory effects of vasoactive catecholamines. Wiener klinische Wochenschrift 2002, 114:752-61.

[29] Oberbeck R, Schmitz D, Wilsenack K, Schuler M, Pehle B, Schedlowski M, Exton MS: Adrenergic modulation of survival and cellular immune functions during polymicrobial sepsis. Neuroimmunomodulation 2004, 11:214-23.

[30] Bone NB, Liu Z, Pittet JF, Zmijewski JW: Frontline Science: D1 dopaminergic receptor signaling activates the AMPK-bioenergetic pathway in macrophages and alveolar epithelial cells and reduces endotoxin-induced ALI. J Leukoc Biol 2017, 101:357-65.

[31] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S: Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. Journal of immunology 1999, 162:3749-52.

[32] Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S: Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999, 11:443-51.

[33] Ishikawa T, Itoh F, Yoshida S, Saijo S, Matsuzawa T, Gonoi T, Saito T, Okawa Y, Shibata N, Miyamoto T, Yamasaki S: Identification of distinct ligands for the C-type lectin receptors Mincle and Dectin-2 in the pathogenic fungus Malassezia. Cell host & microbe 2013, 13:477-88.

[34] Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ: Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. The Journal of biological chemistry 1999, 274:17406-9.

[35] Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA: The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nature immunology 2010, 11:395-402.

[36] Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, Tedin K, Taha MK, Labigne A, Zahringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ, Philpott DJ: Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003, 300:1584-7.

[37] Takeuchi O, Akira S: Pattern recognition receptors and inflammation. Cell 2010, 140:805-20.

[38] Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, Horwood N, Nanchahal J: Alarmins: awaiting a clinical response. The Journal of clinical investigation 2012, 122:2711-9.

[39] Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA, Homer RJ, Goldstein DR, Bucala R, Lee PJ, Medzhitov R, Noble PW: Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nature medicine 2005, 11:1173-9.

[40] Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Gotte M, Malle E, Schaefer RM, Grone HJ: The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. The Journal of clinical investigation 2005, 115:2223-33.

[41] Petrilli V, Dostert C, Muruve DA, Tschopp J: The inflammasome: a danger sensing complex triggering innate immunity. Current opinion in immunology 2007, 19:615-22.

[42] Tamayo E, Gomez E, Bustamante J, Gomez-Herreras JI, Fonteriz R, Bobillo F, Bermejo-Martin JF, Castrodeza J, Heredia M, Fierro I, Alvarez FJ: Evolution of neutrophil apoptosis in septic shock survivors and nonsurvivors. Journal of critical care 2012, 27:415 e1-11.

[43] Alves-Filho JC, Spiller F, Cunha FQ: Neutrophil paralysis in sepsis. Shock 2010, 34 Suppl 1:15-21.

[44] Kovach MA, Standiford TJ: The function of neutrophils in sepsis. Current opinion in infectious diseases 2012, 25:321-7.

[45] Stephan F, Yang K, Tankovic J, Soussy CJ, Dhonneur G, Duvaldestin P, Brochard L, Brun-Buisson C, Harf A, Delclaux C: Impairment of polymorphonuclear neutrophil functions precedes nosocomial infections in critically ill patients. Critical care medicine 2002, 30:315-22.

[46] Cavaillon JM, Adib-Conquy M: Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Critical care 2006, 10:233.

[47] Biswas SK, Lopez-Collazo E: Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in immunology 2009, 30:475-87.

[48] Zhang X, Morrison DC: Lipopolysaccharide structure-function relationship in activation versus reprogramming of mouse peritoneal macrophages. Journal of leukocyte biology 1993, 54:444-50.

[49] Monneret G, Lepape A, Voirin N, Bohe J, Venet F, Debard AL, Thizy H, Bienvenu J, Gueyffier F, Vanhems P: Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock. Intensive care medicine 2006, 32:1175-83.

[50] Singer M, De Santis V, Vitale D, Jeffcoate W: Multiorgan failure is an adaptive, endocrine-mediated, metabolic response to overwhelming systemic inflammation. Lancet 2004, 364:545-8.

[51] Munford RS, Pugin J: Normal responses to injury prevent systemic inflammation and can be immunosuppressive. American journal of respiratory and critical care medicine 2001, 163:316-21.

[52] Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, Hayden DL, Hennessy L, Moore EE, Minei JP, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Brownstein BH, Mason PH, Baker HV, Finnerty CC, Jeschke MG, Lopez MC, Klein MB, Gamelli RL, Gibran NS, Arnoldo B, Xu W, Zhang Y, Calvano SE, McDonald-Smith GP, Schoenfeld DA, Storey JD, Cobb JP, Warren HS, Moldawer LL, Herndon DN, Lowry SF, Maier RV, Davis RW, Tompkins RG, Inflammation, Host Response to Injury Large-Scale Collaborative Research P: A genomic storm in critically injured humans. The Journal of experimental medicine 2011, 208:2581-90.

[53] Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S, Remick DG: The pathogenesis of sepsis. Annual review of pathology 2011, 6:19-48.

[54] Hotchkiss RS, Monneret G, Payen D: Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. The Lancet Infectious diseases 2013, 13:260-8.

[55] Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. N Engl J Med 2003, 348:138-50.

[56] Hotchkiss RS, Opal S: Immunotherapy for sepsis--a new approach against an ancient foe. N Engl J Med 2010, 363:87-9.

[57] Ware LB, Matthay MA: The acute respiratory distress syndrome. N Engl J Med 2000, 342:1334-49.

[58] Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD: Incidence and outcomes of acute lung injury. The New England journal of medicine 2005, 353:1685-93.

[59] Hudson LD, Steinberg KP: Epidemiology of acute lung injury and ARDS. Chest 1999, 116:74S-82S.

[60] Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R: The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. Am J Respir Crit Care Med 1994, 149:818-24.

[61] Acute Respiratory Distress Syndrome N, Brower RG, Matthay MA, Morris A, Schoenfeld D, Thompson BT, Wheeler A: Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. N Engl J Med 2000, 342:1301-8.

[62] Papazian L, Forel JM, Gacouin A, Penot-Ragon C, Perrin G, Loundou A, Jaber S, Arnal JM, Perez D, Seghboyan JM, Constantin JM, Courant P, Lefrant JY, Guerin C, Prat G, Morange S, Roch A, Investigators AS: Neuromuscular blockers in early acute respiratory distress syndrome. N Engl J Med 2010, 363:1107-16.

[63] Guerin C, Reignier J, Richard JC: Prone positioning in the acute respiratory distress syndrome. N Engl J Med 2013, 369:980-1.

[64] Kumar H, Kawai T, Akira S: Pathogen recognition in the innate immune response. The Biochemical journal 2009, 420:1-16.

[65] Matthay MA, Zimmerman GA, Esmon C, Bhattacharya J, Coller B, Doerschuk CM, Floros J, Gimbrone MA, Jr., Hoffman E, Hubmayr RD, Leppert M, Matalon S, Munford R, Parsons P, Slutsky AS, Tracey KJ, Ward P, Gail DB, Harabin AL: Future research directions in acute lung injury: summary of a National Heart, Lung, and Blood Institute working group. Am J Respir Crit Care Med 2003, 167:1027-35.

[66] Matthay MA, Zimmerman GA: Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. American journal of respiratory cell and molecular biology 2005, 33:319-27.

[67] Aggarwal NR, King LS, D'Alessio FR: Diverse macrophage populations mediate acute lung inflammation and resolution. American journal of physiology Lung cellular and molecular physiology 2014, 306:L709-25.

[68] Geiser T, Atabai K, Jarreau PH, Ware LB, Pugin J, Matthay MA: Pulmonary edema fluid from patients with acute lung injury augments in vitro alveolar epithelial repair by an IL-1beta-dependent mechanism. Am J Respir Crit Care Med 2001, 163:1384-8.

[69] D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, Pipeling MR, Brower RG, Tuder RM, McDyer JF, King LS: CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. The Journal of clinical investigation 2009, 119:2898-913.

[70] Kim J, Hematti P: Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. Exp Hematol 2009, 37:1445-53.

[71] Zmijewski JW, Banerjee S, Bae H, Friggeri A, Lazarowski ER, Abraham E: Exposure to hydrogen peroxide induces oxidation and activation of AMP-activated protein kinase. J Biol Chem 2010, 285:33154-64.

[72] Kallet RH, Matthay MA: Hyperoxic acute lung injury. Respiratory care 2013, 58:123-41.

[73] Kelly FJ, Lubec G: Hyperoxic injury of immature guinea pig lung is mediated via hydroxyl radicals. Pediatric research 1995, 38:286-91.

[74] Shi T, Knaapen AM, Begerow J, Birmili W, Borm PJ, Schins RP: Temporal variation of hydroxyl radical generation and 8-hydroxy-2'-deoxyguanosine formation by coarse and fine particulate matter. Occupational and environmental medicine 2003, 60:315-21.

[75] Steinberg KP, Milberg JA, Martin TR, Maunder RJ, Cockrill BA, Hudson LD: Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. Am J Respir Crit Care Med 1994, 150:113-22.

[76] Chesnutt AN, Matthay MA, Tibayan FA, Clark JG: Early detection of type III procollagen peptide in acute lung injury. Pathogenetic and prognostic significance. Am J Respir Crit Care Med 1997, 156:840-5.

[77] Horowitz JC, Cui Z, Moore TA, Meier TR, Reddy RC, Toews GB, Standiford TJ, Thannickal VJ: Constitutive activation of prosurvival signaling in alveolar mesenchymal cells isolated from patients with nonresolving acute respiratory distress syndrome. American journal of physiology Lung cellular and molecular physiology 2006, 290:L415-25.

[78] Mantovani A, Cassatella MA, Costantini C, Jaillon S: Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol 2011, 11:519-31.

[79] Abraham E: Neutrophils and acute lung injury. Crit Care Med 2003, 31:S195-9.

[80] Lee WL, Downey GP: Neutrophil activation and acute lung injury. Curr Opin Crit Care 2001, 7:1-7.

[81] Tadie JM, Bae HB, Jiang S, Park DW, Bell CP, Yang H, Pittet JF, Tracey K, Thannickal VJ, Abraham E, Zmijewski JW: HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. Am J Physiol Lung Cell Mol Physiol 2013, 304:L342-9.

[82] Hampson P, Dinsdale RJ, Wearn CM, Bamford AL, Bishop JRB, Hazeldine J, Moiemen NS, Harrison P, Lord JM: Neutrophil Dysfunction, Immature Granulocytes, and Cell-free DNA are Early Biomarkers of Sepsis in Burn-injured Patients: A Prospective Observational Cohort Study. Annals of surgery 2017, 265:1241-9.

[83] Slaba I, Wang J, Kolaczkowska E, McDonald B, Lee WY, Kubes P: Imaging the dynamic platelet-neutrophil response in sterile liver injury and repair in mice. Hepatology 2015, 62:1593-605.

[84] Herold S, Steinmueller M, von Wulffen W, Cakarova L, Pinto R, Pleschka S, Mack M, Kuziel WA, Corazza N, Brunner T, Seeger W, Lohmeyer J: Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. J Exp Med 2008, 205:3065-77.

[85] Davies NA, Cooper CE, Stidwill R, Singer M: Inhibition of mitochondrial respiration during early stage sepsis. Advances in experimental medicine and biology 2003, 530:725-36.

[86] Brealey D, Singer M: Mitochondrial Dysfunction in Sepsis. Current infectious disease reports 2003, 5:365-71.

[87] Brealey D, Karyampudi S, Jacques TS, Novelli M, Stidwill R, Taylor V, Smolenski RT, Singer M: Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am J Physiol Regul Integr Comp Physiol 2004, 286:R491-7.

[88] Singer M: The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. Virulence 2014, 5:66-72.

[89] Green DE, Zande HD: Universal energy principle of biological systems and the unity of bioenergetics. Proceedings of the National Academy of Sciences of the United States of America 1981, 78:5344-7.

[90] Clapham JC, Arch JR: Thermogenic and metabolic antiobesity drugs: rationale and opportunities. Diabetes, obesity & metabolism 2007, 9:259-75.

[91] Boyer PD, Chance B, Ernster L, Mitchell P, Racker E, Slater EC: Oxidative phosphorylation and photophosphorylation. Annu Rev Biochem 1977, 46:955-66.

[92] Carling D, Thornton C, Woods A, Sanders MJ: AMP-activated protein kinase: new regulation, new roles? The Biochemical journal 2012, 445:11-27.

[93] Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, Cross JR, Jung E, Thompson CB, Jones RG, Pearce EJ: Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood 2010, 115:4742-9.

[94] Fleetwood AJ, Lee MKS, Singleton W, Achuthan A, Lee MC, O'Brien-Simpson NM, Cook AD, Murphy AJ, Dashper SG, Reynolds EC, Hamilton JA: Metabolic Remodeling, Inflammasome Activation, and Pyroptosis in Macrophages Stimulated by Porphyromonas gingivalis and Its Outer Membrane Vesicles. Frontiers in cellular and infection microbiology 2017, 7:351.

[95] Galvan-Pena S, O'Neill LA: Metabolic reprograming in macrophage polarization. Frontiers in immunology 2014, 5:420.

[96] Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P, Cascante M, Bosca L: Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol 2010, 185:605-14.

[97] Mosser DM, Edwards JP: Exploring the full spectrum of macrophage activation. Nature reviews Immunology 2008, 8:958-69.

[98] Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA: Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 2014, 41:14-20.

[99] West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P, Walsh MC, Choi Y, Shadel GS, Ghosh S: TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. Nature 2011, 472:476-80.

[100] Kelly B, O'Neill LA: Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell research 2015, 25:771-84.

[101] Bao Y, Ledderose C, Seier T, Graf AF, Brix B, Chong E, Junger WG: Mitochondria regulate neutrophil activation by generating ATP for autocrine purinergic signaling. The Journal of biological chemistry 2014, 289:26794-803.

[102] Fossati G, Moulding DA, Spiller DG, Moots RJ, White MR, Edwards SW: The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. J Immunol 2003, 170:1964-72.

[103] Sena LA, Chandel NS: Physiological roles of mitochondrial reactive oxygen species. Molecular cell 2012, 48:158-67.

[104] Steinberg GR, Kemp BE: AMPK in Health and Disease. Physiol Rev 2009, 89:1025-78.

[105] Hardie DG, Carling D: The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem 1997, 246:259-73.

[106] Hardie DG, Corton J, Ching YP, Davies SP, Hawley S: Regulation of lipid metabolism by the AMP-activated protein kinase. Biochem Soc Trans 1997, 25:1229-31.

[107] Hawley SA, Selbert MA, Goldstein EG, Edelman AM, Carling D, Hardie DG: 5'-AMP activates the AMP-activated protein kinase cascade, and Ca2+/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. The Journal of biological chemistry 1995, 270:27186-91.

[108] Davies SP, Helps NR, Cohen PT, Hardie DG: 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS letters 1995, 377:421-5.

[109] Oakhill JS, Steel R, Chen ZP, Scott JW, Ling N, Tam S, Kemp BE: AMPK is a direct adenylate charge-regulated protein kinase. Science 2011, 332:1433-5.

[110] Gowans GJ, Hawley SA, Ross FA, Hardie DG: AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. Cell metabolism 2013, 18:556-66.

[111] Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG: Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. Journal of biology 2003, 2:28.

[112] Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D: LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Current biology : CB 2003, 13:2004-8.

[113] Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC: The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proceedings of the National Academy of Sciences of the United States of America 2004, 101:3329-35.

[114] Mungai PT, Waypa GB, Jairaman A, Prakriya M, Dokic D, Ball MK, Schumacker PT: Hypoxia triggers AMPK activation through reactive oxygen species-mediated activation of calcium release-activated calcium channels. Molecular and cellular biology 2011, 31:3531-45.

[115] Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L: Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Current biology : CB 2000, 10:1247-55.

[116] Wu SB, Wei YH: AMPK-mediated increase of glycolysis as an adaptive response to oxidative stress in human cells: implication of the cell survival in mitochondrial diseases. Biochimica et biophysica acta 2012, 1822:233-47.

[117] Musi N, Fujii N, Hirshman MF, Ekberg I, Froberg S, Ljungqvist O, Thorell A, Goodyear LJ: AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. Diabetes 2001, 50:921-7.

[118] Corton JM, Gillespie JG, Hawley SA, Hardie DG: 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? European journal of biochemistry 1995, 229:558-65.

[119] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001, 108:1167-74.

[120] Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB: Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. Diabetes 2006, 55:2256-64.

[121] Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Iyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS, Frevert E: Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. Cell Metab 2006, 3:403-16.

[122] Jensen TE, Ross FA, Kleinert M, Sylow L, Knudsen JR, Gowans GJ, Hardie DG, Richter EA: PT-1 selectively activates AMPK-gamma1 complexes in mouse skeletal muscle, but activates all three gamma subunit complexes in cultured human cells by inhibiting the respiratory chain. The Biochemical journal 2015, 467:461-72.

[123] Bultot L, Jensen TE, Lai YC, Madsen AL, Collodet C, Kviklyte S, Deak M, Yavari A, Foretz M, Ghaffari S, Bellahcene M, Ashrafian H, Rider MH, Richter EA, Sakamoto K: Benzimidazole derivative small-molecule 991 enhances AMPK activity and glucose uptake induced by AICAR or contraction in skeletal muscle. American journal of physiology Endocrinology and metabolism 2016, 311:E706-E19.

[124] Guigas B, Viollet B: Targeting AMPK: From Ancient Drugs to New Small-Molecule Activators. Exs 2016, 107:327-50.

[125] Lin J, Handschin C, Spiegelman BM: Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab 2005, 1:361-70.

[126] Jager S, Handschin C, St-Pierre J, Spiegelman BM: AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A 2007, 104:12017-22.

[127] Inoki K, Zhu T, Guan KL: TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003, 115:577-90.

[128] Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ: AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 2008, 30:214-26.

[129] Shackelford DB, Vasquez DS, Corbeil J, Wu S, Leblanc M, Wu CL, Vera DR, Shaw RJ: mTOR and HIF-1alpha-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome. Proc Natl Acad Sci U S A 2009, 106:11137-42.

[130] Kovacic S, Soltys CL, Barr AJ, Shiojima I, Walsh K, Dyck JR: Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart. The Journal of biological chemistry 2003, 278:39422-7.

[131] Suzuki T, Bridges D, Nakada D, Skiniotis G, Morrison SJ, Lin JD, Saltiel AR, Inoki K: Inhibition of AMPK catabolic action by GSK3. Molecular cell 2013, 50:407-19.

[132] Park DW, Jiang S, Liu Y, Siegal GP, Inoki K, Abraham E, Zmijewski JW: GSK3beta-dependent inhibition of AMPK potentiates activation of neutrophils and macrophages and enhances severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2014, 307:L735-45.

[133] Liu Z, Dai X, Zhu H, Zhang M, Zou MH: Lipopolysaccharides Promote S-Nitrosylation and Proteasomal Degradation of Liver Kinase B1 (LKB1) in Macrophages in Vivo. The Journal of biological chemistry 2015, 290:19011-7.

[134] Jian MY, Alexeyev MF, Wolkowicz PE, Zmijewski JW, Creighton JR: Metformin-stimulated AMPK-alpha1 promotes microvascular repair in acute lung injury. Am J Physiol Lung Cell Mol Physiol 2013, 305:L844-55.

[135] Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, Abraham E: Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008, 295:L497-504.

[136] Zmijewski JW, Lorne E, Zhao X, Tsuruta Y, Sha Y, Liu G, Siegal GP, Abraham E: Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. Am J Respir Crit Care Med 2008, 178:168-79.

[137] Vida G, Pena G, Kanashiro A, Thompson-Bonilla Mdel R, Palange D, Deitch EA, Ulloa L: beta2-Adrenoreceptors of regulatory lymphocytes are essential for vagal neuromodulation of the innate immune system. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2011, 25:4476-85.

[138] Galkin A, Moncada S: Modulation of the conformational state of mitochondrial complex I as a target for therapeutic intervention. Interface focus 2017, 7:20160104.

[139] Clementi E, Brown GC, Feelisch M, Moncada S: Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proceedings of the National Academy of Sciences of the United States of America 1998, 95:7631-6.

[140] Chang AH, Sancheti H, Garcia J, Kaplowitz N, Cadenas E, Han D: Respiratory substrates regulate S-nitrosylation of mitochondrial proteins through a thiol-dependent pathway. Chemical research in toxicology 2014, 27:794-804.

[141] Thumm M, Egner R, Koch B, Schlumpberger M, Straub M, Veenhuis M, Wolf DH: Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. FEBS letters 1994, 349:275-80.

[142] Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y: Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. The Journal of cell biology 1992, 119:301-11.

[143] Harding TM, Morano KA, Scott SV, Klionsky DJ: Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. The Journal of cell biology 1995, 131:591-602.

[144] Glick D, Barth S, Macleod KF: Autophagy: cellular and molecular mechanisms. The Journal of pathology 2010, 221:3-12.

[145] Cuervo AM: Chaperone-mediated autophagy: Dice's 'wild' idea about lysosomal selectivity. Nat Rev Mol Cell Biol 2011, 12:535-41.

[146] Levine B, Kroemer G: Autophagy in the pathogenesis of disease. Cell 2008, 132:27-42.

[147] Mizushima N, Levine B, Cuervo AM, Klionsky DJ: Autophagy fights disease through cellular self-digestion. Nature 2008, 451:1069-75.

[148] Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, Jimenez-Sanchez M, Korolchuk VI, Lichtenberg M, Luo S, Massey DC, Menzies FM, Moreau K, Narayanan U, Renna M, Siddiqi FH, Underwood BR, Winslow AR, Rubinsztein DC: Regulation of mammalian autophagy in physiology and pathophysiology. Physiological reviews 2010, 90:1383-435.

[149] Choi AM, Ryter SW, Levine B: Autophagy in human health and disease. The New England journal of medicine 2013, 368:651-62.

[150] Luciani A, Villella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, Gavina M, Pulze L, Giardino I, Pettoello-Mantovani M, D'Apolito M, Guido S, Masliah E, Spencer B, Quaratino S, Raia V, Ballabio A, Maiuri L: Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. Nature cell biology 2010, 12:863-75.

[151] Patel AS, Lin L, Geyer A, Haspel JA, An CH, Cao J, Rosas IO, Morse D: Autophagy in idiopathic pulmonary fibrosis. PloS one 2012, 7:e41394.

[152] West AP, Shadel GS, Ghosh S: Mitochondria in innate immune responses. Nature reviews Immunology 2011, 11:389-402.

[153] Jin SM, Youle RJ: PINK1- and Parkin-mediated mitophagy at a glance. Journal of cell science 2012, 125:795-9.

[154] Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, Youle RJ: PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. The Journal of cell biology 2014, 205:143-53.

[155] Lazarou M: Keeping the immune system in check: a role for mitophagy. Immunology and cell biology 2015, 93:3-10.

[156] Lemasters JJ: Variants of mitochondrial autophagy: Types 1 and 2 mitophagy and micromitophagy (Type 3). Redox biology 2014, 2:749-54.

[157] Chu CT, Zhu J, Dagda R: Beclin 1-independent pathway of damage-induced mitophagy and autophagic stress: implications for neurodegeneration and cell death. Autophagy 2007, 3:663-6.

[158] Behr MA, Schurr E: Cell biology: A table for two. Nature 2013, 501:498-9.

[159] Evans TD, Sergin I, Zhang X, Razani B: Target acquired: Selective autophagy in cardiometabolic disease. Sci Signal 2017, 10.

[160] Rubinsztein DC, Codogno P, Levine B: Autophagy modulation as a potential therapeutic target for diverse diseases. Nature reviews Drug discovery 2012, 11:709-30.

[161] Morris HR: Genetics of Parkinson's disease. Ann Med 2005, 37:86-96.

[162] Manzanillo PS, Ayres JS, Watson RO, Collins AC, Souza G, Rae CS, Schneider DS, Nakamura K, Shiloh MU, Cox JS: The ubiquitin ligase parkin mediates resistance to intracellular pathogens. Nature 2013, 501:512-6.

[163] Mehanna R, Jankovic J: Respiratory problems in neurologic movement disorders. Parkinsonism & related disorders 2010, 16:628-38.

[164] Schmid D, Munz C: Innate and adaptive immunity through autophagy. Immunity 2007, 27:11-21.

[165] Gunst J: Recovery from critical illness-induced organ failure: the role of autophagy. Critical care 2017, 21:209.

[166] Richy FF, Sabido-Espin M, Guedes S, Corvino FA, Gottwald-Hostalek U: Incidence of lactic acidosis in patients with type 2 diabetes with and without renal

impairment treated with metformin: a retrospective cohort study. Diabetes care 2014, 37:2291-5.

[167] Lalau JD: Lactic acidosis induced by metformin: incidence, management and prevention. Drug safety 2010, 33:727-40.

[168] Bailey CJ, Turner RC: Metformin. The New England journal of medicine 1996, 334:574-9.

[169] Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, Glasauer A, Dufour E, Mutlu GM, Budigner GS, Chandel NS: Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. eLife 2014, 3:e02242.

[170] Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM, Hardie DG: Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. Cell metabolism 2010, 11:554-65.

[171] Zhang CS, Li M, Ma T, Zong Y, Cui J, Feng JW, Wu YQ, Lin SY, Lin SC: Metformin Activates AMPK through the Lysosomal Pathway. Cell metabolism 2016, 24:521-2.

[172] He L, Wondisford FE: Metformin action: concentrations matter. Cell metabolism 2015, 21:159-62.

[173] Varis E, Pettila V, Poukkanen M, Jakob SM, Karlsson S, Perner A, Takala J, Wilkman E, Group FS: Evolution of Blood Lactate and 90-Day Mortality in Septic Shock. A Post Hoc Analysis of the FINNAKI Study. Shock 2017, 47:574-81.

[174] Doenyas-Barak K, Beberashvili I, Marcus R, Efrati S: Lactic acidosis and severe septic shock in metformin users: a cohort study. Critical care 2016, 20:10.

[175] Liu Z, Bone N, Jiang S, Park DW, Tadie JM, Deshane J, Rodriguez CA, Pittet JF, Abraham E, Zmijewski JW: AMP-activated protein kinase and Glycogen Synthase Kinase 3beta modulate the severity of sepsis-induced lung injury. Mol Med 2015.

APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 14-Sep-2016

TO: Zmijewski, Jaroslaw

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 14-Sep-2016.

Protocol PI: Zmijewski, Jaroslaw

Title: AMPK Activation and Acute Lung Injury

Sponsor: National Heart, Lung, and Blood Institute/NIH/DHHS

Animal Project Number (APN): IACUC-09509

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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