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Uncoupling of IL-6 signaling and LC3-associated phagocytosis drives immunoparalysis during sepsis

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Highlights

- ERK signaling regulates NADPH-oxidase mediated activation of LAP
- IL-6/JAK-2/Ninein axis controls microtubule-mediated ERK trafficking to the LAPosome
- Loss of IL-6 signaling inhibits LAP, leading to impaired pathogen killing
- IL-6 supplementation restores LAP defects and sepsis-induced immunoparalysis

Summary

Immune deactivation of phagocytes is a central event in the pathogenesis of sepsis. Herein, we identify a master regulatory role of IL-6 signaling on LC3-associated phagocytosis (LAP) and reveal that uncoupling of these two processes during sepsis induces immunoparalysis in monocytes/macrophages. In particular, we demonstrate that activation of LAP by the human fungal pathogen *Aspergillus fumigatus* depends on ERK1/2-mediated phosphorylation of p47phox subunit of NADPH oxidase. Physiologically, autocrine IL-6/JAK2/Stat3 axis orchestrates microtubule organization and dynamics regulating ERK recruitment to the phagosome and LC3⁺ phagosome (LAPosome) formation. In sepsis, loss of IL-6 signaling specifically abrogates microtubule-mediated trafficking of ERK, leading to defective activation of LAP and impaired killing of bacterial and fungal pathogens by monocytes/macrophages, which can be selectively restored by IL-6 supplementation. Our work uncovers a molecular pathway linking IL-6 signaling with LAP and provides insight into the mechanisms underlying immunoparalysis in sepsis.

Keywords

Aspergillus fumigatus, LAP, LC3-associated phagocytosis, phagosome, IL-6, JAK, NADPH oxidase, extracellular-signal-kinase regulated (ERK), microtubules, sepsis, immunoparalysis

Introduction

Sepsis, a complex and heterogeneous disease caused by a deregulated inflammatory response to an infectious insult, remains a leading cause of death worldwide (Reinhart et al., 2017; Singer et al., 2016; van der Poll et al., 2017). Numerous clinical trials on immunomodulation in sepsis have failed to improve patient outcome, emphasizing the need for deeper mechanistic insight on pathogenesis (Arnold, 2018; Peters van Ton et al., 2018; Rello et al., 2018). In recent years, it has been realized that many patients who survive the initial sepsis episode enter a prolonged state of immune deactivation, termed sepsis-induced immunoparalysis, accounting for treatment failures and death due to secondary infections by opportunistic pathogens (Boomer et al., 2011; Hotchkiss et al., 2013).

As opposed to “immunological tolerance,” a physiological compensatory mechanism that confers a balanced inflammatory response to an infectious agent, sepsis-induced immunoparalysis is characterized by impaired activation of essential immune effector pathways in myeloid phagocytes (Cheng et al., 2016; Domínguez-Andrés et al., 2019; van der Poll et al., 2017) and loss of their microbicidal activity (Boomer et al., 2011; Chiswick et al., 2015; Döcke et al., 1997; Hotchkiss et al., 2013; van der Poll et al., 2017). At the molecular level, supra-physiological induction of pathways regulating “immunological tolerance,” results in cytokine hypo-responsiveness and is a central molecular event of immune deactivation in sepsis (Hotchkiss et al., 2013; van der Poll et al., 2017). In particular, ROS-mediated super-induction of activating transcription factor 3 (ATF3) suppress innate cytokines, predominantly IL-6, and accounts for heightened susceptibility to secondary infection by opportunistic bacterial and fungal pathogens (Hoetzenecker et al., 2011). Aberrant ATF3 expression in sepsis is also induced via metabolic reprogramming of myeloid phagocytes (Bambouskova et al., 2018; Domínguez-Andrés et al., 2019). Furthermore, epigenetic modulation of myeloid progenitors induced by sepsis leads to persistent immune deactivation of professional phagocytic cells (Wen et al., 2008; Zhang et al., 2016). Immunotherapy with cytokines, such as IFN- γ and GM-CSF, partially reverses cytokine hypo-responsiveness and restores the microbicidal activity of sepsis monocytes (Arnold, 2018; Döcke et al., 1997; Leentjens et al., 2012; Peters van Ton et al., 2018; van der Poll et al., 2017). However, successful implementation of cytokine therapies in human patients with sepsis requires understanding the underlying signaling defects that account for immunoparalysis in the individual patient.

LC3 associated phagocytosis (LAP), a phagosome biogenesis pathway utilizing part of the autophagy machinery in response to certain pattern-recognition receptors (PRRs), promotes phagolysosomal fusion and killing of an expanding list of pathogens by macrophages (Heckmann and Green, 2019; Martinez et al., 2015; Sanjuan et al., 2007). Besides, LAP regulates the anti-inflammatory activity of macrophages (Chu et al., 2016; Heckmann and Green, 2019; Heckmann et al., 2019; Martinez et al., 2011, 2016). LAP utilizes a distinct signaling pathway from canonical autophagy and is regulated by Rubicon (Martinez et al., 2015). In particular, NADPH-oxidase-complex-mediated ROS production is a fundamental requirement for LC3⁺ phagosome (LAPosome) formation (Martinez et al., 2015).

Currently, there is no single biological marker to define sepsis immunoparalysis, and the underlying molecular mechanisms remain at least in part obscure. *Aspergillus fumigatus*, an airborne saprophytic mold shaping immunological tolerance in human respiratory epithelia

(Bacher et al., 2016), has become a model pathogen to study immune deactivation in sepsis (Benjamim et al., 2003, 2005; Hoetzenecker et al., 2011). Interestingly, life-threatening respiratory infections caused by this opportunistic fungus are increasingly encountered in patients recovering from bacterial and viral sepsis (Bae et al., 2020; Bartoletti et al., 2020; Bassetti et al., 2017; Colombo et al., 2017; Dimopoulos et al., 2003; Meersseman et al., 2004). We and others have shown the crucial role of LAP in protective immunity against *Aspergillus* and other human pathogens (Akoumianaki et al., 2016; Kyrnizi et al., 2018, 2013; Martinez et al., 2015; Oikonomou et al., 2016), (de Luca et al., 2014). However, the molecular mechanisms of crosstalk of cytokine signaling and LAP are poorly understood. In addition, the role of LAP in the pathogenesis of sepsis is currently unknown.

Using *Aspergillus* as a model pathogen, we identify defective activation of LAP as a hallmark feature of immune deactivation of phagocytes in septic patients and mice. In particular, we discover a unique role of autocrine IL-6/JAK2/Stat3 axis on the regulation of microtubule-dependent ERK recruitment to the phagosome for LAPosome formation. Next, we demonstrate that loss of IL-6 signaling during sepsis selectively disrupts microtubule dynamics regulating ERK-dependent activation of LAP, leading to impaired control of bacterial and fungal pathogens by monocytes/macrophages. IL-6 specifically restores microbicidal activity of myeloid phagocytes in sepsis patients and mice, in a LAP-dependent way. Collectively, our findings link IL-6 signaling with LAP, with an important role in the development of immunoparalysis of sepsis, which can be explored as a therapeutic target.

Results

Defective activation of LAP in monocytes is a dominant feature of immunoparalysis in human patients with sepsis

Given the essential role of LAP in killing of *Aspergillus* by phagocytes (Akoumianaki et al., 2016; Kyrnizi et al., 2013, 2018; Martinez et al., 2015), we explored whether this pathway is modulated by sepsis. We obtained monocytes from 38 consecutive patients admitted to intensive care unit (ICU) with community-acquired septic shock. Functional studies were performed on the day of patient admission (day 1) and upon recovery from the infectious episode (day 7) (Figure 1A). We used melanin-deficient (albino) conidia of *Aspergillus fumigatus* $\Delta pksP$ mutant as model bioparticles that induce a robust activation of LAP (Akoumianaki et al., 2016; Kyrnizi et al., 2013, 2018; Martinez et al., 2015) and compared immune responses of monocytes from sepsis patients with those induced in monocytes of healthy individuals (controls). Importantly, killing of wild-type *Aspergillus* conidia is also dependent on activation of LAP upon cell wall melanin removal inside monocytes/macrophages (Akoumianaki et al., 2016; Kyrnizi et al., 2013, 2018; Martinez et al., 2015). Therefore, we additionally assessed killing of the isogenic wild-type *A. fumigatus* clinical isolate (ATCC46645) by monocytes of sepsis patients versus controls. In parallel, we analyzed clinical and microbiological characteristics and the outcome of patients to explore whether classical features of sepsis immunoparalysis (e.g., secondary infections) are associated with impaired LAP responses (Tables S1–S3). LAP was assessed by counting the percentage of LC3⁺ *Aspergillus*-containing phagosomes in monocytes by confocal microscopy (Figure S1) (Akoumianaki et al., 2016; Kyrnizi et al., 2013, 2018; Martinez et al., 2015). LAP responses following sepsis recovery (day 7; when patients are at increased risk for development of secondary infections) segregated patients in two distinct groups (Figure S1). When compared

with healthy individuals (n = 19), group A included patients with intact LAP responses (n = 15, P = NS versus healthy control group), and group B included patients with defective activation of LAP (n = 23, p < 0. 0001 versus healthy control group and versus patients with intact LAP) (Figure 1B). These differences in LAP responses were less apparent on day 1 of sepsis (Figure S1). Because LAP is dependent on NADPH oxidase (Martinez et al., 2015), we assessed NADPH oxidase activation in monocytes of sepsis patients by measuring p47phox translocation to the phagosome membrane. Notably, monocytes from patients with LAP defects displayed impaired recruitment of p47phox to the phagosome (Figures 1C and S1). Accordingly, we found evidence of phagosome maturation arrest in monocytes of patients with LAP defects following *Aspergillus* infection, suggested by the lack of acquisition of CD63 (a marker of phagolysosomal fusion and acidification) (Meersseman et al., 2004), (Figures 1D and S1).

Defective LAP responses in monocytes of patients recovering from sepsis (day 7) were significantly associated with impaired killing of conidia of a clinical isolate of *Aspergillus* (ATCC 46645) (Figures 1E and S1), and our melanin-deficient *Aspergillus* mutant used as a model for phagocytosis (Figure S1). In contrast, monocytes from patients with compromised LAP responses had no apparent defects on the level of phagocytosis of conidia of melanin-deficient $\Delta pksP$ *Aspergillus* strain (Figure S1). In addition, there was no difference in the percentage of CD16⁺ monocytes and the level of CD163 expression between patients with intact versus defective LAP responses (Figure S2). Of interest, HLA-DR expression were decreased in LAP-defective monocytes (Figure S2).

An impaired cytokine response of monocytes following *ex vivo* re-stimulation with TLR agonists is a prominent feature of immune deactivation in sepsis (Hotchkiss et al., 2013; van der Poll et al., 2017). Specifically, suppressed IL-6 production has a major causative role in the development of sepsis-induced immunoparalysis (Cenci et al., 2001; Hoetzenecker et al., 2011). Monocytes of patients with LAP defect on day 7 had a significant decrease of IL-6 production following overnight stimulation with *A. fumigatus* when compared with control healthy monocytes and monocytes of patients with intact LAP responses, which was proportional to the severity of the underlying LAP defect (Figure 1F; Table S2); production of other pro-inflammatory cytokines was decreased in monocytes of patients with LAP defects versus those with intact LAP but was not statistically significant (Figure S2).

Development of secondary infections is a hallmark clinical feature of sepsis-induced immunoparalysis (Boomer et al., 2011; Hotchkiss et al., 2013; van der Poll et al., 2017). Notably, patients with LAP defect had a higher clinical severity of sepsis on the day of admission in the ICU (APACHE II score) (Figure 1G) and a significant 3-fold increase in cumulative risk for secondary infections as compared with patients with intact LAP (Figure 1H; Tables S1–S3). Collectively, defective activation of LAP in monocytes of patients with sepsis is associated with classical immunological and clinical features of immunoparalysis.

Localized ERK1/2 signaling on the phagosome regulates NADPH oxidase-dependent activation of LAP and is inhibited in sepsis

Impaired phagosomal localization of p47phox was a striking upstream signaling defect in monocytes/macrophages of sepsis patients with a LAP defect (Figures 1B and 1C). To gain further insights into defective LAP activation in sepsis, we explored the signaling pathway regulating p47phox phosphorylation. We observed that infection of monocytes with *Aspergillus* conidia induced early and selective phosphorylation of p47phox on Ser345 (Figures 2A and S3); in contrast, PMA, a classical PKC activator, induced the phosphorylation of p47phox on distinct serine sites (Ser315, Ser328) (Figures 2A and S3). Since phosphorylation of p47phox on Ser345 is regulated by ERK1/2 signaling in human neutrophils (Boussetta et al., 2010; Dang et al., 2006; El-Benna et al., 2009; Makni-Maalej et al., 2013), we evaluated the role of ERK1/2 signaling on NADPH oxidase-mediated activation of LAP by *Aspergillus* in monocytes. Importantly, ERK1/2 signaling in monocytes was rapidly activated within minutes of infection by *Aspergillus* (Figures 2B and S3). In addition, immunostaining revealed an endosomal pattern of distribution of ERK in monocytes, with early and sustained ERK recruitment and phosphorylation on the phagosome following *Aspergillus* infection (Figures 2C and S3). Importantly, inhibition of ERK1/2 phosphorylation upon monocyte treatment with UO126 (Figures 2AB and S3), a specific MEK1/2 inhibitor (Favata et al., 1998), significantly reduced p47phox phosphorylation at Ser345 (Figure 2B), p47phox recruitment to the phagosome (Figure 2D), NADPH oxidase complex assembly—as demonstrated by inhibition of phagosomal localization of p22phox membrane subunit (Figure 2E)—intracellular ROS production (Figure 2F), and LAPosome formation (Figure 2G). Accordingly, inhibition of ERK1/2 signaling significantly decreased the killing of *Aspergillus* conidia by monocytes (Figure 2H). Importantly, pharmacological inhibition of Class III PI3K (VPS34) complex by wortmannin did not impair ERK recruitment to the phagosome (Figure S3). Collectively, these studies reveal an essential role of ERK signaling in NADPH-oxidase-dependent activation of LAP by *Aspergillus*. Monocytes of two representative patients recovering from sepsis with LAP defect (Figure 2I) had impaired trafficking of p47phox to the phagosome (Figure 2J) and blockade on ERK-dependent phosphorylation of p47phox at Ser345 (Figures 2K and S3). Accordingly, ERK trafficking to the phagosome was impaired in patients with LAP defect compared with a healthy control (Figure 2K); while ERK1/2 phosphorylation was not always impaired (Pt-2; Figure S3). Collectively, these findings reveal a pronounced defect in ERK recruitment to the phagosome of sepsis patients with impaired activation of LAP.

Autocrine IL-6 signaling regulates ERK trafficking and LAPosome formation independent of ERK1/2 phosphorylation

IL-6 production is defective in phagocytes during immunoparalysis in sepsis (Hoetzenecker et al., 2011). We observed that defective IL-6 production in sepsis monocytes was significantly associated with LAP blockade (Figure 1F; Table S2). In contrast, inhibition of LAP was not associated with reduction in the expression of genes regulating ERK-dependent activation of LAP (Data S1). To explore the role of IL-6 on regulation of LAP, we evaluated phagosome responses in bone-marrow-derived macrophages (BMDMs), of IL-6 knockout (IL-6^{-/-}) and control (IL-6^{+/+}) mice infected with *Aspergillus*; BMDMs are typically used for studies of LAP in myeloid phagocytes of mice (Akoumianaki et al., 2016; Kyrnizi et al., 2013, 2018; Martinez et al., 2015; Oikonomou et al., 2016), (de Luca et al., 2014). We observed that NADPH-oxidase-mediated activation of LAP was significantly impaired in IL-6^{-/-} BMDMs as compared with control IL-6^{+/+} BMDMs. In particular, there was a significant decrease on localization of the

membrane-associated (p22phox) (Figure 3A) and cytosolic (p47phox) (Figure 3B) subunits of NADPH oxidase complex to the phagosome, and defective LAPosome formation in the IL-6^{-/-} versus control (IL-6^{+/+}) BMDMs (Figure 3C). In addition, IL-6^{-/-} BMDMs had significant defects in acquisition of markers of phagosome acidification (Figure 3D), phagolysosomal fusion (Figures 3E and 3F), and in the ability to kill conidia of a wild-type clinical isolate of *Aspergillus fumigatus* (Figure 3G). Accordingly, pulmonary infection of IL-6^{-/-} mice with wild-type *Aspergillus* conidia resulted in significant increase in fungal load in the lungs as compared with infection of control IL-6^{+/+} mice (Figures 3H and S4), without evidence of invasive fungal disease in histopathology (Figure S4).

Next, we assessed activation of ERK1/2 signaling by *Aspergillus* in BMDMs of IL-6^{-/-} versus IL-6^{+/+} mice. Of interest, western blot analysis of protein lysates obtained from BMDMs of IL-6^{-/-} versus IL-6^{+/+} revealed no appreciable differences in total ERK1/2 levels and the degree of ERK1/2 phosphorylation induced by *Aspergillus* (Figures 3I and 3J). In sharp contrast, we found a significant defect in ERK1/2 phosphorylation and recruitment on *Aspergillus*-containing phagosomes of IL-6^{-/-} versus IL-6^{+/+} BMDMs (Figure 3K), which was rapidly restored within 15 min of IL-6 supplementation in culture media (Figure 3L). Furthermore, in contrast to other cytokines, IL-6 supplementation selectively enhanced defective killing of *Aspergillus* conidia by IL-6^{-/-} BMDMs (Figure 3M). Collectively, these studies reveal a previously uncharacterized mechanism of action of IL-6 signaling on ERK trafficking to the phagosome and LAPosome formation.

Defects in autocrine IL-6/JAK2/Stat3 axis disrupt microtubule dynamics regulating phagosomal trafficking of ERK and LAPosome formation

Next, we evaluated the mechanism of regulation of phagosomal trafficking of ERK by IL-6 signaling. The phagosome maturation process is regulated by microtubule dynamics (Blocker et al., 1997; Harrison et al., 2003). In particular, physical clustering of dynein motors into lipid micro-domains on the phagosome membrane leads to rapid directed transport of the phagosome toward microtubule minus ends to promote fusion with lysosomes and pathogen degradation (Rai et al., 2016). Therefore, we assessed whether the process of ERK recruitment to the phagosome is also regulated by microtubules. We found that the disruption of microtubule assembly via depolymerization induced by nocodazole or inhibition of microtubule dynamics following treatment of BMDMs with the microtubule-stabilizing agent taxol resulted in abolished ERK localization to *Aspergillus*-containing phagosomes (Figure 4A). Therefore, we specifically assessed the effect of IL-6 on organization of the microtubule network and phagosome transport. Notably, immunostaining of uninfected IL-6^{-/-} BMDMs for β -tubulin revealed major defects in the size of the microtubule organizing center (centrosome) at the steady state, as compared with control IL-6^{+/+} BMDMs, a defect that was reversed within 15 min of IL-6 supplementation in IL-6^{-/-} BMDMs (Figure 4B). We also found that centripetal transport of *Aspergillus*-containing phagosomes from the cell periphery to the microtubule minus end (microtubule organization center) was inhibited in IL-6^{-/-} BMDMs, as compared with control IL-6^{+/+} BMDMs, a defect that was restored within minutes of IL-6 supplementation (Figure 4C–4E; Videos S1 and S2). Importantly, IL-6^{-/-} BMDMs had no apparent defect in phagocytosis of different pathogens, including *Aspergillus* and *Pseudomonas*, and biotin beads (Figure S5).

To investigate the signaling pathway of IL-6-dependent regulation of microtubule dynamics and phagosome trafficking we evaluated the effect of inhibition of JAK/STAT signaling pathway downstream of IL-6 receptor on phagosomal localization of ERK, phagosome movement and LAP. Of interest, inhibition of JAK2 with Ruxolitinib, a specific JAK1/2 inhibitor (Quintás-Cardama et al., 2010) resulted in a significant, dose-dependent inhibition of phagosome transport toward the microtubule minus end (Figure 4F), leading to blockade in phagosomal localization of ERK (Figure 4G), LAPosome formation (Figure 4H) and fungal killing (Figure 4I). In contrast, inhibition of the phosphorylation of STAT3, which is downstream of JAK2, with the use of a specific STAT3 inhibitor (S3I-201) (Siddiquee et al., 2007) had no apparent effect on ERK trafficking to the phagosome (Data S2).

To further gain mechanistic insight on regulation of microtubule organizing center by IL-6/JAK2 signaling, we explored γ -tubulin expression, a master regulator of the centrosome nucleation, in IL-6^{-/-} BMDMs. We found no differences in γ -tubulin perinuclear localization in the area of centrosomes of IL-6^{+/+} versus IL-6^{-/-} BMDMs by immunostaining (Figures 4J and 4K). Next, we focused on the pattern of perinuclear localization of Ninein, a master regulatory protein of microtubule anchoring to the centrosome that has been shown to be phosphorylated by JAK2 (Jay et al., 2015). Of interest, we found a significant decrease in perinuclear expression of Ninein in IL-6 KO BMDMs, accompanied by loss of co-localization of ninein with JAK2, which was rapidly restored within min of IL-6 supplementation (Figures 4L and 4M). Collectively, these findings demonstrate a previously uncharacterized role of the pathway IL-6/JAK2/Ninein signaling on microtubule cytoskeleton dynamics, which then regulates phagosome transport and ERK-mediated LAPosome formation.

Sepsis-induced susceptibility to fungal infections is associated with LAP blockade in mouse monocytes/macrophages

To further explore the underlying mechanisms of LAP defect in sepsis, we developed a physiologically relevant mouse model of polymicrobial sepsis (peritonitis) by inducing different degrees of disease severity (mild sepsis, <5% mortality rates; severe sepsis, >50% mortality rates) (Figures 5A and 5B). Severe sepsis selectively compromised the ability of mice to control superinfection with *Aspergillus*, as evidenced by a significantly higher fungal load in the lungs of mice recovering from severe sepsis (day 7) (Figure 5C), when compared with mice recovering from mild sepsis or SHAM-operated mice; *Aspergillus* infection in mice recovering from severe sepsis (hereafter sepsis) was specifically associated with extensive inflammatory immunopathology (Figure S6) and invasive fungal growth (Figures 5D and S6). In line with the *in vivo* studies, defective activation of LAP following *Aspergillus* infection was induced in splenic monocytes, BMDMs, and alveolar macrophages (AMs) of mice recovering from sepsis, in contrast to SHAM-operated mice (Figures 5E, 5F, and S7). Furthermore, sepsis-induced LAP defect in BMDMs was associated with diminished ROS production in response to *Aspergillus* (Figure 5G) and phagosome maturation arrest, as evidenced by (1) defective accumulation of fluorescein isothiocyanate-labeled dextran (FITC-Dextran) to the phagosome following labeling of lysosomes (Figures 5H and 5I), (2) impaired phagosome acidification assessed by VATPase staining (Figure 5J), and (3) defective phagosomal localization of the lysosomal markers cathepsin-D (Figure 5K) and LAMP-1 (Figures 5L and S7). Finally, sepsis BMDMs displayed significant loss of fungicidal activity against conidia of wild-type *Aspergillus* (Figure 5M) and diminished IL-6 production upon re-stimulation with fungal conidia (Figure S7). Overall, these

findings reveal that sepsis-induced immune deactivation in murine monocytes/macrophages is associated with defective activation of LAP.

Loss of IL-6 signaling in mice and human patients with sepsis selectively impairs ERK trafficking, LAPosome formation, and fungal killing

Next, we assessed whether LAP blockade in sepsis macrophages is mechanistically linked with defects in IL-6-dependent trafficking of ERK to the phagosome. We found a profound defect in centrosome organization and microtubule assembly at the baseline in BMDMs obtained from mice with severe sepsis, a phenotype reminiscent of microtubule cytoskeleton abnormalities in IL-6^{-/-} BMDMs (Figure 6A). Indeed, sepsis-induced defects in the size of microtubule organizing center in uninfected BMDMs were rapidly restored following IL-6 supplementation (Figure 6A). Accordingly, BMDMs obtained from mice with severe sepsis displayed significant defects on phagosome transport following infection with *Aspergillus*, which were restored within minutes of IL-6 treatment (Figure 6B).

In line with these results, ERK trafficking to *Aspergillus*-containing phagosomes was abolished in BMDMs of mice recovering from severe sepsis in contrast to control SHAM treated mice (Figures 6C and 6D). Instead, impaired phagosomal ERK recruitment was not always accompanied by defective ERK phosphorylation by *Aspergillus* (Data S2). Of interest, IL-6 supplementation resulted in complete restoration of phagosomal localization of ERK (Figures 6C and 6D) and LAPosome formation (Figure 6E) in BMDMs obtained from mice with severe sepsis. Furthermore, as opposite to other cytokines (TNF, IFN- γ), IL-6 treatment selectively enhanced defective killing of *Aspergillus* by sepsis BMDMs (Figure 6F).

Similar to the findings in macrophages of mice following sepsis recovery, we found defects in microtubule anchoring and organization at the centrosome and microtubule-mediated phagosome transport in monocytes of patients with sepsis-induced inhibition of LAP (Figure 6G). Importantly, IL-6 supplementation in culture media of human monocytes with sepsis-induced inhibition of LAP fully restored defective killing activity against *Aspergillus* (Figure 6H); instead, IL-6 treatment had no effect in fungicidal activity of healthy control monocytes. These studies reveal a previously uncharacterized role of IL-6 signaling on ERK-mediated activation of LAP in monocyte/macrophage with direct pathophysiological and therapeutic implications for immunoparalysis in sepsis.

IL-6-induced restoration of microbicidal activity of myeloid phagocytes in sepsis depends on LAP

In order to establish a causative relationship between loss of IL-6 signaling, LAP blockade, and sepsis immunoparalysis, we explored the effect of IL-6 supplementation on microbicidal activity of sepsis macrophages in mice with genetic defect of Atg5. BMDMs from mice with genetic deletion of Atg5 (*LysMCre*^{+/+}; *Atg5flox/flox* mice; hereafter Atg5^{-/-} BMDMs), an autophagy protein crucial for LAPosome formation (Akoumianaki et al., 2016; Martinez et al., 2015), had no apparent defects on microtubule cytoskeleton organization (Figure 7A); however, Atg5^{-/-} BMDMs displayed broad defects on phagosome biogenesis, comparable with those induced by

severe sepsis (Figures 7B–7E). Notably, IL-6 supplementation resulted in full reversal of sepsis-induced defects in killing of *Aspergillus* conidia by Atg5^{+/+} BMDMs but failed to restore killing defects in Atg5^{-/-} BMDMs following sepsis (Figure 7F).

Next, we evaluated the physiological importance of IL-6/LAPosome pathway in macrophage antimicrobial activity against *Pseudomonas*, a bacterial pathogen classically associated with secondary infections due to sepsis-induced immunoparalysis (Boomer et al., 2011; van der Poll et al., 2017) including our patient cohort (Table S1). We initially verified that killing of a reference clinical isolate of *Pseudomonas aeruginosa* by macrophages depends on IL-6 signaling (Figure 7G) and LAP (Figure 7H). Next, we tested the effect of IL-6 supplementation on bactericidal capacity of BMDMs differentiated from control versus LAP-defective mice recovering from severe sepsis. Importantly, IL-6 supplementation significantly improved killing of *Pseudomonas* by BMDMs obtained from Atg5^{+/+} (control) BMDMs following recovery from severe sepsis (Figure 7I). In contrast, IL-6 failed to restore bactericidal activity of Atg5^{-/-} BMDMs following sepsis (Figure 7I). Collectively, these studies lead to a new mechanism of regulation of LAP by IL-6 signaling with physiological importance in pathogenesis of sepsis immunoparalysis.

Discussion

Integration of signals from PRRs and cytokines to phagocytosis has a crucial role in successful control of infection by macrophages (Pauwels et al., 2017). In particular, PRR-mediated activation of the LAP pathway regulates phagosome maturation and confers protective immunity against a range of pathogens (Akoumianaki et al., 2016; Heckmann and Green, 2019; Kymizi et al., 2013, 2018; Martinez et al., 2015). Likewise, specific cytokines transcriptionally induce the expression of antimicrobial effectors to enhance the microbicidal capacity of macrophages (Forbester et al., 2018; Jung and Robinson, 2014; Shen et al., 2018; Subramanian Vignesh et al., 2013; Via et al., 1998). Moreover, reciprocal interactions of cytokines and LAP are required for an optimal anti-inflammatory function of macrophages (de Luca et al., 2014; Oikonomou et al., 2016). However, a mechanistic link between cytokine signaling and LAP has not yet been identified.

Herein, we employ sepsis as a model human disease to explore physiologically important interactions of cytokine signaling and LAP. Our studies in humans and mice “identify LAP blockade in phagocytes as a core immunological feature of sepsis-induced immunoparalysis.” Moreover, we discover “a role for the autocrine IL-6/JAK2 axis in microbicidal activity of phagocytes via regulation of LAP, with direct implications in immune deactivation of sepsis.”

In our initial studies, we found that defective LAP is a prominent feature of phagocyte dysfunction in patients recovering from sepsis, who display all classical features of immunoparalysis (Hotchkiss et al., 2013; van der Poll et al., 2017). In view of the absence of biological measures of immunoparalysis induced by sepsis, inhibition of LAP should be regarded as an immunological feature of this pathological process. Apart from monocytes/macrophages, neutrophils are major effectors of innate immune response in sepsis (Hotchkiss et al., 2013; van

der Poll et al., 2017). Therefore, the role of LAP defects in immune deactivation of neutrophils in sepsis should be investigated.

We also identified a selective mechanism of p47phox phosphorylation at Ser345 in monocytes during fungal infection, a phosphorylation site regulated by MAPK signaling (Dang et al., 2006; El-Benna et al., 2009; Makni-Maalej et al., 2013). This reveals a major role of ERK1/2 signaling on NADPH oxidase-mediated activation of LAP both physiologically and in sepsis, independently of VPS-34 complex formation on the phagosome. Because cytokine deregulation has a major role in the pathogenesis of sepsis (van der Poll et al., 2017), we evaluated the direct role of cytokine signaling on ERK-dependent activation of LAP. We focused on IL-6, a signature cytokine of sepsis-induced immunosuppression and related susceptibility to aspergillosis (Cenci et al., 2001; Hoetzenecker et al., 2011). Surprisingly, we observed that although p47phox-mediated activation of NADPH oxidase was abolished in IL-6 knockout (KO) BMDMs during fungal infection, upstream ERK1/2 phosphorylation remained intact. In contrast, ERK1/2 recruitment on the phagosome was abolished in IL-6 KO BMDMs, which explained the downstream blockade in NADPH oxidase-dependent activation of LAP. These findings confirmed our hypothesis on distinctive pathways regulating ERK phosphorylation and trafficking on the phagosome and revealed a central regulatory role for IL-6 signaling on endosomal trafficking of ERK and LAPosome formation.

Importantly, we discovered that IL-6 KO BMDMs display profound disorganization of microtubule organizing center at steady state, associated with impaired LAP-dependent killing of pathogens without an apparent defect in phagocytosis. Subsequently, we found that microtubule-dependent phagosome movement and ERK trafficking were regulated by JAK2, independently of STAT3. Of interest, both STAT3 (Jay et al., 2015; Ma and Sayeski, 2007) and JAK2 (Morris et al., 2017; Ng et al., 2006) are involved in microtubule organization dynamics via interactions with distinct signaling pathways. Evermore, JAK2 phosphorylates and is negatively regulated by the centrosomal protein ninein (Jay et al., 2015). In particular, Ninein is a master regulator of microtubule anchoring to the centrosome. Accordingly, inhibition of JAK2 has been shown to selectively impair the size of microtubule organizing center without affecting the process of centrosome nucleation, a function regulated by γ -tubulin (Jay et al., 2015). Indeed, our findings on diminished Ninein expression and co-localization with JAK2 in perinuclear regions of IL-6 KO macrophages without an apparent defect in topology of γ -tubulin are in line with previous studies (Ma and Sayeski, 2007; Rai et al., 2016; Via et al., 1998).

In proof-of-concept studies, we demonstrated that monocytes/macrophages isolated from septic conditions display defects in microtubule cytoskeleton organization, ERK trafficking, LAPosome formation, and pathogen killing. These are specifically related to loss of IL-6 signaling, as they were all restored within minutes of IL-6 supplementation. Furthermore, we revealed that IL-6 induced restoration of LAP defects in sepsis has broad implications in phagocyte immunity against major bacterial and fungal pathogens. Of interest, previous studies report on the restoration of sepsis-induced immune deactivation in monocytes following several days of immunotherapy with IFN- γ (Döcke et al., 1997). It would be important to investigate the effects of prolonged immunotherapy with IFN- γ on IL-6/LAP axis. Alternatively, other cytokines may regulate phagosome biogenesis or trigger canonical autophagy (e.g., xenophagy) responses against certain microbial pathogens (e.g., intracellular bacteria) independently of NADPH oxidase signaling and LAP (Sokolovska et al., 2013). In addition, whether unique intracellular

trafficking of IL-6 as compared with other cytokines may have a role on the specialized role of this cytokine on regulation of ERK-dependent activation of LAP deserves exploitation (Murray et al., 2005). Collectively, our study identifies an unexplored physiological pathway linking autocrine IL-6/JAK2/Stat3 axis, cytoskeleton organization, and LAP with the microbicidal activity of myeloid phagocytes and offers mechanistic insight in immunoparalysis of sepsis.

Our findings have also broad implications for understanding emerging mechanisms of iatrogenic immunosuppression predisposing to opportunistic infections. In particular, recent clinical studies report an alarming increase in cases of severe bacterial and fungal diseases in patients receiving small molecule kinase inhibitors (SMKIs), in the absence of additional immunosuppressive conditions. Notably, SMKIs targeting ERK1/2 and JAK/STAT signaling have been recently associated with clinical cases of invasive aspergillosis (Chamilos et al., 2018a, 2018b; Dioverti et al., 2018; Lussana et al., 2018). Preclinical studies also demonstrate compromised killing capacity of phagocytes against several pathogens, including *Aspergillus*, following pharmacological or genetic inhibition of IL-6, JAK1/2, and ERK (Cenci et al., 2001; Dubourdeau et al., 2006; Espinosa et al., 2017; Taylor et al., 2016). Finally, IL-6 is an appealing therapeutic target for restoration of inflammatory immunopathology induced by cytokine storm in COVID 19 (Moore and June, 2020). Importantly, superinfections by bacterial and fungal pathogens are increasingly encountered in patients recovering from COVID-19 (Bartoletti et al., 2020). Therefore, our findings provide novel mechanistic view on the emergence of secondary infections caused by opportunistic pathogens in patients with receiving immunomodulatory therapies with inhibitors of IL-6 and JAK/STAT signaling pathways.

Furthermore, our findings lead to a model of phagosome biogenesis that explains the synergistic mechanism of immunosuppressive action of corticosteroid therapy in patients receiving SMKIs, as a result of dual inhibition of two major pathways regulating LAP, PRRs and cytokine signaling (Kymrzi et al., 2013). Understanding genetic and epigenetic factors regulating LAP responses could have important implications on risk stratifications for susceptibility to infection of these patients. Overall, our work provides the scientific rationale for the development of personalized cytokine-based immunotherapies for sepsis and/or other conditions of immune deregulation, aiming at the restoration of LAP-dependent effector functions and anti-inflammatory pathways in phagocytes.

STAR Methods

Key resources table

REAGENT RESOURCE	or SOURCE	IDENTIFIER
Antibodies		
Monoclonal Anti-beta-Tubulin antibody produced in mouse	Sigma-Aldrich	Cat# T4026; RRID: AB_477577
Mouse monoclonal Anti-Actin Antibody, clone C4	Millipore	Cat# MAB1501; RRID: AB_2223041
Mouse monoclonal Anti-LC3 (microtubule-associated protein 1 light chain 3B) antibody	Nanotools	Cat# 0231-100/LC3-5F10; RRID: AB_2722733
Rabbit Anti-LC3 polyclonal, Unconjugated antibody	Novus	Cat# NB 100-2220; RRID: AB_578334
Rabbit polyclonal LC3B Antibody	Novus	Cat# NB600-1384; RRID: AB_669581
Rabbit mAb IL-6 Cell (D5W4V) XP®	Cell Signaling	Cat#12912; RRID: AB_2798059
Mouse Anti-CD107a monoclonal Antibody, Unconjugated, Clone H4A3	BD Biosciences	Cat# 555798; RRID: AB_396132
Mouse Anti-CD63 monoclonal Antibody, Unconjugated, Clone H5C6	BD Biosciences	Cat# 556019; RRID: AB_396297
Mouse	Santa Cruz	Cat#sc-17845; RRID: AB_627986

REAGENT RESOURCE	or SOURCE	IDENTIFIER
monoclonal p47- phox (D-10) antibody	Biotechnolog y	
Mouse monoclonal p47phox antibody	BD Biosciences	Cat# 610354; RRID: AB_397744
Rabbit polyclonal p22-phox (FL- 195) antibody	Santa Cruz Biotechnolog y	Cat#sc-20781; RRID: AB_2090309
Mouse monoclonal LAMP-1 (E-5) antibody	Santa Cruz Biotechnolog y	Cat# sc-17768; RRID: AB_626851
Rabbit polyclonal Rab 5B (A-20) antibody	Santa Cruz Biotechnolog y	Cat# sc-598; RRID: AB_2175453
Mouse monoclonal Cathepsin D (D- 7)	Santa Cruz Biotechnolog y	Cat# sc-377299
Rabbit Calnexin antibody	Novus	Cat# NB100-1974; RRID: AB_10001873
Rabbit Anti- ATP6V1B2 Polyclonal Antibody, Unconjugated	Abcam	Cat# ab73404; RRID: AB_1924799
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb antibody	Cell Signaling	Cat# 4695; RRID: AB_390779
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (20G11) Rabbit mAb antibody	Cell Signaling	Cat# 4376; RRID: AB_331772
Jak2 (D2E12) XP Rabbit mAb antibody	Cell Signaling	Cat# 9367; RRID: AB_1904103
Rabbit polyclonal anti-GFP	Minotech	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER	
Rabbit polyclonal anti-PhosphoSer315-p47 ^{phox} antibody	Boussetta et al., 2010	N/A	
Rabbit polyclonal anti-PhosphoSer328-p47 ^{phox} antibody	Boussetta et al., 2010	N/A	
Rabbit polyclonal anti-PhosphoSer345-p47 ^{phox} antibody	Dang et al., 2006	N/A	
Ninein antibody (F-5)	Santa Cruz Biotechnology	Cat# sc-376420, RRID:AB_11151570	
PerCP anti-human HLA-DR	Biolegend	Cat# RRID: AB_893574	307628
APC anti-human CD14	Biolegend	Cat# RRID: AB_830680	325607
Deposited data			
Original Western Blot and FACS gating	This study	https://data.mendeley.com/datasets/cgzsvpmpzc/draft?a=f204d5e0-349e-4781-914c-96fd2a0f74d8	
RNA-seq data	This study	GEO database (GEO access number: GSE174183)	
Chemicals, peptides, and recombinant proteins			
Ruxolitinib	CAYMAN CHEMICAL COMPANY	11609; CAS: 941678-49-5	
Recombinant Murine IL-6	PEPROTEC H	216-16	
2',7'-Dichlorofluorescein diacetate	Sigma-Aldrich	D6883; CAS: 4091-99-0	
U0126	Calbiochem	662005; CAS: 109511-58-2	
Propidium Iodide	Sigma-Aldrich	P4170; CAS: 25535-16-4	
Fluorescein isothiocyanate-dextran	Sigma-Aldrich	46945; CAS: 60842-46-8	
Alexa Fluor TM 633 NHS Ester	ThermoFisher Scientific	A20005	

REAGENT RESOURCE	or SOURCE	IDENTIFIER
(Succinimidyl Ester)		
Nocodazole	Sigma-Aldrich	M1404; CAS: 31430-18-9
Paxene (paclitaxel 6mg/ml)	IVAX	N/A
S3I-201	Sigma-Aldrich	SML0330; CAS: 501919-59-1

Critical commercial assays

Mouse IL-6 ELISA Deluxe kit	MAX BioLegend	Cat:430304
Human IL-6 Uncoated kit	Elisa Invitrogen	REF:88-7066-22

Experimental models: Cell lines

NCTC clone 929 [L cell, L-929, derivative of Strain L]	ATCC	CCL-1
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Experimental models: Organisms/strains

<i>Aspergillus fumigatus</i>	ATCC	ATCC 46645
Mouse: <i>IL6</i> ^{+/+} of 129×C57BL/6 genetic background	(Venihaki et al., 2001)	N/A
Mouse: <i>IL6</i> ^{-/-} of 129×C57BL/6 genetic background	(Venihaki et al., 2001)	N/A
Mouse: C57BL/6 (B6)	The Jackson Laboratory	000664
Mouse: GFP-LC3	RIKEN BioResource Center	RBRC00806

Software and algorithms

GraphPad Prism 5	GraphPad Software	https://www.graphpad.com/
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REAGENT RESOURCE	or SOURCE	IDENTIFIER
Endnote x9	Endnote Software	https://endnote.com/
ImageJ	Image Software	^J https://imagej.nih-gov.proxy.insermbiblio.inist.fr/ij/
Other		
Graphical Abstract	BioRender	https://app.biorender.com

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Georgios Chamilos (hamilos@imbb.forth.gr).

Materials availability

All the data that support the findings of this study will be made available upon request made to the Lead Contact.

Data and code availability

The published article includes all datasets generated or analyzed during this study. The RNAseq data have been deposited in the GEO database (GEO access number: GSE174183). Original FACS analysis and western blots data were deposited at Mendeley at: <https://data.mendeley.com/datasets/cgzsvpmpzc/draft?a=f204d5e0-349e-4781-914c-96fd2a0f74d8>

Experimental model and subject details

Human study subjects and clinical specimens

All consecutive adult patients admitted to the ICU of a tertiary center Hospital (University Hospital of Crete) from April 2014 to December 2017 with the clinical diagnosis of community acquired septic shock, and a high index of disease severity (APACHE II score ≥ 8) were eligible for the study. Additional sepsis patients were enrolled between December 2019 and April 2020. The diagnosis of septic shock was based on Standardized Criteria (Singer et al., 2016) as follows: 1) an identifiable site of infection and at least two of the signs of systemic inflammatory response syndrome (SIRS); and 2) arterial blood pressure of < 90 mmHg despite adequate fluid resuscitation and requiring vasopressor therapy. Additional patients with septic shock admitted to the medical ICU of Cochin Hospital (Paris, France) were enrolled in 2017.

Exclusion criteria were an age less than 18 years, pregnancy, do-not-resuscitate orders on admission, hematologic malignancy, immunodeficiency defined as HIV infection, presence of

bone marrow or solid organ transplantation, daily corticosteroid therapy greater than 0.5 mg/kg prednisone equivalent, cytokine-blocking agents, chemotherapy or any other immunosuppressive treatments. We enrolled healthy volunteers as controls for functional studies.

Approval for the collection of clinical information and tissue samples from septic patients and control healthy individuals was obtained from the Ethics Committee of the University Hospital of Heraklion, Crete, Greece (5159/2014 and 10925/2018) from the appropriate Ethics Committee in France (CPP Ile-de France 3, ref #S.C. 3351). The patient or next of kin and each healthy volunteer provided written informed consent in accordance with the Declaration of Helsinki. Blood was collected on the day of sepsis diagnosis (Day 1) and later on following sepsis recovery (Day 7).

Data collection

Baseline data

Baseline data collection included demographics (age and physician-assigned gender), comorbidities, and immunosuppressive medications.

Characteristics of the primary infection

The primary site of infection and microbiological data on the pathogens isolated were recorded. We also recorded the APACHE II score, and the SOFA scores on the day of admission in the ICU and at Day 7.

Secondary infections

Secondary infection in septic shock patients was defined as a new infection acquired more than 48 hours after admission to the ICU. The diagnosis of a secondary infection was performed according to the criteria of the Centers for Disease Control and Prevention (CDC, 2008) (Horan et al., 2008). Infection was differentially diagnosed from colonization according to the CDC criteria and required one or more new antibiotics. The time, site and pathogen isolated for the secondary infection were recorded, and the analysis was restricted to the first episode of secondary infection at the same site.

Endpoints

Mortality, as well as hospital and ICU length of stay were collected.

Functional studies

Blood was immediately processed upon collection, CD14⁺ monocytes were isolated, stimulated with *Aspergillus* conidia and fixed on coverslips for immunostaining and analysis. Total RNA from 1 million unstimulated CD14⁺ monocytes lysed in Trizol reagent (Invitrogen) was kept in -80°C. Evaluation of LC3⁺ phagosomes and other markers of phagosome biogenesis was performed in batches of paired samples of each individual patient (Day 1, Day 7) as compared to

the LAPosome formation in monocytes obtained from healthy volunteers (control) from the blood bank of the Hospital.

Mouse models

C57BL/6 mice (obtained from the IMBB Institute, Crete), IL6^{-/-} and IL6^{+/+} mice of 129×C57BL/6 genetic background (Venihaki et al., 2001), GFP-LC3 mice (RIKEN BioResource Center), *Atg5* conditional KO mice generated by crossing *Atg5*^{fllox/fllox} mice (RIKEN BioResource Center; (Akoumianaki et al., 2016)) and VavCre^{+/+} mice (Akoumianaki et al., 2016), were maintained in grouped cages in a High-Efficiency Particulate Air-filtered environmentally controlled virus-free facility (24°C, 12/12 h light/dark cycle), and fed by standard chow diet and water ad libitum. All experiments were approved by the local ethics committee of the University of Crete Medical School, Greece in line with the corresponding national and European Union legislation (animal protocols 07/05/14-6167 and 17/07/2017-147075).

Polymicrobial sepsis Model in mice

Pathogen-free, male C57BL/6, 8-10 weeks old (25-30 gr) were subjected to cecal ligation and puncture (CLP) polymicrobial sepsis model (Benjamim et al., 2003, 2005). In brief, mice were anesthetized prior to surgery, by intraperitoneal injection of a ketamine/xylazine mixture. Under sterile conditions, a longitudinal skin midline incision was made and the cecum was exposed to the surface. The cecum was partially ligated (60% of the cecum in reference to the distal pole) with a 4-0 silk suture without disrupting the intestinal continuity. The ligated cecum was punctured through and through with a 21-gauge needle three times (six punctures in total) in order to achieve severe sepsis conditions. For mild sepsis, only one puncture was performed. Sham-operated mice underwent the same surgery but without cecal ligation and puncture and were used as controls. Six hours after induction of polymicrobial sepsis, sham or CLP operated mice were started on antibiotic treatment with 10mg/kg Imipenem/cilastatin. The antibiotic was administered every 12 hours for a period of three days. On Day 7 following recovery from sepsis, sham and CLP mice were challenged by intratracheal installation of 5×10^7 *A. fumigatus* conidia. After 48 hours of infection, mice were euthanized, their lungs were harvested and homogenized in phosphate buffer saline. The fungal burden was evaluated by CFU plating of lung homogenates.

Microorganisms and culture conditions

All *A. fumigatus* strains used ($\Delta pksP$ and the isogenic wild type strain ATCC46645) have been described previously (Akoumianaki et al., 2016). All strains were grown on YAG agar plates for 3 days at 37°C. Fungal conidia (spores) were harvested by gentle shaking in the presence of sterile 0.1% Tween 20 in PBS, washed twice with PBS, filtered through a 40µm-pore-size cell strainer (Falcon) to separate conidia from contaminating mycelium, counted by a hemocytometer, and suspended at a concentration of 10^8 spores/mL. When indicated, the conidia were labeled with FITC or Alexa Fluor 633 succinimidyl ester dye (Invitrogen) as previously described (Akoumianaki et al., 2016). Briefly, freshly harvested conidia ($5 \times 10^7/2$ mL in 100mM NaHCO₃, pH 8.3) were incubated with FITC (Sigma Aldrich, 0.1 mg/mL final concentration) or Alexa Fluor 633 succinimidyl ester dye (Invitrogen, 1.7 mg/mL) at 37°C for 2h and washed by centrifugation three times in PBS-0.1% Tween 20. Inactivation of fungal conidia was done by

exposure to 4% FA (2 h, room temperature) following by treatment with glycine (100 mM in PBS) and three washes in PBS and verified by CFU plating. Importantly, we have previously validated that FA-inactivation of *A. fumigatus* conidia does not affect LAPosome formation and PRR signaling activation (Akoumianaki et al., 2016).

A single colony of *Pseudomonas aeruginosa* clinical isolate (ATCC 27853) isolated from LB plates was grown at a mid-logarithmic phase in LB liquid media the day of infection. Bacterial cells were washed with PBS, adjusted to 0.5 MacFarlane (10^8 bacteria/ml) and immediately used for infection experiments.

Method details

Isolation and stimulation of primary human monocytes

Healthy volunteers without any known infectious or inflammatory disorders donated blood. Monocytes from sepsis patients or healthy controls were isolated from PBMCs using magnetic bead separation with anti-CD14 coated beads (MACS Miltenyi Germany) according to the protocol supplemented by the manufacturer. The monocytes were resuspended in RPMI 1640 culture medium supplemented with Hepes 10 mM, penicillin-streptomycin 1%, l-glutamine 1%, sodium pyruvate 1%, glucose 25 mM and fetal bovine serum 10%. The cells were counted, and their number was adjusted to 2×10^6 /mL. A total of 2×10^5 monocytes per condition in a final volume of 100 μ L were allowed to adhere to sterile polylysine coated glass coverslips (\varnothing 13 mm) for 1 h followed by stimulation with *A. fumigatus* conidia at a multiplicity of infection (MOI) of 3:1 at 37°C for the indicated time point. After stimulation, cells were washed twice with PBS to remove medium and non-phagocytosed spores and cells were fixed for 15 min in 4% paraformaldehyde or 4% formaldehyde (w/o MeOH). Subsequently, the coverslips were washed with PBS followed by an extra step of fixation with ice-cold methanol for 10 min at -20°C in case of immunostaining for LC3, after which were stored in PBS at 4°C until immunofluorescence staining.

Immunofluorescence staining

For immunofluorescence imaging, fixed cells were blocked for 15 minutes with 2% BSA, 0.1% saponin in PBS, incubated for 1 h with the indicated primary antibody, washed twice in PBS, stained by the appropriate secondary AlexaFluor secondary Ab (Molecular Probes), followed by DNA staining with 1 μ M TOPRO-3 iodide (642/661; Invitrogen). For ERK staining coverslips were incubated overnight with the primary antibody at 4°C. After the washing steps, slides were mounted in Prolong Gold antifade media (Molecular Probes). Images were acquired using a laser-scanning spectral confocal microscope (TCS SP8; Leica), LCS Lite software (Leica), and a 40 \times Apochromat 1.25 NA oil objective using identical gain settings. A low fluorescence immersion oil (11513859; Leica) was used, and imaging was performed at room temperature. Serial confocal sections at 0.5 μ m steps within a z-stack spanning a total thickness of 10 to 12 μ m of the cell were taken, and 3D images were generated using the LCS Lite software to assess for internalized conidia contained within phagosomes. Unless otherwise stated, mean projections of image stacks were obtained using the LCS Lite software. Phagosomes surrounded by a rim of fluorescence of the indicated protein-marker were scored as positive, according to established protocols in our lab. At least 200 phagosomes were analyzed for each experimental condition in

three independent experiments, in a double blinded fashion by the same investigator (T.A.). Assessment of phagolysosomal fusion was performed in BMDMs pre-loaded with FITC-Dextran. Briefly, BMDMs were pre-loaded with FITC-Dextran for 4h, media was replaced for 2h to allow for labeling of lysosomes, and phagolysosomal fusion was assessed following fungal infection based on the accumulation of FITC-Dextran at the phagosomes.

In addition, phagocytosis rate and phagocytosis index were assessed in primary human monocytes at 30 min of infection with FITC-labeled *A. fumigatus* (MOI 3:1). The phagocytic index was expressed with the following formula: (total number of engulfed cells/total number of counted macrophages) \times (number of macrophages containing engulfed cells/total number of counted macrophages) \times 100.

Live imaging

BMDMs were loaded with FITC-Dextran (5 μ M final concentration, Invitrogen) for 4h washed with PBS cells counted and seeded in chambers for live imaging (627870 Greiner) in HBSS (w/o red phenol, 2 mM CaCl₂, 1 mM MgCl₂, 20 mM Hepes). After adhesion of the cells, infections with the indicated *A. fumigatus* strain were performed just before live imaging. The acquisition was performed in SP8 Leica converted microscope with 40 \times Apochromat 1.25 NA water objective using identical imaging setting, and high speed live imaging with the use of a resonant scanner.

Measurement of ROS production

ROS measurements were performed by means of a dichlorofluorescein assay (Akoumianaki et al., 2016). The Stock solution of dichlorofluorescein diacetate (DCFH-DA) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM. Human monocytes (2×10^5 /well) or mouse BMDMs (1×10^5 /well) were plated on 96-well round bottom plates, incubated at 37°C for 30 min and accordingly stimulated for 1h with *A. fumigatus* conidia (MOI 10:1) or zymosan (50 μ g/ml) in the presence of DCFH-DA added to a final concentration of 10 μ M during the last 30 min. After 30 min of exposure, the well content was transferred to vials and the fluorescence of the cells was measured by flow cytometry. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Generation of murine BMDMS

BMDMs were generated by culturing bone marrow cells obtained from 8 to 12 weeks old female mice in DMEM, supplemented with L929 cell-conditioned medium (30%). The resulting cultures consisted of macrophages (>95% purity), as determined by staining for F4/80 and flow cytometry.

Isolation of splenic monocytes

Mouse monocytes were isolated from single cell suspension of spleens obtained from control and septic mice. Briefly, splenic cells were incubated for 30 min with a Ly6C biotinylated antibody and monocytes were isolated with magnetic streptavidin beads.

Killing of *A. fumigatus* by monocytes/macrophages

Primary human monocytes (2×10^5 /well) or BMDMs (1×10^5 /well) were plated onto 96-well round bottom plates for 1h, and subsequently infected with the indicated *A. fumigatus* strain at an MOI of 1:10 (conidia: target cell ratio) for 1 h at 37°C with or without the indicated concentration of JAK2 inhibitor (ruxolitinib) or STAT3 inhibitor (S3I-201) added 30 min before infection. Medium containing non-adherent, non-phagocytosed conidia was removed, wells were washed three times using warm PBS, and new media with or without the presence of the corresponding inhibitor were added. Monocytes were then allowed to kill conidia for 6h before intracellular conidia were harvested by lysis of monocytes with 0.5% Triton-X. The process of cellular lysis was confirmed by light microscopy and the killing of *A. fumigatus* conidia was assessed by CFU plating. Each condition was performed in quadruplicate with monocytes obtained from 4 different donors. In other experiments killing of FITC-labeled conidia by BMDMs was assessed by viability staining with Propidium Iodide (PI). Briefly, murine BMDMs were allowed to ingest FITC-labeled *A. fumigatus* conidia at an MOI of 1:1 for 1 h at 37°C in 24 well plates. BMDMs were allowed to kill conidia for 6 h before intracellular conidia were harvested by sonication (Akoumianaki et al., 2016). Next, cell lysates were stained with Propidium Iodide (PI) to assess the viability of *A. fumigatus* conidia. FITC labeled PFA-killed or live *A. fumigatus* conidia served as controls for PI staining.

Killing of *Pseudomonas* by BMDMs

BMDMs (2×10^5 per condition) from IL-6^{-/-} (KO) or control IL-6^{+/+} mice were seeded overnight in 96 well flat bottom plates and infected with *Pseudomonas aeruginosa* (ATCC 27853 grown at a mid-logarithmic phase) in antibiotic free media at a MOI of 10:1 (target: effector ratio) with or without supplementation of culture media with IL-6 at a final concentration of 10 ng/ml. Cells were extensively washed at 1h of infection with warm PBS and a cell-impermeable antibiotic (gentamicin 300 µg/ml) was added in culture media for 30 min to kill remaining extracellular bacteria. The amount of phagocytosed bacteria at 1h of infection was assessed following cell lysis by CFU plating. Bacterial killing by BMDMs was assessed by CFU counts of intracellular bacteria at 12h of infection.

Western blot analysis

Primary human monocytes or murine BMDMs were stimulated with *Aspergillus fumigatus* conidia or PMA for the indicated time points at MOI 10:1 with or without the presence of the indicated inhibitor. Cells were washed once in PBS prior to lysis in 1% NP-40 containing RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0,25% sodium deoxycholate, 1 mM NaF, 1 mM Na₃VO₄, 1mM PMSF plus a mixture of protease inhibitors [Roche Molecular Biochemicals]). For analysis of p47phox phosphorylation, a mixture of phosphatase inhibitors (PhosSTOP; Roche Molecular Biochemicals) was added to the lysis buffer. Cell lysis was performed on ice for 30 minutes, and samples were centrifuged. After protein estimation of supernatants, addition of SDS sample buffer and boiling, samples were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Western blotting was performed according to the instructions of the manufacturer using the indicated primary antibodies: Secondary antibodies used in western blotting were purchased from Cell Signaling

(anti-rabbit HRP, anti-goat HRP) as well as Millipore (anti-mouse HRP). The blots were developed using chemiluminescence (ECL; Thermo Scientific).

Measurement of cytokines

Supernatants of stimulated human monocytes were collected after overnight culture and were stored at -20°C until analysis. Cytokine levels in the supernatants were determined by using ELISA kits for human IL-1 β , IL-1Ra, and TNF (R&D Systems) and IL-6 (Sanquin) according to the manufacturer's instructions.

RNA extraction

Monocytes isolated from different groups of sepsis patients stratified by LAP responses were lysed in Trizol reagent and stored at -80°C . Total RNA was extracted from cells using the QIAGEN RNeasy RNA extraction kit, using on-column DNaseI treatment. Ribosomal RNA was removed using the riboZero rRNA removal kit.

Library preparation for RNA-seq

RNA samples were shipped to the sequencing facility. RNA integrity was measured using Agilent TapeStation System. All samples had good qualities (RIN score ≥ 8). RNA libraries were prepared to enrich for polyA tail-RNA using Bioscientific NEXTflex-Rapid-Directional mRNA-seq kits. RNA libraries were prepared with the Caliper SciClone system. The libraries were sequenced using the NextSeq500 High Output kit V2 (Illumina) for 75 cycles.

RNA-seq data analysis

Raw RNA-seq reads were aligned to the human genome version 37 using STAR (v2.5.1b) (Dobin et al., 2013) HTSeq-count package (v0.61) was used to count and normalize the number of reads mapped to each gene (Anders et al., 2015). Differential expression was determined using DEseq2 (v.1.12.4) (Love et al., 2014). For subsets of genes involved in autophagy, LAP, IL-6 signaling and ERK pathways (refer to Figure S7), normalized counts were log transformed and comparisons between patient groups was performed using One-way ANOVA and corrected for the number of genes tested (Bonferroni) ($\text{FDR} \leq 0.05$ and fold change > 1.5). The RNAseq data have been deposited in the GEO database (GEO access number: GSE174183).

Quantification and statistical analysis

For in vivo results, each n value represents an individual experiment and mouse. For the human in vitro experiments, each n refers to a separate independent experiment. The number of experiments for each figure can be found in the figure legends. The data were expressed as mean \pm SEM. Data were tested for Gaussian distribution using a Shapiro-Wilk normality analysis. For normally distributed data two-sided unpaired Student's t test was used and for non-Gaussian data two sided unpaired Mann-Whitney tests were used to compute statistical significance between two groups. One-way ANOVA with the indicated post hoc test was used for multiple comparisons ($P < 0.05$ was considered statistically significant). Survival analysis and studies on

the cumulative probability of infection in patients over time were performed by log-rank (Mantel–Cox) test. Analysis was performed using the GraphPad Prism software (version VII). All *in vitro* experiments were performed at least in triplicate and replicated at least twice.

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Author contributions

T.A. performed and analyzed most of the experiments in human patients and mice, designed and established protocols on the *in vivo* model of sepsis, live imaging studies, and assays for evaluation of microtubule organization, and participated in the writing of the manuscript. R.B. and M.G. performed experiments on human samples. M.G. performed analysis of cytokine from sepsis patients. D.G., K.V., F.P., and E.D. collected and analyzed clinical data and provided samples from patients with sepsis; M.V. provided the IL-6^{-/-} mice and analyzed data; J.E.-B. provided reagents and analyzed data; K.T.T.L. and V.K. performed RNA-seq analysis; F.L.v.d.V. and M.G.N. were involved in the design of experiments on human studies, analyzed data, and participated in the writing of the manuscript; E.D. performed histopathological studies. J.-P.L. analyzed data and provided discussions and suggestions throughout the study; G.C. conceived and supervised the study, was involved in the design and evaluation of all of the experiments, and wrote the manuscript along with comments from co-authors.

Declaration of interests

The authors declare no competing interests.

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DECLARATION OF INTERESTS

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Figure legends

Figure 1. LAP blockade in monocytes is a hallmark of immunoparalysis in patients with sepsis. (A) Outline of the study protocol. (B) Monocytes obtained from healthy controls or patients upon sepsis recovery (day 7) were stimulated for 30 min with conidia of *A. fumigatus* melanin-deficient $\Delta pksP$ strain and analyzed for LC3+ phagosome (LAPosome) formation. One-way ANOVA, and Tukey's multiple comparisons. (C and D) Comparative analysis of (C) p47phox and (D) CD63 recruitment on the phagosome of monocytes stimulated as in (B). One-way ANOVA, Tukey's multiple comparisons. (E) Correlation between LAPosome formation and clearance of *Aspergillus* conidia (ATCC 46645, wild-type strain) by human monocytes obtained from sepsis patients (day 7; n = 9) and healthy controls (n = 5). Killing was assessed at 24 h of infection by CFU counting. Pearson correlation coefficient, two tailed. (F) IL-6 production in culture supernatants of monocytes following overnight stimulation with conidia of melanin-deficient *Aspergillus* ($\Delta pksP$ strain). Student's t test. (G) Differences in Apache II score of patients with intact LAP versus defective LAP responses. Unpaired Student's t test. (H) Kaplan Mayer curves on the cumulative probability of secondary infections over time (n = 43 patients; n = 54 infectious episodes). Log-rank test. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 2. ERK signaling on the phagosome regulates NADPH oxidase-mediated activation of LAP. (A) Monocytes obtained from healthy individuals were left untreated or stimulated with PMA or conidia of *Aspergillus* $\Delta pksP$ strain (MOI 10:1) and phosphorylation of p47phox was determined in cell lysates by immunoblot analysis. (B) Human monocytes stimulated with *Aspergillus* as in (A) with or without the presence of the MEK1/2 inhibitor UO126 and phosphorylation of ERK1/2 and p47phox (Ser345) was determined in cell lysates by immunoblot analysis. (C) Kinetics of ERK recruitment and representative fluorescent image of ERK localization to the phagosome in monocytes at 30 min of infection with *Aspergillus* $\Delta pksP$ strain. Scale bar, 5 μ m. (D and E) Data on quantification of p47phox⁺ (D) and p22phox⁺ (E) phagosomes in human monocytes stimulated with *Aspergillus* with or without UO126 as in (B). Unpaired Student's t test. (F) Human monocytes were left untreated or stimulated with *Aspergillus* for 1 h with or without UO126, and intracellular ROS production was determined. (G) Data on quantification of LC3⁺ phagosomes in human monocytes stimulated as in (D). Unpaired Student's t test. (H) Killing of *Aspergillus* $\Delta pksP$ conidia by human monocytes infected as in d for 24 h and assessed by CFU counts. Unpaired Student's t test. (I–K) (I) Data on quantification of LC3⁺ (I), p47phox⁺ (J), and ERK⁺ (K) phagosomes in monocytes obtained from a healthy individual (HC) and two patients following recovery from septic shock (day 7) stimulated as in (D). Unpaired Student's t test. Representative fluorescent images of ERK localization in phagosomes are shown. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 3. Autocrine IL-6 signaling regulates ERK trafficking to the phagosome for LAP-mediated fungal killing. (A–F) Data on quantification of (A) p22phox⁺, (B) p47phox⁺, (C) LC3⁺, (D) VATPase⁺, (E) cathepsin-D⁺, and (F) FITC-Dextran⁺ phagosomes in BMDMs from

IL-6^{-/-} (KO) or control IL-6^{+/+} mice infected with *Aspergillus ΔpksP* strain for 15 min (A–C) or 1 h (D–F). Unpaired Student's t test. (G) BMDMs from IL-6^{-/-} (KO) or control IL-6^{+/+} mice were infected with conidia of *Aspergillus* wild-type strain (ATCC 46645) for 6 h and killing was assessed by counting the viability of intracellular conidia. Unpaired Student's t test. (H) Fungal loads in lung homogenates from IL-6^{-/-} (KO) (n = 9) or control IL-6^{+/+} (n = 9) mice infected via intratracheal administration of wild-type *Aspergillus fumigatus* (ATCC 46645) assessed by CFU counts at 48 h of infection. Mann-Whitney test. (I) BMDMs from IL-6^{-/-} (KO) or control IL-6^{+/+} mice were left untreated or stimulated with *Aspergillus* for 15 min and phosphorylation of ERK1/2 was determined in cell lysates by immunoblot analysis. (J) Quantification of relative density of p-ERK1/2 to total ERK1/2 in blots from four different experiment performed as in (I). Mann-Whitney test. (K) Representative fluorescent images of ERK phosphorylation (p-ERK) and ERK localization on the phagosome of BMDMs stimulated as in (J). Scale bar, 5 μm. (L) Data on quantification of ERK⁺ phagosomes in BMDMs of IL-6^{-/-} (KO), IL-6^{+/+} (control), or IL-6 (KO) mice following IL-6 supplementation in culture media upon infection with *Aspergillus* (30 min). One-way ANOVA, Tukey's multiple comparisons. (M) BMDMs from IL-6^{-/-} (KO) or control IL-6^{+/+} mice were infected with conidia of *Aspergillus ΔpksP* strain or wild-type strain (ATCC 46645) with or without supplementation of culture media with IL-6, TNF, or IFN-γ, and killing was assessed by counting the viability of intracellular conidia at the indicated time point. One-way ANOVA, Tukey's multiple comparisons. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 4. IL-6/JAK2/ninein autocrine axis regulates microtubule-dependent ERK trafficking and LAPosome formation. (A) Data on quantification of ERK⁺ phagosomes in BMDMs obtained from C57BL/6 (B6) mice left untreated or treated with nocodazole (5 μM) or taxol (5 μM). One-way ANOVA, and Tukey's multiple comparisons. (B) Representative fluorescent images of β-tubulin in uninfected BMDMs from IL-6^{-/-} (KO), IL-6^{+/+} (control), or IL-6 KO mice following IL-6 supplementation in culture media. Scale bar, 5 μm. (C) Representative fluorescent images of β-tubulin in BMDMs from IL-6^{-/-} (KO), IL-6^{+/+} (control), or IL-6 KO mice following IL-6 supplementation, upon infection with *Aspergillus ΔpksP* strain (MOI 3:1, 30 min). Scale bar, 5 μm. (D) Representative time-lapse images of *Aspergillus* phagosome transport in BMDMs of IL-6^{+/+} (control) or IL-6^{-/-} (KO) mice. Scale bar, 5 μm. (E) Data on quantification of the transport of *Aspergillus*-containing phagosomes from the minus end of microtubules to the organizing center of BMDMs analyzed from the experiment performed in (C). One-way ANOVA and Tukey's multiple comparisons. (F–I) Data on quantification of (F) *Aspergillus*-containing phagosomes transport, (G) ERK⁺ phagosomes, (H) LAPosome formation, and (I) fungal killing, in BMDMs obtained from B6 mice left untreated, or treated with JAK2 inhibitor (ruxolitinib) and infected with *Aspergillus ΔpksP* strain. One-way ANOVA and Tukey's multiple comparisons. (J) Data on quantification of γ-tubulin relative fluorescent intensity at the microtubule organizing center of uninfected BMDMs from IL-6^{-/-} (KO), IL-6^{+/+} (control), or IL-6 KO mice following IL-6 supplementation in culture media for 15 min. (K) Data on quantification of ninein relative fluorescent intensity at the microtubule organizing center of uninfected BMDMs from IL-6^{-/-} (KO), IL-6^{+/+} (control), or IL-6 (KO) mice following IL-6 supplementation. One-way ANOVA and Tukey's multiple comparisons. (L) Representative fluorescent images of γ-tubulin from experiment performed in (J). Scale bar, 5 μm. (M) Representative fluorescent images of immunostaining for ninein and JAK2 in uninfected BMDMs from IL-6^{-/-} (KO), IL-6^{+/+} (control), or IL-6 (KO) mice following IL-6 supplementation. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 5. Increased susceptibility of mice recovering from sepsis to fungal disease is associated with LAP blockade in monocytes/macrophages. (A) Outline of the mouse model of bacterial peritonitis (CLP). (B) Survival rates of C57BL/6 (B6) mice subjected to different degree of severity of polymicrobial sepsis (CLP) or SHAM treated (control) mice. (C) Fungal loads of C57BL/6 (B6) mice infected via intratracheal administration of *Aspergillus* (ATCC46645 strain) following the recovery from mild, severe sepsis or SHAM treatment (day 7), assessed by CFU counts at 48 h. Mann-Whitney test. (D) Histopathology (GMS staining) from representative tissue sections of lungs from B6 mice on day 3 of infection with *Aspergillus* wild-type strain as in (C) following recovery from severe sepsis (hereafter sepsis) or SHAM treatment. Magnification $\times 400$. (E) Representative immunofluorescent images of LAPosome formation in splenic monocytes from B6 and BMDMs from GFP-LC3 transgenic mice stimulated for 30 min with *Aspergillus* (melanin-deficient $\Delta pksP$ strain) following recovery from severe sepsis (sepsis) or SHAM (control). (F) Data on quantification of LC3⁺ phagosomes in monocytes, BMDMs, or alveolar macrophages (AMs) stimulated as in (E). One-way ANOVA and Tukey's multiple comparisons. (G) Intracellular ROS production in BMDMs from mice following recovery from sepsis (day 7) or control SHAM treatment. (H) Assessment of phagolysosomal fusion in BMDMs pre-loaded with FITC-dextran and infected for 4 h as in (G). (I) Representative fluorescence images are shown in (I). Scale bar, 5 μm . (J–L) Data on quantification of VATPase⁺ (J), cathepsin D⁺ (K), and LAMP-1⁺ (L) phagosomes in BMDMs from B6 mice stimulated as in (E). Unpaired Student's t test. (M) Data on killing of *Aspergillus* (ATCC 46645 strain) by BMDMs obtained from B6 mice following recovery from sepsis or SHAM treatment. Unpaired Student's t test. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 6. Restoration of IL-6 signaling defects in mice and human patients recovering from sepsis reverses LAP blockade and impaired fungal clearance. (A) Representative fluorescent images of β -tubulin in uninfected BMDMs from B6 mice following SHAM treatment or recovery from sepsis with or without IL-6 supplementation. Scale bar, 5 μm . (B) Data on quantification of the transport of *Aspergillus*-containing phagosomes ($\Delta pksP$ strain) in BMDMs obtained from experiments performed as in (A). One-way ANOVA and Tukey's multiple comparisons. (C) Representative images of ERK recruitment on the phagosome of BMDMs (30 min) obtained from experiments performed as in (B). Scale bar, 5 μm . (D and E) Data on quantification of ERK⁺ (D) and LC3⁺ (E) phagosomes in BMDMs from experiment performed in (C). One-way ANOVA and Tukey's multiple comparisons. (F) BMDMs from SHAM treated mice, or mice recovering from sepsis (day 7) were infected with the indicated *Aspergillus* strain, with or without supplementation with IL-6, TNF, or IFN- γ . Killing of intracellular conidia was assessed by counting of viable (germinating) conidia. One-way ANOVA and Tukey's multiple comparisons. (G) Representative fluorescent images of β -tubulin in monocytes from a healthy control (HC) individual, and patients recovering from sepsis at 30 min of infection with *Aspergillus* $\Delta pksP$ strain. Scale bar, 5 μm . (H) Monocytes obtained from sepsis patients and healthy controls (HC) were infected with conidia of *Aspergillus fumigatus* (ATCC 46645 strain), with or without IL-6 supplementation and killing of conidia was assessed by CFU counting at 24 h. Unpaired Student's t test. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 7. Loss of IL-6 signaling in sepsis results in increased susceptibility to bacterial and fungal pathogens, specifically via inhibiting LAP. (A) Representative immunostaining of β -tubulin in BMDMs obtained from uninfected Atg5^{+/+} (control) and Atg5^{-/-} (KO) mice. (B–D) Data on quantification of (B) FITC-Dextran⁺, (C) VATPase⁺, and (D) LAMP-1⁺ phagosomes in control and Atg5 KO BMDMs upon infection with *ΔpksP Aspergillus* strain (1 h). Unpaired Student's t test. (E) Killing of *Aspergillus* conidia by control and Atg5 KO BMDMs assessed at 6 h. Unpaired Student's t test. (F–I) (F) BMDMs obtained from control and Atg5 KO mice following recovery (day 7) from sepsis or SHAM (control) treatment were infected with *Aspergillus* with or without IL-6 supplementation and killing of conidia was assessed at 6 h one-way ANOVA and Tukey's multiple comparisons. (G) BMDMs from IL-6 KO or control mice were infected with *Pseudomonas aeruginosa* (ATCC 27853 strain). The number of phagocytosed bacteria at 1 h was assessed by CFU plating. Bacterial killing by BMDMs was assessed by CFU counts of intracellular bacteria at 12 h of infection. One-way ANOVA and Tukey's multiple comparisons. (H) Killing of *Pseudomonas aeruginosa* (ATCC 27853 strain) following infection of control and Atg5 KO BMDMs assessed as in (H). Unpaired Student's t test. (I) BMDMs obtained from control and Atg5 KO mice following recovery (day 7) from sepsis or SHAM (control) treatment were infected with *Pseudomonas aeruginosa* with or without IL-6 supplementation and bacterial killing was assessed as in (H). One-way ANOVA and Tukey's multiple comparisons post hoc test. ****p < 0.00001, ***p < 0.0001, **p < 0.001, *p < 0.01.