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

Graphical abstract



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 sepsisinduced immunoparalysis

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In brief

The mechanisms of sepsis-induced immunoparalysis are molecularly unexplored. Akoumianaki et al. discovered that loss of IL-6 signaling in monocytes/macrophages of sepsis patients and mice disrupts microtubule dynamics regulating ERK-dependent activation of LC3-associated phagocytosis (LAP) and results in impaired pathogen killing, which is reversible upon IL-6 supplementation. These findings have broad implications in pathogenesis and therapeutics of immune deactivation in sepsis.







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

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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/Ninein 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.

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-y 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; Kyrmizi 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.

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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/Ninein 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; Kyrmizi 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 communityacquired 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 ApksP mutant as model bioparticles that induce a robust activation of LAP (Akoumianaki et al., 2016; Kyrmizi 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; Kyrmizi et al., 2013, 2018; Martinez et al., 2015). Therefore, we additionally assessed killing of the isogenic wildtype 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; Kyrmizi 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

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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

Δ*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* (Δ*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 < 0.001.



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 Δ*pksP* strain. Scale bar, 5 μm.

(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.

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 cyto-kines was decreased in monocytes of patients with 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 oxidasemediated 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

⁽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 < 0.001.



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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* $\Delta pksP$ 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.

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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; Kyrmizi 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 IL6^{-/-} mice with wild-type Aspergillus conidia resulted in significant increase in fungal load in the lungs as compared with infection of control IL6^{+/+} mice (Figures 3H and S4), without evidence of invasive fungal disease in histopathology (Figure S4).

Next, we assessed activation of ERK1/2 signaling by Asperaillus 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/Ninein 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 microtubulestabilizing 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-depended 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

⁽K) Representative fluorescent images of ERK phosphorylation (p-ERK) and ERK localization on the phagosome of BMDMs stimulated as in (J). Scale bar, 5 μ m. (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* $\Delta pksP$ 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 < 0.001.



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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 μ M) or taxol (5 μ M). 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 μ m.

(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* $\Delta pksP$ strain (MOI 3:1, 30 min). Scale bar, 5 μ m.

(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.

IL-6/JAK2/Ninein signaling on microtubule cytoskeleton dynamics, which then regulates phagosome transport and ERKmediated 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 5l), (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, sepsisinduced 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*



⁽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 Δ*pksP* 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 μ m. (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 < 0.001, *p < 0.01.



CellPress

Α

CLP

Differentiate BM cells



В

· · · SHAM → Mild sepsis

Stimulate BMDMs

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.

Cell Host & Microbe

• SHAM

v

Mild sepsis

Severe sepsis

С

Article



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/LA-Posome 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; Kyrmizi 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 cvtokines 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

⁽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 ×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 Δ*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 $\mu m.$

⁽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 < 0.001, *p < 0.001.





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 (\DeltapksP strain) in BMDMs obtained from experiments performed as in (A). Oneway 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.

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/Ninein 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 phar-



macological 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 (Kyrmizi 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**

Detailed methods are provided in the online version of this paper and include the following:

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(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 < 0.001, *p < 0.001.

⁽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 μ m.



Article



Figure 7. Loss of IL-6 signaling is 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 $\Delta pksP$ 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 sevesepsis) 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.



- Measurement of cytokines
- RNA extraction
- Library preparation for RNA-seq
- RNA-seq data analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j. chom.2021.06.002.

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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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(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.



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STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	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 (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 monoclonal p47-phox (D-10) antibody	Santa Cruz Biotechnology	Cat#sc-17845; RRID: AB_627986
Mouse monoclonal p47phox antibody	BD Biosciences	Cat# 610354; RRID: AB_397744
Rabbit polyclonal p22-phox (FL-195) antibody	Santa Cruz Biotechnology	Cat#sc-20781; RRID: AB_2090309
Mouse monoclonal LAMP-1 (E-5) antibody	Santa Cruz Biotechnology	Cat# sc-17768; RRID: AB_626851
Rabbit polyclonal Rab 5B (A-20) antibody	Santa Cruz Biotechnology	Cat# sc-598; RRID: AB_2175453
Mouse monoclonal Cathepsin D (D-7)	Santa Cruz Biotechnology	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
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 (F-5) antibody	Santa Cruz Biotechnology	Cat# sc-376420, RRID:AB_11151570
PerCP anti-human HLA-DR	Biolegend	Cat# 307628 RRID: AB_893574
APC anti-human CD14	Biolegend	Cat# 325607 RRID: AB_830680
Deposited data		
Original Wester 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	PEPROTECH	216-16
2',7'-Dichlorofluorescin diacetate	Sigma-Aldrich	D6883; CAS: 4091-99-0
U0126	Calbiochem	662005: CAS: 109511-58-2

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Continued				
REAGENT or RESOURCE	SOURCE	IDENTIFIER		
Propidium Iodide	Sigma-Aldrich	P4170; CAS: 25535-16-4		
Fluorescein isothiocyanate-dextran	Sigma-Aldrich	46945; CAS: 60842-46-8		
Alexa Fluor™ 633 NHS Ester (Succinimidyl Ester)	ThermoFisher Scientific	A20005		
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 MAX Deluxe kit	BioLegend	Cat:430304		
Human IL-6 Uncoated Elisa kit	Invitrogen	REF:88-7066-22		
Experimental models: Cell lines				
NCTC clone 929 [L cell, L-929, derivative of Strain L]	ATCC	CCL-1		
Experimental models: Organisms/strains				
Aspergillus fumigatus	ATCC	ATCC 46645		
Mouse: IL6 ^{+/+} of 129×C57BL/6 genetic background	(Venihaki et al., 2001)	N/A		
Mouse: IL6 ^{-/-} 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/		
Endnote x9	Endnote Software	https://endnote.com/		
ImageJ	Image J Software	https://imagej.nih.gov/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 \geq 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: 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 lle-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*^{flox/flox} mice (RIKEN Bio-Resource 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/xy-lazine 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 5x10⁷ *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 10⁸ 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 × 10⁷/2 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 (10⁸ 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 µL were allowed to adhere to sterile polylysine coated glass coverslips (Ø 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× 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) × (number of macrophages containing engulfed cells/total number of counted macrophages) × 100.

Live imaging

BMDMs were loaded with FITC-Dextran (5 μM 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× 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 µg/ml) in the presence of DCFH-DA added to a final concentration of 10 µM 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 x 10⁵/well) or BMDMs (1 x 10⁵/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 × 10⁵ 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 μ g/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*

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-1β, 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 DNasel 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) (FDR<=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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Supplemental information

Uncoupling of IL-6 signaling

and LC3-associated phagocytosis

drives immunoparalysis during sepsis

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Fig. S1







After sepsis (Day 7)

 ∇

0

1250

1000

750

500

After sepsis (Day 7) Intact LAP Δ **Defective LAP** ∇ 2000 Δ 1500 IL-1B (pg/mL) 1000 500 0 After sepsis (Day 7)

• •

 $\Delta \pm 2$

400

300

200



-₩ 0

40

20



 ∇

⊽ ⊽⊽ ₹

v ▼

- △ Intact LAP
- ▽ Defective LAP



After sepsis (Day 7)

Fig. S1, related to Fig. 1. Studies on phagosome biogenesis and monocyte effector functions in sepsis patients with different LAP responses.

(a) Primary human CD14⁺ monocytes obtained on the day of sepsis diagnosis (Day 1) and upon sepsis recovery (Day 7) were stimulated for 30 min with FITC-labeled conidia of *A. fumigatus* melanin-deficient $\Delta pksP$ strain (MOI 3:1), fixed, stained for LC3 and analyzed by confocal microscopy. Data on differences in LAPosome formation in monocytes of 38 consecutive patients over time (Day 1 vs. Day 7) are shown. Segregation of LAP responses in two distinct groups of patients (intact LAP vs. defective LAP) on Day 7 is shown with different colors.

(b) Data on quantification of LC3⁺, p47phox⁺, and CD63⁺ phagosomes in monocytes obtained from a patient with defective LAP vs. a patient with intact LAP responses following recovery from sepsis (Day 7), stimulated as in (a) are presented as mean \pm SEM. At least > 200 phagosomes were counted for each phagosome marker per patient. ***p < 0.0001, **p < 0.001, * p < 0.01, unpaired Student's t test.

(c) Representative immunofluorescent images of LC3⁺ phagosome (LAPosome) formation, p47phox, and CD63 lysosomal protein recruitment to the phagosome of monocytes obtained from the patients analyzed in b are shown. FITC-labeled *A. fumigatus* conidia are shown in magenta; LC3, p47phox, and CD63 were stained with Alexa⁵⁵⁵ secondary antibody and are shown in green. Scale bar, 5 µm.

(d) CD14⁺ primary human monocytes obtained from healthy control (HC) individuals or sepsis patients with LAP defects were infected with conidia of wild type *Aspergillus fumigatus* (ATCC46645) or the melanin-deficient *A. fumigatus* strain $\Delta pksP$ at a MOI 1:10 upon sepsis recovery (Day 7). Killing of intracellular conidia was assessed following cell lysis at 24h by CFU counting. *p < 0.01, unpaired Student's t test.

(e) Phagocytosis rate and phagocytosis index of *Aspergillus* conidia by monocytes of HC or patients with sepsis stratified based on LAP responses upon stimulation at a MOI of 1:1 for 1h with conidia of $\Delta pksP$ melanin-deficient strain.

(f) Cytokine (TNF, IL-1β, IL-1Ra) production in culture supernatants following overnight stimulation of monocytes of patients with or without LAP defects with *Aspergillus* conidia, stimulated as in Fig. 1f. Each symbol represents mean value of each individual patient.

Fig. S2





С

HLA-DR MFI

△ Intact LAP

▽ Defective LAP



d

% CD163⁺ monocytes

△ Intact LAP

▽ Defective LAP



Fig. S2 related to Fig. 1. Flow cytometry analysis in monocytes of sepsis patients with different LAP responses.

(a) Gating strategy for analysis of human monocyte subsets in the peripheral blood of patients with sepsis.

(b) Differences in the percentage of CD14⁺CD16⁺ monocytes in a representative cohort of sepsis patients with intact or defective LAP responses. Each symbol represents mean value of the percentage of CD14⁺CD16⁺ monocytes of an individual patient.

(c) Differences in the percentage and mean fluorescence intensity (MFI) of HLA-DR and CD163 expression in CD14⁺ monocytes obtained from sepsis patients with intact or defective LAP responses. * p < 0.05, unpaired Student's t test.















Fig. S3 related to Fig. 2. Studies on mechanism of activation of ERK signaling in monocytes from healthy controls (HC) and sepsis patients.

(a) ERK⁺ phagosomes in monocytes infected with *Aspergillus* with or without pretreatment with the MEK1/2 inhibitor UO126 presented as mean \pm SEM of one out of three independent experiments performed in triplicate. **p < 0.001, *p < 0.01, unpaired Student's t test.

(b) Representative immunofluorescence images of ERK1/2 phosphorylation on *Aspergillus*-containing phagosomes of human monocytes from HC stimulated as in (b). p-ERK was stained with the use of secondary Alexa⁴⁸⁸ antibody (green). White arrows demonstrate p-ERK localization on the phagosome.

(c, d) Primary human monocytes from HC were stimulated as in (a) and either left untreated or treated with wortmannin (250 nM), a VPS34 (PI3K class III) inhibitor, added at 5 min of infection. LAPosome formation (c) and ERK recruitment (d) at *Aspergillus* phagosomes were evaluated at 30 min of infected and presented as mean \pm SEM of one out of three independent experiments performed in triplicate. ***p < 0.0001, unpaired Student's t test. Representative immunofluorescence images of ERK recruitment to the phagosome (white arrows) are shown. ERK stained with Alexa⁵⁵⁵ secondary antibody is shown in green and FITC-labeled *Aspergillus* conidia are shown in magenta. Scale bar, 5 µm.

(e) Primary human monocytes obtained from HC or patients following recovery from sepsis with evidence of LAP blockade and ERK phagosomal trafficking defects shown in **Fig. 2 (i-k)**, were left untreated or stimulated for 20 minutes with *Aspergillus*. Phosphorylation of ERK1/2, p47phox (Ser345) and total ERK1/2 were determined in cell lysates by immunoblot analysis. Calnexin was used as loading control.



Fig. S4 related to Fig. 3. Histopathological analysis of *Aspergillus* infection in IL6^{-/-} (KO) and control IL-6^{+/+} mice.

IL-6^{-/-} (KO) or control IL-6^{+/+} mice infected *via* intratracheal administration of a standardized inoculum (5 × 10⁷ conidia per mice) of wild type *Aspergillus fumigatus* (ATCC 46645). Mice were sacrificed on Day 3 of infection with *Aspergillus* and representative histopathological sections were stained with H&E or GMS stain to demonstrate fungal elements. In the lungs of IL-6^{-/-} (KO) mice there is evidence of increased numbers of *Aspergillus* dormant and swollen conidia as compared to control IL-6^{+/+} mice. Few germinating conidia are present in the lung of IL-6^{-/-} (KO) mice (shown in inset). Magnification X 400



IL-6+/+

Lysotracker Red



С

Figure S5 related to Fig. 3 and Fig. 4. Analysis of phagocytic capacity in IL6^{-/-} (KO) and control IL-6^{+/+} BMDMs.

(a) BMDMs from IL-6^{-/-} (KO) or control (IL-6^{+/+}) mice were infected for 1h with wild type *Aspergillus fumigatus* (ATCC 46645) as in Fig. 3c. Phagocytosis rate and index was assessed by confocal microscopy.

(b) Phagocytosis of *Pseudomonas aeruginosa* (ATCC 27853 strain) following infection of control (IL-6^{+/+}) and IL-6 KO (IL-6^{-/-}) BMDMs as in Fig. 7h was assessed at 1h. **(c)** BMDMs from IL-6^{-/-} (KO) or control (IL-6^{+/+}) mice were stimulated with 3 μ m size streptavidin beads coated with biotin (biotin beads) for 1h, stained with Lysotracker Red (to discriminate intracellular particles) and phagocytosis rate was assessed by confocal microscopy. Representative images are shown. Scale bar, 5 μ m



b



Fig. S6 related to Fig. 5. Histopathological analysis of *Aspergillus* pneumonia in mice recovering from sepsis.

C57BL/6 (B6) mice subjected to severe polymicrobial sepsis (sepsis) or SHAM treated (control) mice were infected via intratracheal administration of a standardized inoculum (5×10^7 conidia per mice) of *Aspergillus fumigatus* wild type clinical isolate (ATCC46645) on Day 7 of recovery from sepsis or SHAM treatment. Mice were sacrificed on Day 3 of infection with *Aspergillus* and representative histopathological sections were stained with **(a)** H&E or **(b)** Grocott-Gomori's Methenamine Silver (GMS) stain to demonstrate fungal elements.

(a) In sepsis mice there is evidence of extensive inflammatory infiltrates by neutrophils and mononuclear phagocytes, consistent with consolidative pneumonia. Localized inflammatory infiltrates are present in SHAM treated mice infected with *Aspergillus*.

(b) Representative lungs sections from sepsis mice with evidence of *Aspergillus* germinating conidia in shown in red arrows. H&E, original magnification X 100. GMS, original magnification X 400

a Murine alveolar macrophages







С



- d SHAM
 - △ Severe sepsis

е

- SHAM
- △ Severe sepsis

P = NS



Fig. S7 related to Fig. 5 and Fig. 6. Studies on phagosome maturation and IL-6 production in control and sepsis mice.

(a) GFP-LC3 mice were SHAM-treated or subjected to severe or mild bacterial peritonitis and upon recovery (Day 7) infected intratracheally with 5×10^7 conidia of melanin-deficient *Aspergillus* $\Delta pksP$ strain. Alveolar macrophages (AMs) were isolated from bronchoalveolar lavage at 4h of infected, fixed and analyzed by confocal imaging. Representative fluorescent images of GFP-LC3⁺ phagosomes from SHAM-treated mice or mice recovering from severe sepsis are shown. LAPosome formation is shown with white arrows. Scale bar 5 µm.

(b) Data on quantification of LC3⁺ phagosomes in monocytes, BMDMs or alveolar macrophages (AMs) stimulated as in (a) and analyzed by confocal microscopy, are presented as mean \pm SEM of three independent experiments. ***p < 0.0001, **p < 0.001, one-way ANOVA and Tukey's multiple comparisons post hoc test.

(c) Representative fluorescent images from BMDMs of control SHAM treated mice or mice recovering from severe sepsis (Day 7) following infection with florescent-labeled *Aspergillus* conidia (shown in magenta), fixation and immunostaining for LAMP1, VATPase, and Cathepsin-D stained with Alexa⁵⁵⁵ secondary antibody (shown in green). Selective localization of each phagosome maturation marker in BMDMs of SHAM treated as compared to BMDMs of severe sepsis mice is evident as a rim of fluorescence surrounding *Aspergillus*-containing phagosomes (white arrows).

(d) IL-6 serum levels in mice following recovery from SHAM treatment or severe sepsis. (e) IL-6 production in culture supernatants of BMDMs obtained on Day 7 following recovery from SHAM treatment or severe sepsis, left untreated or following overnight left stimulated with melanin-deficient *Aspergillus* $\Delta pksP$ strain at a MOI of 10:1. *p < 0.01, unpaired Student's t test

Table S1 related to Fig. 1. Analysis of secondary infections in patients admitted in the Intensive Care Unit (ICU) with community acquired septic shock

Patient #	% LAPosomes (D7)	LAP status	Secondary Infection	Type of Infection	Type of Pathogen	Day of infection	ICU Center
1	55	Intact LAP	Yes	VAP	Pseudomonas aeruginosa	9	University of Crete
4	53	Intact LAP	Yes	VAP	Klebsiella pneumoniae	7	University of Crete
5	48	Intact LAP	No		I I I I I I I I I I I I I I I I I I I	0	University of Crete
6	53	Intact LAP	No			0	University of Crete
7	52	Intact LAP	No			0	University of Crete
8	51	Intact LAP	No			0	University of Crete
14	53	Intact LAP	Yes	VAP	Serratia marcescens	10	University of Crete
16	47	Intact LAP	No			0	University of Crete
23	49	Intact LAP	No			0	University of Crete
24	53	Intact LAP	No			0	University of Crete
25	46	Intact LAP	No			0	University of Crete
26	49	Intact LAP	Yes	CVC-related bacteremia	Acinetobacter baumannii	12	University of Crete
29	52	Intact LAP	No			0	University of Crete
30	51	Intact LAP	No			0	University of Crete
31	46	Intact LAP	No			0	University of Crete
2	36	Defective LAP	Yes	CVC-related bacteremia	Acinetobacter baumannii	8	University of Crete
3	32	Defective LAP	Yes		Acinetobacter baumannii	10	University of Crete
9	33	Defective LAP	No			0	University of Crete
11	39	Defective LAP	Yes	VAP	Stenotrophomonas maltophilia	6	University of Crete
11	39	Defective LAP	Yes	Bacteremia	Enterococcus spp.	8	University of Crete
12	38	Defective LAP	Yes	VAP-bacteremia	Pseudomonas aeruginosa	29	University of Crete
12	38	Defective LAP	Yes	Bacteremia	Klebsiella pneumoniae	42	University of Crete
13	38	Defective LAP	No			0	University of Crete
18	33	Defective LAP	No			0	University of Crete
22	38	Defective LAP	Yes	CVC-related candidemia	Candida tropicalis	19	University of Crete
27	34	Defective LAP	No			0	University of Crete
28	31	Defective LAP	Yes	VAP- bacteremia	Acinetobacter baumannii	14	University of Crete
32	42	Defective LAP	No			0	University of Crete
36	39	Defective LAP	No			0	Cochim Hospital, Paris
10	21	Defective LAP	No			0	University of Crete
15	23	Defective LAP	Yes	CVC-related bacteremia	Pseudomonas aeruginosa	3	University of Crete
17	14	Defective LAP	Yes	CVC-related bacteremia	Staphylococcus epidermidis	10	University of Crete
19	16	Defective LAP	Yes	New sepsis episode (UTI)	Escherichia coli	21	University of Crete
20	20	Defective LAP	Yes	Sepsis, CVC-related bacteremia	Escherichia coli	16	University of Crete
20	20	Defective LAP	Yes	CVC-related bacteremia	CoNS	25	University of Crete
21	8	Defective LAP	Yes	CVC-related bacteremia	Acinetobacter baumannii	18	University of Crete
21	8	Defective LAP	Yes	CVC-related candidemia	Candida tropicalis	35	University of Crete
21	8	Defective LAP	Yes	Bacteremia	Klebsiella pneumoniae	85	University of Crete
33	18	Defective LAP	Yes	VAP	Klebsiella pneumoniae	18	University of Crete
34	24	Defective LAP	No	N/A		0	University of Crete
35	18	Defective LAP	Yes	CVC-related candidemia	Candida spp.	16	University of Crete
37	28	Defective LAP	No	N/A		0	Cochim Hospital, Paris
38	16	Defective LAP	No	N/A		0	Cochim Hospital, Paris
39	42	Defective LAP	Yes	VAP	Acinetobacter baumannii	13	University of Crete
39	42	Defective LAP	Yes	CVC-related candidemia	Candida parapsilosis	44	University of Crete
39	42	Defective LAP	Yes	CVC-related bacteremia	Acinetobacter baumannii	45	University of Crete
39	42	Defective LAP	Yes	VAP	Pseudomonas aeruginosa	53	University of Crete
40	31	Defective LAP	Yes	Bacteremia	Pseudomonas aeruginosa	7	University of Crete
41	26	Defective LAP	Yes	VAP-bacteremia	Acinetobacter baumannii	38	University of Crete
42	22	Defective LAP	Yes	VAP	Acinetobacter baumannii	7	University of Crete
42	22	Defective LAP	Yes	Bacteremia	Klebsiella pneumoniae	11	University of Crete
42	22	Defective LAP	Yes	New sepsis episode	olymicrobial intraabdominal sepsi	s 13	University of Crete
43	36	Defective LAP	No			0	University of Crete

Table S2 related to Fig. 1. Analysis of cytokine responses in monocytes of sepsis patients with different degree of LAP responses following stimulation with *Aspergillus*

	Pt # V LC3+ phagosomes (<u>IL-1b (D1)</u>	• IL-1b (D7)	<u>IL-1Ra (D1)</u>	IL-1Ra (D7)	TNF (D1)	TNF (D7)	<u>IL-6 (D1)</u>	<u>IL-6 (D7)</u>
			N1 / A	N / A		N / A	N1 / A		N1 / A
ŀ	1 55	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	4 53	628	608	12867	33996	150	196	8805	9017
	5 48	6 3	167	1112/	27565	122	306	313 475	6 96
	b 53	230	202	50943	34027	433	625	1/5	409
ŀ	/ 52	2107	610	58893	/1051	410	433	8805	2554
2	8 51	<u>1</u> β4	421	14772	27110	11 6	387	751	1406
-	14 53	B 9	481	10400	9764	<mark>7</mark> 8	792	<mark>6</mark> 27	3817
	16 47	N/A	1705	N/A	40311	N/A	819	N/A	4000
-	23 49	1022	665	18037	<u>97</u> 55	1150	821	10708	2265
-	24 53	246	<u>21</u> 8	10060	10653	328	429	1331	<u>12</u> 75
-	25 46	154	553	8888	22520	532	257	1379	5716
-	26 49	293	42	8968	3637	1053	228	2333	208
1	29 52	18	87	6989	780	<mark>8</mark> 6	<mark>171</mark>	251	9
:	30 51	<mark>1</mark> 29	<u>17</u> 0	12865	16643	202	<u>13</u> 3	166	<mark>4</mark> 56
:	31 46	308	329	19896	20314	305	444	<mark>89</mark> 0	<mark>4</mark> 86
1	2 36	386	277	30890	36056	148	<u>12</u> 1	6873	3252
:	3 32	160	<mark>3</mark> 9	28352	8061	<mark>12</mark> 9	89	<mark>7</mark> 10	31
9	9 33	144	175	40561	25577	550	124	372	<mark>3</mark> 65
:	11 39	133	76	19060	14355	407	245	<mark>8</mark> 55	99
:	12 38	39	168	12857	39417	78	194	221	12 <mark>45</mark>
	13 38	N/A	592	N/A	21152	N/A	565	N/A	4000
	18 33	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	22 38	761	285	25869	27836	1023	397	12000	2713
	27 34	170	750	6805	10060	381	902	2295	2794
1	28 31	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1	32 42	704	107	6897	7529	672	133	1105	228
1	36 39	81	87	N/A	N/A	153	87	119	37
-	10 21	50	122	26976	92412	223	851	301	765
-	15 23	N/A	89	N/A	14433	N/A	78	N/A	195
	17 14	N/A	N/A	, N/A	N/A	, N/A	N/A	N/A	N/A
	19 16	N/A	, N/A	, N/A	, N/A	, N/A	, N/A	N/A	, N/A
	20 20	N/A	195	N/A	22456	N/A	136	N/A	<mark>6</mark> 33
ŀ	21 8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	33 18	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	34 24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	35 18	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	27 28	15	16	N/A	N/A	68	12	20	16
	28 16	77	03		N/A	182	101	187	03
-14	JU 10	I /				LUJ		101	

Table S3 related to Fig. 1. Analysis of demographic, clinical and microbiological characteristics of patients with septic shock

Pt #	% LAPosomes (D7)	SEX	AGE	SEPSIS SOURCE	Identified Pathogen	APACHE II score	SOFA D1	Sofa D7	days in ICU	Mortality in ICU
1	55	F	71	Pneumonia	Proteus spp.	24	14	9	42	YES
4	53	F	65	Intra-abdominal		19	6	3	48	YES
5	48	Μ	84	Intra-abdominal		23	10	6	23	NO
6	53	М	70	Intra-abdominal		21	5	4	9	NO
7	52	F	49	Intra-abdominal		8	3	2	5	NO
8	51	F	47	Intra-abdominal		21	9	5	21	NO
14	53	М	85	Intra-abdominal		18	8	15	16	YES
16	47	F	43	Urinary tract infection		26	15	3	9	NO
23	49	Μ	68	Intra-abdominal		21	16	15	12	YES
24	53	F	58	Intra-abdominal		23	11	1	7	NO
25	46	М	84	Intra-abdominal		19	9	8	14	YES
26	49	М	74	Intra-abdominal		14	8	13	13	YES
29	52	Μ	79	Intra-abdominal		23	8	10	21	NO
30	51	М	79	Pneumonia	Klebsiella pneumoniae	21	10	4	9	NO
31	46	Μ	26	Urinary tract infection	Providencia spp.	20	13	5	8	NO
2	36	Μ	46	Intraabdominal		18	7	7	21	NO
3	32	М	68	Intraabdominal		40	7	7	14	YES
9	33	Μ	45	Intraabdominal		16	8	2	9	NO
11	39	М	71	Pneumonia		31	10	9	21	NO
12	38	F	58	Pneumonia		18	8	11	48	YES
13	38	F	50	Intra-abdominal		13	7	7	11	NO
18	33	М	78	Pneumonia-empyema	Serratia marcescens	12	5	4	44	NO
22	38	Μ	74	Pneumonia-bacteremia	Klebsiella pneumoniae	23	10	12	17	YES
27	34	Μ	37	Intra-abdominal		21	12	10	17	NO
28	31	М	66	Pneumonia-bacteremia	Streptococcus pneumoniae	35	12	13	22	NO
32	42	М	63	Intra-abdominal		16	9	4	4	NO
36	39	М	71	Intra-abdominal-bacteremia	Pseudomonas aeruginosa, Enterococccus faecalis	37	15	N/A	26	NO
10	21	М	66	Pneumonia	Escherichia coli	31	13	6	14	NO
15	23	F	42	Streptococcal Toxic Shock Syndrome	Streptococcus pyogenes	22	12	9	12	NO
17	14	М	72	Intra-abdominal		31	6	1	5	NO
19	16	М	69	Urinary tract infection-meningitis	Escherichia coli	30	14	7	20	NO
20	20	F	87	Intra-abdominal		21	11	9	16	YES
21	8	М	54	Intra-abdominal		27	9	11	104	YES
33	18	F	49	Intra-abdominal	Escherichia coli	13	12	8	23	NO
34	24	Μ	57	Intra-abdominal		17	9	4	10	NO
35	18	М	56	Skin and Soft Tissue Infection (Fasciitis)		17	10	5	11	NO
37	28	F	65	Pneumonia		39	14	N/A	12	NO
38	16	F	59	Pneumonia		29	7	N/A	16	NO
39	42	М	79	Pneumonia		23	7	8	85	YES
40	31	М	79	Pneumonia		24	10	10	10	YES
41	26	М	76	Pneumonia	Pseudomonas aeruginosa	40	17	21	7	YES
42	22	М	57	Intra-abdominal		14	3	8	53	YES
43	36	F	42	Urinary tract infection	Proteus spp.	19	15	15	17	NO

Data S1





Data S1 related to Fig. 1. RNAseq analysis of selected genes in human sepsis monocytes.

Transcriptomics analysis of genes regulating (a) general autophagy, (a, b) LAP, and (c) Cytokine signaling in unstimulated monocytes of sepsis patients with or without defective LAP responses following sepsis recovery (Day 7). Data on expression of individual genes are expressed with whisker boxes. Distributions of the RNAseq datasets using Box plot representations. "Box plots representing expression level distributions (log10 of normalized counts) for all genes in the different groups of patients.





Data S2 related to Fig. 4 and Fig. 6. Studies on activation of ERK1/2 signaling in BMDMs of mice recovering from sepsis.

(a) Data on kinetics of ERK recruitment in the phagosome of BMDMs obtained from C57BL/6 (B6) mice at different time point of infection with *Aspergillus* conidia (MOI 3:1), presented as as mean ± SEM of three independent experiments.

(b) BMDMs from C57BL/6 (B6) mice left untreated, or treated with STAT3 inhibitor (S3I-201) and infected as in Fig. 4f. Data on quantification of ERK⁺ phagosomes are presented as mean ± SEM of one out of three independent experiments.

(c) BMDMs from SHAM treated mice or mice recovering from severe sepsis (CLP1, 2) were left untreated or infected for 20 min with *Aspergillus* and phosphorylation of ERK1/2 was determined in cell lysates by immunoblot analysis. ERK1/2 was used as loading control.

Movie S1 related to Fig. 4. Time-lapse video of *Aspergillus* phagosome transport in BMDMs of IL-6^{+/+} (control) mice. Overlaid in cyan is the trajectory of a representative phagosome from the beginning of the time series to the last shown frame.

Movie S2 related to Fig. 4. Time-lapse video of *Aspergillus* phagosome transport in BMDMs of IL-6^{-/-} (KO) mice. Overlaid in green is the trajectory of a representative phagosome from the beginning of the time series to the last shown frame.