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The helminth product ES-62 protects against septic shock via Toll-like receptor 4–dependent autophagosomal degradation of the adaptor MyD88

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Sepsis is one of the most challenging health problems worldwide. Here we found that phagocytes from patients with sepsis had considerable upregulation of Toll-like receptor 4 (TLR4) and TLR2; however, shock-inducing inflammatory responses mediated by these TLRs were inhibited by ES-62, an immunomodulator secreted by the filarial nematode *Acanthocheilonema viteae*. ES-62 subverted TLR4 signaling to block TLR2- and TLR4-driven inflammatory responses via autophagosome-mediated downregulation of the TLR adaptor-transducer MyD88. *In vivo*, ES-62 protected mice against endotoxic and polymicrobial septic shock by TLR4-mediated induction of autophagy and was protective even when administered after the induction of sepsis. Given that the treatments for septic shock at present are inadequate, the autophagy-dependent mechanism of action by ES-62 might form the basis for urgently needed therapeutic intervention against this life-threatening condition.

The incidence of sepsis increased considerably over the past few decades; this was most probably due to a combination of aging in the population, an increase in the number of invasive clinical procedures and the use of immunosuppressants[1–3]. Indeed, sepsis is one of the most common causes of admission to intensive care units worldwide[1–3]. In the USA alone, around three-quarters of a million cases of sepsis occur every year, and despite all efforts to the contrary, the fatality rate for sepsis remains between 30% and 50% (ref. 4). The initiation of sepsis is commonly triggered by bacterial infection. Host responses to bacterial infection are mediated mainly by the innate immune system, in particular by neutrophils and monocytes-macrophages[5,6], which express pattern-recognition receptors that bind the conserved molecular structures called 'pathogen-associated molecular patterns' that are shared by microorganisms[6–8]. Stimulation of pattern-recognition receptor signaling pathways by pathogen-associated molecular patterns such as lipopolysaccharide (LPS) initiates the secretion of inflammatory cytokines and other proinflammatory mediators that promote both the elimination of infectious agents and the induction of tissue repair[7–10]. However, excessive production of inflammatory mediators can lead to tissue damage and septic shock[11–13]. Thus, aberrant hyperproduction of particular proinflammatory mediators, such as tumor necrosis factor (TNF), interleukin 1β (IL-1β), macrophage migration–inhibitory factor (MIF) and high-mobility group box 1 (HMGB1) protein, associated with signaling mediated by pathogen-associated molecular patterns via the pattern-recognition receptors Toll-like receptor 2 (TLR2) and TLR4 (refs. 11–15), results in systemic inflammation that triggers the pathological outcomes of sepsis, including systemic vascular leakage, tissue injury, multiorgan failure and death[4,5,11,12]. ES-62, a well-characterized, homogeneous, phosphorylcholine-containing glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae* associates with TLR4 and subverts its proinflammatory functions in mouse macrophages, dendritic cells and mast cells[16,17]. This ability of ES-62 to inhibit LPS-triggered production and secretion of proinflammatory mediators prompted us to investigate its therapeutic potential in septic shock caused by bacteria.

RESULTS

ES-62 blocks endotoxin-triggered signaling

TLR4 is constitutively expressed on the surface of most cells of the immune system; we found that after incubation of macrophages derived from healthy humans with ES-62, it localized together with and bound to TLR4 (**Fig. 1a**,**b**). Such ES-62–TLR4 complexes were internalized, and surface expression of TLR4 on macrophages was substantially downregulated by 2 h after ES-62 exposure (**Fig. 1c**). Consistent with that, preincubation of macrophages with ES-62 also inhibited LPS-mediated activation of the inflammatory transcription factor NF-κB (**Fig. 1d**), as well as the release of various proinflammatory mediators, including TNF, IL-1β, IL-6, MIP-1α

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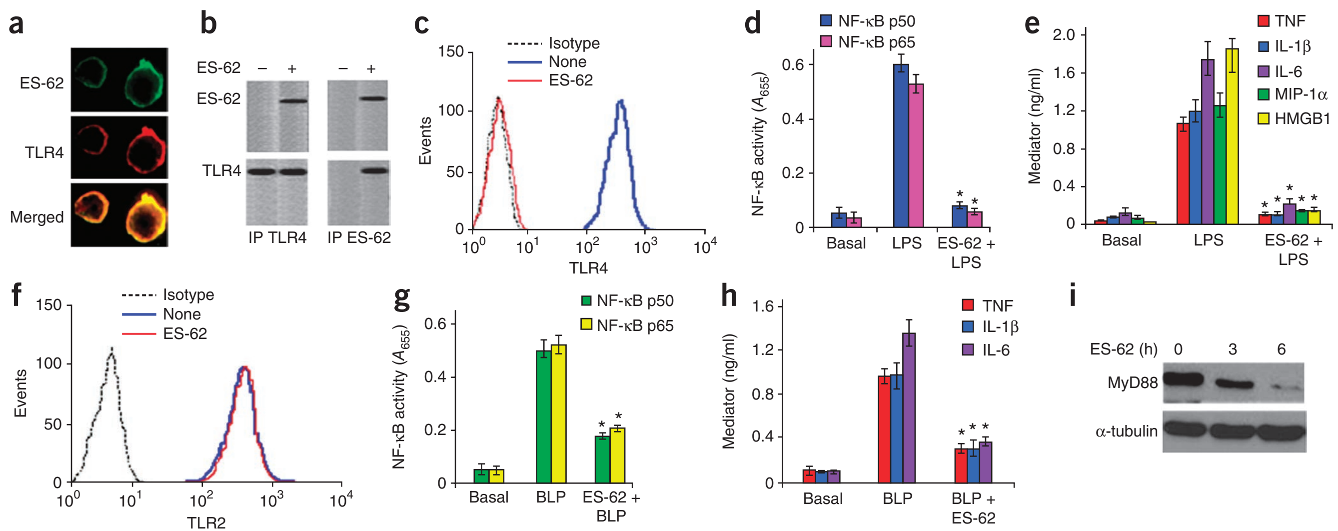

Figure 1 Effects of ES-62 on human macrophages. (**a**) Confocal microscopy of the colocalizion of ES-62 and TLR4 in primary human macrophages treated for 10 min with ES-62. Original magnification, ×40. (**b**) Immunoblot analysis of immune complexes containing TLR4 and ES-62 in primary human macrophages treated for 10 min with vehicle (–) or ES-62 (+), followed by immunoprecipitation (IP) of TLR4 (left) or ES-62 (right). (**c**) TLR4 expression in macrophages incubated for 2 h with (ES-62) or without (None) ES-62. Isotype, isotype-matched control antibody. (**d**) NF-κB activity in cells cultured with medium alone (Basal) or stimulated for 30 min with LPS after no pretreatment (LPS) or pretreatment for 2 h with ES-62 (ES-62 + LPS). (**e**) Release of proinflammatory mediators 24 h after the addition of PBS (Basal) or LPS to macrophages pretreated as in **d**. (**f**) TLR2 expression in macrophages incubated for 6 h with or without ES-62. (**g**) NF-κB activity in cells cultured with medium alone (Basal) or stimulated for 30 min with BLP after no pretreatment (BLP) or pretreatment for 6 h with ES-62 (ES-62 + BLP). (**h**) Release of cytokines 24 h after the addition of PBS (Basal) or BLP to macrophages pretreated as in **g**. (**i**) MyD88 expression in untreated control macrophages (0) and in macrophages treated for 3 h or 6 h with ES-62 (above lanes); α-tubulin serves as a loading control. $*P < 0.01$, versus LPS-induced control macrophages (Student's *t*-test). Data are from one experiment representative of at least three independent experiments (mean ± s.d. of triplicates in **d**,**e**,**g**,**h**).

and HMGB1 (**Fig. 1e**). Although exposure to ES-62 did not lead to the downregulation of cell surface expression of TLR2 at 2 h after incubation (**Fig. 1f**), cells pretreated with ES-62 had significantly less activation of NF-κB and production of proinflammatory mediators after treatment with the TLR2 agonist BLP (**Fig. 1g,h**). We therefore investigated the potential mechanisms by which ES-62 influenced not only TLR4 signals but also TLR2 signals and found that treatment with ES-62 led to downregulation of the key signal transducer MyD88 (**Fig. 1i**). This effect was selective, as ES-62 did not similarly modulate expression of the adaptor protein Mal (TIRAP; **Supplementary Fig. 1a**). Moreover, this immunomodulatory action of ES-62 reflected subversion of classical TLR4 signaling, as LPS did not downregulate either MyD88 or Mal under these conditions (**Supplementary Fig. 1a**). Confocal microscopy established not only that such differences in the regulation of MyD88 expression similarly occurred in mouse bone marrow–derived macrophages but also that although MyD88 expression was dispersed throughout the cell in resting and LPS-stimulated cells, the residual MyD88

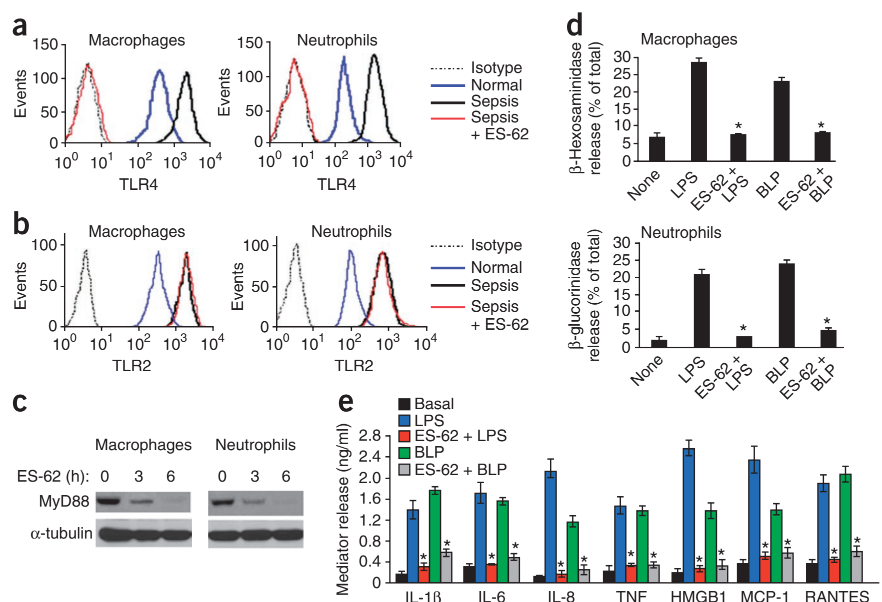

Figure 2 Effect of ES-62 on phagocytes from patients with sepsis. (**a**,**b**) Expression of TLR4 (**a**) and TLR2 (**b**) in macrophages and neutrophils from healthy volunteers (Normal) and patients with sepsis, left untreated (Sepsis) or treated for 3 h with ES-62 (Sepsis + ES-62). (**c**) MyD88 in macrophages and neutrophils obtained from patients with sepsis and left untreated (0) or pretreated for 3 h or 6 h with ES-62 (above lanes). (**d**) Degranulation of macrophages and neutrophils obtained from patients with sepsis and cultured for 60 min with PBS (None; basal) or with LPS or BLP after no pretreatment (LPS or BLP) or after pretreatment for 6 h with ES-62 (ES-62 + LPS or ES-62 + BLP), assessed as the release of β-hexosaminidase (macrophages) or β-glucorinidase (neutrophils). (**e**) Release of proinflammatory mediators from macrophages 24 h after treatment of cells as in **d**. $*P < 0.01$, ES-62 versus control (Student's *t*-test). Data are from one experiment representative of at least three independent experiments (mean and s.d. of triplicates in **d**,**e**).

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expression resulting from ES-62 treatment seemed to be restricted to a perinuclear location (**Supplementary Fig. 1b**). Quantitative laser-scanning cytometry confirmed that whereas over a period of 16 h, LPS did not downregulate MyD88 (**Supplementary Fig. 2a**–**c**), ES-62 downregulated MyD88 expression substantially in bone marrow–derived macrophages (**Supplementary Fig. 2d**,**e**).

Upregulation of TLRs in phagocytes during sepsis

We next analyzed the responses of infiltrating macrophages and neutrophils obtained from the peritoneal cavity and peripheral blood of patients with sepsis. For this study we enrolled 30 patients with severe sepsis as well as 15 healthy volunteer control subjects. The main diagnoses in the patients were systemic bacterial infection (n = 12; 40%), intra-abdominal infection (n = 12; 40%) and trauma (n = 6; 20%). Neutrophils and macrophages from both anatomical sites of the patients had higher cell surface expression of both TLR4 and TLR2 than did those from healthy people (**Fig. 2a**,**b** and data not shown). Those data were consistent with published clinical findings indicating upregulation of TLRs on neutrophils and monocytes-macrophages during the early stages of sepsis[18,19]. Nevertheless, and consistent with the effect of ES-62 on macrophages from the healthy humans described above, ES-62 also induced downregulation of the expression of TLR4 (**Fig. 2a**) and MyD88 (**Fig. 2c**) but not of TLR2 (**Fig. 2b**) in neutrophils and macrophages from patients with sepsis. Consistent with that, pretreatment of these cells with ES-62 prevented LPS- and BLP-triggered degranulation (**Fig. 2d**), as well as the secretion of various proinflammatory mediators (**Fig. 2e**). Collectively, the data reported so far (**Figs. 1** and **2**) suggested that ES-62 may block sepsis-mediated proinflammatory responses by downregulating the expression of TLR4 and MyD88, the latter being a key regulator of TLR responses.

Mechanism of ES-62-induced downregulation of TLR4 and MyD88

As the association of ES-62, but not of LPS, with TLR4 seemed to lead to downregulation of TLR4 at the cell surface and consequent degradation of MyD88, we investigated whether these TLR4 ligands induced different vesicular trafficking as a switch mechanism to trigger anti-inflammatory versus proinflammatory signaling, respectively. Whereas treatment with ES-62 induced the trafficking of TLR4 and MyD88 to early endosomes containing the early endosomal marker EEA-1 within 20 min, it resulted in their localization in late endosomes and/or lysomomes containing the lysosome marker LAMP-1 at later time points (by 1 h). In contrast, in LPS-treated cells, TLR4 and MyD88 recycled to the cell surface via the early endosomes but, notably, did not localize to the late endosomes and/or lysosomes, even up to 4 h after stimulation (**Fig. 3a**,**b** and data not shown). Consistent with that, LPS induced downregulation of TLR4 from the cell surface of macrophages at early time points (≤2 h) but not late time points (overnight), and analysis of permeabilized cells confirmed that this early surface downregulation did not reflect TLR4 degradation but instead reflected its internalization and, over time, recycling to the cell surface (**Supplementary Fig. 3**).

We therefore hypothesized that ES-62 mediated the trafficking of TLR4 and MyD88 to lysosomes for degradation by subverting the TLR4-mediated regulation of autophagy[20], which functions both to target intracellular proteins for degradation and as an effector of TLR-mediated innate and adaptive immune responses[20,21]. Thus, to address whether ES-62 promotes the sequestration and degradation of proinflammatory TLR signaling complexes in an autophagy-dependent manner, we investigated whether there were differences in the way LPS and ES-62 induced vesicles containing LC3, a marker of autophagosome formation[22]. Indeed, although LPS did not induce vesicular localization of LC3 (**Fig. 3c**), in ES-62-treated cells there was redistribution of the diffuse cytosolic expression of LC3 to a classical autophagosome-punctuate or vesicular pattern[22] and also colocalization of TLR4 and MyD88 to such LC3-containing vesicles (**Fig. 3c**).

To provide further evidence of the involvement of autophagy in ES-62-mediated degradation of MyD88, we did additional sets of experiments. First, electron microscopy over a period of 8 h showed various stages of autophagosome formation and then resolution in primary human macrophages exposed to ES-62 (**Fig. 4a**). Additionally, in primary mouse macrophages, small interfering RNA (siRNA)-mediated silencing of *Atg5* and *Atg7* (**Fig. 4b**), genes encoding molecules with critical roles in autophagosome formation, nearly completely abrogated the ES-62-induced degradation of MyD88 (**Fig. 4c**). Third, because complete and effective autophagy is a function of efficient

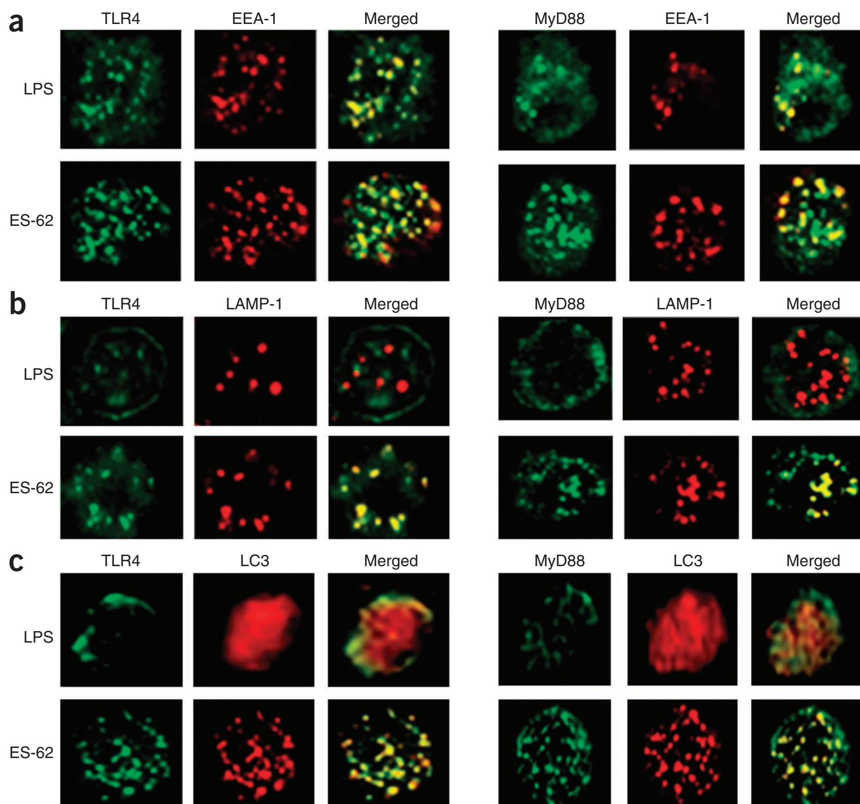

Figure 3 ES-62 targets TLR4 and MyD88 to early and late endosomes. Microscopy of the intracellular vesicle colocalization of TLR4 and MyD88 trafficking into EEA-1$^+$ early endosomes (20 min; **a**), LAMP-1$^+$ late endosomes and/or lysosomes (1 h; **b**) and LC3$^+$ autophagosomes (3 h; **c**) in human macrophages exposed to LPS or ES-62. Original magnification, ×40. Data are representative of at least three independent experiments.

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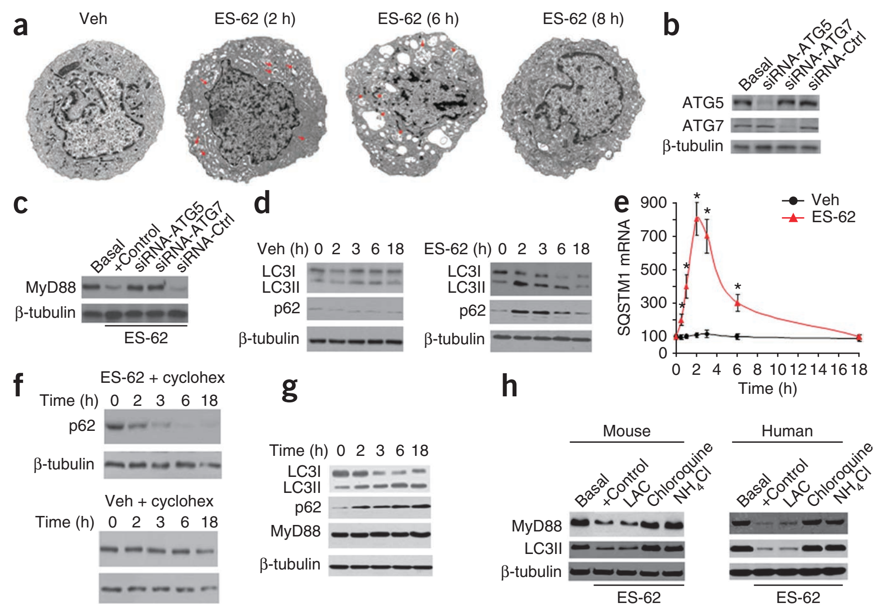

Figure 4 ES-62 induces autophagosome formation. (**a**) Electron microscopy of human macrophages treated with vehicle (Veh) or ES-62 (time, above images). Red arrows indicate double-membraned vacuole structures. (**b**) Immunoblot analysis of the expression of ATG5 and ATG7 in primary mouse macrophages left untreated (Basal) or treated with siRNA specific for ATG5 (siRNA-ATG5) or ATG7 (siRNA-ATG7) or control siRNA (siRNA-Ctrl). (**c**) Immunoblot analysis of MyD88 expression in primary mouse macrophages pretreated for 6 h with medium (Basal) or ES-62 (+Control) in the presence or absence of siRNA as in **b**. (**d**) Immunoblot analysis of the expression of LC3I, LC3II and p62 in primary mouse macrophages treated for 0–18 h (above lanes) with vehicle or ES-62. (**e**) Expression of p62 (SQSTM1) mRNA in primary mouse macrophages treated with vehicle or ES-62. (**f**) Expression of p62 protein in primary mouse macrophages treated with ES-62 or vehicle in the presence or absence (0) of cycloheximide (cyclohex). (**g**) Immunoblot analysis of the expression of LC3I, LC3II, p62 and MyD88 in cells treated with ES-62 (time, above lanes) in the presence of E64D and pepstatin A. (**h**) Immunoblot analysis of MyD88 and LC3II in human or mouse macrophages left untreated (Basal) or pretreated for 6 h with ES-62 in the presence or absence (+Control) of lactacystin (LAC), chloroquine or NH_4Cl. β-tubulin serves as a loading control (**b**–**d** and **f**–**h**). *$P < 0.01$, ES-62 versus vehicle (Student's *t*-test). Data are from one experiment representative of at least three independent experiments (mean ± s.d. of triplicates in **d**).

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autophagic flux, we investigated the effect of ES-62 on the expression of p62 (also known as the 'sequestrome' or SQSTM1), as p62 binds to LC3II, the cleaved and post-translationally modified form of LC3 that increases in expression during autophagy, and this p62-LC3II complex is degraded in cells undergoing efficient autophagy[23]. Indeed, analysis of the kinetics of p62 expression in primary mouse macrophages after exposure to ES-62 indicated early accumulation of p62 together with LC3II, followed at later time points by degradation of the two proteins (**Fig. 4d**). The early accumulation of p62 protein expression reflected rapid and transient upregulation of p62 mRNA (**Fig. 4e**), but analysis in the presence of the protein-synthesis inhibitor cycloheximide showed that this upregulation masked concomitant degradation of p62 (**Fig. 4f**), indicative of a highly dynamic and efficient autophagic flux. Finally, although inhibitors of lysosomal degradation (E64D and pepstatin A; **Fig. 4g**) and modifiers of lysosomal pH (chloroquine and NH_4Cl; **Fig. 4h**) rescued MyD88, p62 and LC3II from degradation induced by ES-62, the proteosomal inhibitor lactacystin had no effect on the biological activity of ES-62 in mouse cells (**Fig.4h**, left), which provided further evidence that ES-62-mediated MyD88 degradation involves autophagosome-mediated mechanisms. This mechanism of action by which ES-62 desensitizes TLR-MyD88 signaling was also present in primary human macrophages (**Fig. 4h**, right).

ES-62 protects mice from endotoxic shock

To determine whether ES-62 could protect organisms from pathogen product–mediated proinflammatory responses *in vivo*, we investigated if it diminished inflammation and lethal shock in mouse models of sepsis. In our first study, we induced LPS-associated endotoxemia in mice and monitored death over time. Treatment of mice with ES-62, starting 2 h before LPS administration, resulted in dose-dependent protection and, notably, 100% of the mice survived endotoxemia when given ES-62 at a dose of 2 μg per mouse, whereas none of the control mice pretreated with vehicle survived (**Fig. 5a** and **Supplementary Fig. 4a**). Analysis of tissue samples from the mice pretreated with ES-62 showed substantial suppression of LPS-induced pathology in terms of both infiltration of cells of the immune system and damage to the lungs and liver (**Fig. 5b**), as well as lower serum concentrations of TNF, IL-1β,

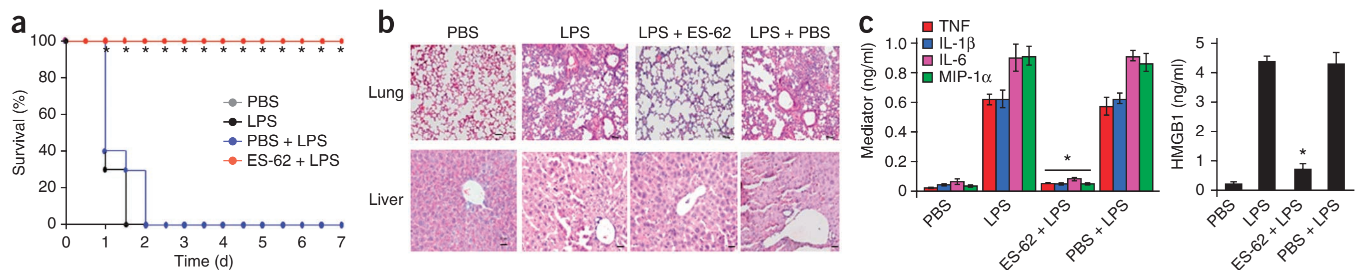

Figure 5 ES-62 protects against endotoxic shock. (**a**) Survival of PBS-injected control mice (PBS), and mice given no pretreatment (LPS) or pretreated with PBS (PBS + LPS) or ES-62 (ES-62 + LPS) 2 h before injection with a lethal dose of LPS. (**b**) Lung and liver sections from mice treated as in **a**, assessed 12 h after treatment. Scale bars, 50 μm. (**c**) TNF, IL-1β, IL-6, MIP-1α and HMGB1 in serum from mice treated as in **a** (n = 10 mice per group), assessed 12 h after treatment. *$P < 0.01$, compared with control mice given LPS (Student's *t*-test). Data are from one experiment representative of at least three independent experiments (mean and s.d. in **c**).

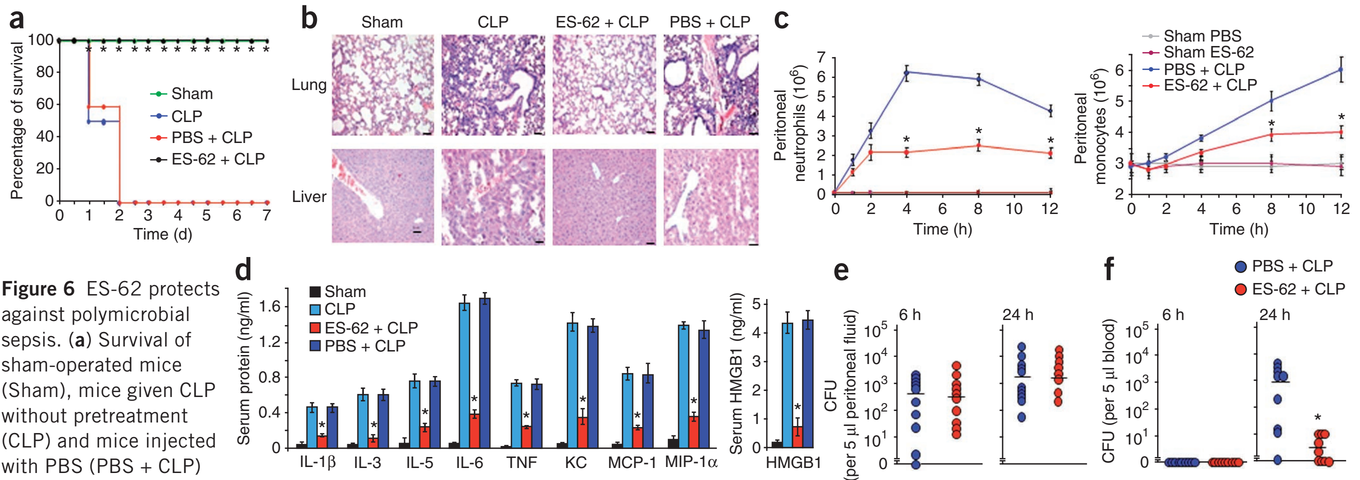

Figure 6 ES-62 protects against polymicrobial sepsis. (**a**) Survival of sham-operated mice (Sham), mice given CLP without pretreatment (CLP) and mice injected with PBS (PBS + CLP) or ES-62 (ES-62 + CLP) starting 2 h before CLP. (**b**) Lung and liver sections from mice treated as in **a**, assessed 12 h after surgery. Scale bars, 50 µm. (**c**) Infiltration of peritoneal neutrophils (left) and monocytes (right) after surgery in mice treated as in **a**. (**d**) IL-1β, IL-3, IL-5, IL-6, TNF, chemokine CXCL1 (KC), MCP-1, MIP-1α and HMGB1 in serum of mice treated as in **a**, assessed 12 h after surgery. (**e**,**f**) Bacteria in the peritoneal fluid (**e**) and peripheral blood (**f**) of mice injected with PBS or ES-62 starting 2 h before CLP, assessed 6 h or 24 h after surgery. CFU, colony-forming units. Each symbol represents an individual mouse ($n = 10$ mice per group); small horizontal bars indicate the mean. $^*P < 0.01$, compared with PBS pretreatment and CLP (Student's *t*-test). Data are from one experiment representative of at least three independent experiments (mean ± s.d. in **c**,**d**).

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IL-6, MIP-1α and HMGB1 (**Fig. 5c**). We observed the same protective effect in a BLP-induced model of shock (data not shown).

ES-62 protects mice from polymicrobial sepsis

Our data showing that ES-62 was protective against bacterial product–induced shock were consistent with the well-established role of TLR4 and TLR2 in the pathogenesis of septic shock[18,19]. However, although it is an informative model, experimental endotoxic shock reproduces human sepsis only in part. This is because it does not involve the replication and dissemination of bacteria, which results in the production of pathogenic inflammation by simultaneous triggering of multiple pattern-recognition receptors, including TLR2, TLR4 and TLR9 (refs. 18,19). Moreover, it was possible that, ultimately, the anti-inflammatory effects of ES-62 could be detrimental to the host by impairing the ability of the immune system to fight such bacterial infections, as noted for treatment with antibody to TNF[24,25]. We therefore investigated whether ES-62 protects mice from septic shock due to polymicrobial peritonitis and sepsis without compromising the host's ability to fight infection; for this we used a model of cecal ligation and puncture (CLP) that resembles the scenario of human infectious sepsis[26].

In a prophylactic multidose study similar to that of endotoxin-induced shock, ES-62 at a dose of 2 µg per mouse was sufficient to fully protect the mice from CLP-induced death (**Fig. 6a** and **Supplementary Fig. 4b**) and multiorgan inflammatory damage (**Fig. 6b**). This was accompanied by significantly fewer peritoneal neutrophils and macrophages (**Fig. 6c**), as well as by lower serum concentrations of a wide range of cytokines and chemokines and also HMGB1 (**Fig. 6d**). There was no substantial difference between untreated and ES-62-treated mice in their peritoneal bacteria counts (**Fig. 6e**). Notably, however, bacteria were significantly less abundant in the blood of ES-62-treated mice than in that of untreated mice (**Fig. 6f**). These results suggest that ES-62 diminishes inflammatory responses without compromising control of bacterial infection. Indeed, it seemed to aid control, perhaps by preventing neutropenia; notably, neutrophils are obligatory for effective killing of bacteria in the blood, and it has been suggested that an optimal concentration of neutrophils is required for the clearance of bacteria in suspension[27]. Perhaps consistent with the idea that ES-62 prevents CLP-driven neutropenia, analysis of the production of nitric oxide showed that whereas high concentrations of nitric oxide were present in the bronchoalveolar lavage fluid but not in the plasma after CLP, treatment with ES-62 resulted in an inverse profile of nitric oxide production (**Supplementary Fig. 4c**). This was in agreement with the pattern of migration of neutrophils and monocytes (**Fig. 6c**), cells that are probably the main producers of this effector under these disease and therapeutic conditions.

ES-62-triggered autophagy mediates protection *in vivo*

Our data demonstrating that ES-62 prevented TLR2- and TLR4-driven proinflammatory responses by downregulating MyD88, together with the finding that ES-62 protected mice from the lethal systemic inflammation triggered by various polymicrobial ligands, agreed with published *in vitro* studies showing that in mouse macrophages, ES-62 not only disrupts TLR4 signaling but also inhibits TLR2- and TLR9-driven, but not MyD88-independent TLR3-driven, proinflammatory responses in a TLR4-dependent manner[16]. To further prove that the mechanism by which ES-62 exerts its protective functions *in vivo* was through such subversion of TLR4 signaling, we subjected *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ mice to CLP. Published studies have shown that deletion of either *Tlr4* or *Tlr2* confers only a very small degree of protection from polymicrobial sepsis, whereas deletion of MyD88 is more protective[28]. Thus, consistent with the hypothesis that ES-62 was acting to suppress signaling from multiple TLRs by triggering degradation of MyD88 via TLR4, ES-62 was unable to provide *Tlr4*$^{-/-}$ mice protection from CLP but was able to confer on *Tlr2*$^{-/-}$ mice full protection from the lethal outcome of polymicrobial sepsis (**Fig. 7a**). To address whether such ES-62-mediated subversion of TLR4 signaling *in vivo* also reflected induction of autophagy, we exploited *in vivo* siRNA silencing[29,30] to knock down the expression of *Atg5* and *Atg7* in this model (**Fig. 7b**). After silencing of *Atg5* or *Atg7 in vivo*, ES-62 was no longer able to exert protective effects against CLP (**Fig. 7c**). Collectively, these data demonstrated that *in vivo*, ES-62 acts via TLR4 and, similar to our *in vitro* data, that it signals via an autophagosome-dependent mechanism to subvert TLR responses

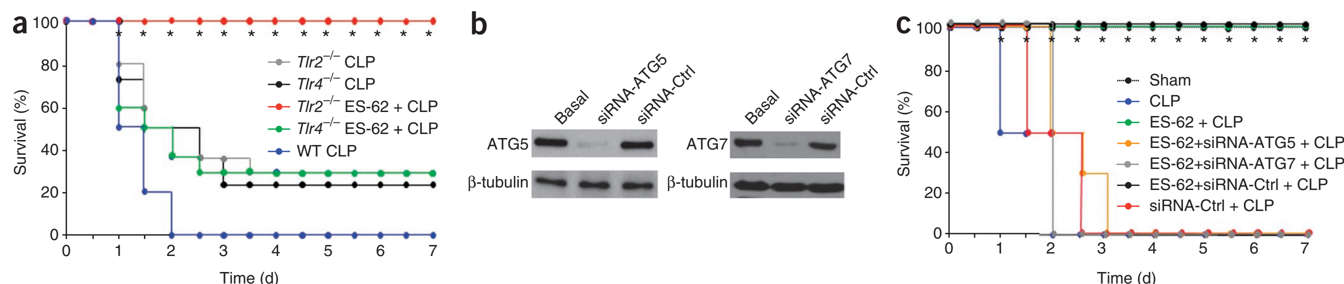

Figure 7 ES-62 protects via autophagy *in vivo*. (**a**) Survival of wild-type (WT), *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice given CLP without pretreatment (CLP) or injected with ES-62 (ES-62 + CLP) starting 2 h before CLP. (**b**) Immunoblot analysis of the expression of ATG5 (left) and ATG7 (right) in PBMCs from mice treated *in vivo* with PBS (Basal), siRNA specific for ATG5 (siRNA-ATG5) or ATG7 (siRNA-ATG7) or control siRNA (siRNA-Ctrl); β-tubulin serves as a loading control. (**c**) Survival of sham-operated mice, mice given CLP without pretreatment, mice injected with ES-62 starting 2 h before CLP, and mice pretreated with siRNA specific for ATG5 or ATG7 or control siRNA and injected with ES-62 or saline starting 2 h before CLP (key, top to bottom). *$P < 0.01$, compared with CLP without pretreatment (Student's *t*-test). Data are from a single experiment representative of three independent experiments with ten mice per group.

(**Supplementary Fig. 5**) to protect mice from the lethal inflammation that is invariably fatal in experimental sepsis.

Therapeutic potential of ES-62 in polymicrobial sepsis

We next investigated whether ES-62 was protective when administered 1, 3 or 6 h after CLP. Notably, ES-62 still conferred 100% protection from polymicrobial sepsis when given 1 h after CLP (**Fig. 8a**), and we also observed substantial protection when we first administered ES-62 at 3 h (80% survival) or even 6 h (70% survival) after CLP (**Fig. 8a**). However, protection was considerably diminished at later time points (20% survival at 8 h) and was lost completely by 12 h after CLP (data not shown). Nevertheless, it is clear that ES-62 can be highly effective even when injected after the onset of sepsis. Consistent with that, analysis of blood samples from vehicle-treated control mice and mice treated with ES-62 at various time points associated with protection after CLP showed that mice treated with ES-62 had significantly lower plasma concentrations of TNF, IL-1β, IL-6, MIP-1α and HMGB1 (**Fig. 8b,c**). Furthermore, histological examination of the lungs and liver showed that mice given CLP and treated therapeutically with ES-62 had much less inflammation and tissue damage than did vehicle-treated control mice (**Fig. 8d**). Injection of ES-62 into naive mice did not affect the number of circulating leukocytes (data not shown); thus, treatment with ES-62 was sufficient to lower systemic TNF, IL-1β, IL-6, MCP-1α and HMGB1 and to decrease cellular infiltrates at the site of inflammation below amounts lethal to the host without causing leukopenia.

Finally, in an attempt to reproduce the clinical scenario, we used a broad-spectrum antibiotic, as patients diagnosed as having septic shock are immediately treated with a course of such antibiotics at a high dose for up to 3 d. Although early use of antibiotics is essential to the treatment of sepsis, such antibiotic treatment must be applied with caution. This is especially true because the very high doses required to control the overwhelming infection in patients with septic shock can be administered for only a short time period because of the potential

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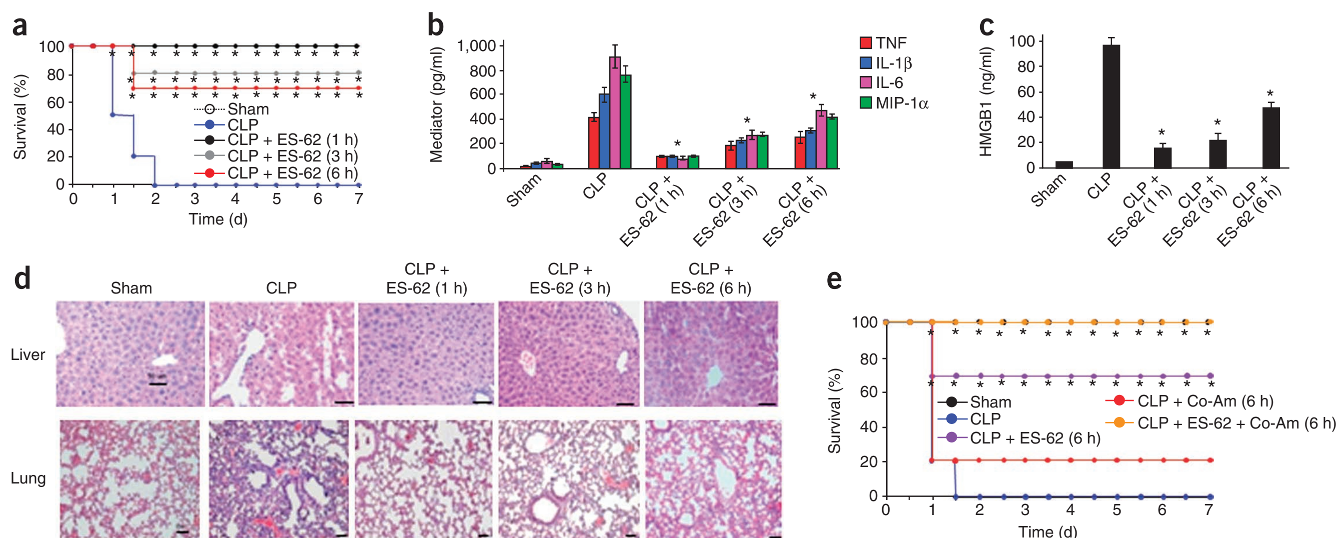

Figure 8 Therapeutic role for ES-62 in polymicrobial sepsis. (**a**) Survival of sham-operated mice, mice given CLP without additional treatment and mice injected with ES-62 starting 1 h, 3 h or 6 h after CLP (time, key). (**b**,**c**) TNF, IL-1β, IL-6 and MIP-1α (**b**) and HMGB1 (**c**) in the serum of mice treated as in **a**, assessed 12 h after surgery. (**d**) Hematoxylin and eosin staining of liver and lung sections from mice treated as in **a**, assessed 12 h after surgery. Scale bars, 50 μm. (**e**) Survival sham-operated mice, mice given CLP without additional treatment and mice injected with ES-62 or amoxicillin–clavulanic acid (Co-Am) alone or together starting 6 h after CLP ($n = 10$ mice per group). *$P < 0.01$, compared with CLP without pretreatment (Student's *t*-test). Data are from one experiment representative of three independent experiments (error bars (**b**,**c**), s.d.).

for severe hepatotoxicity. Hence, new (combination) therapies that potentially diminish this risk are desirable. Injection of a combination of amoxicillin and clavulanic acid at a dose of 20 mg per kg body weight (the dose of this antibiotic now in use clinically) 6 h after CLP provided 20% protection from death (**Fig. 8e**). However, combination therapy with ES-62 plus amoxicillin–clavulanic acid at 6 h after CLP provided 100% protection from death (**Fig. 8e**).

DISCUSSION

It is well established that TLR signaling in response to overwhelming bacterial infection leads to the hypersecretion of proinflammatory mediators associated with shock. Autophagy is a homeostatic mechanism designed mainly to promote cell survival and function and thus acts to provide protection from a range of pathologies, including degenerative disorders and cancer[20,21]. However, there is increasing evidence that autophagy can also serve various roles in the regulation of innate and adaptive immune responses, in particular in promoting the clearance of microbial infection and preventing autoimmunity[20,21]. For example, during microbial infection, TLR-mediated induction of autophagy has been indicated to be an antimicrobial mechanism. This is true in terms of not only the elimination of pathogens by innate cells but also the delivery of cytosolic pathogen-associated molecular patterns to endosomal TLRs. Moreover, autophagy can also be involved in the development of protective adaptive immune responses both through the promotion of antigen presentation and as autophagy, an effector of T helper type 1 (T_H1)-T_H2 polarization. Indeed, whereas T_H1 cytokines such as interferon-γ and TNF promote autophagy and the clearance of bacteria, this response is suppressed by T_H2 cytokines such as IL-4 and IL-13 (refs. 18,19). It was therefore unexpected that TLR signaling (either directly via TLR ligands or indirectly via culture with bacteria) has been found to be not associated with autophagosome formation in primary macrophages[31], but this has now been suggested to reflect the autophagy-suppressing effects of NF-κB activation[21]. Indeed, blockade of autophagy with the phosphatidylinositol-3-OH kinase inhibitor 3-methyladenine has been demonstrated to result in the hyperinduction of IL-1β in response to LPS, and such aberrant production of this proinflammatory cytokine has also been found in autophagy-defective macrophages deficient in ATG16L1 and ATG7 (ref. 31). Moreover, although TLR ligands have been found to substantially upregulate p62 (STSQM1) in a MyD88-dependent manner in primary human keratinocytes, this synthesis of p62 is required mainly for NF-κB activation and inflammation[32]. Thus, whereas siRNA-mediated silencing of genes encoding molecules critical for autophagy (beclin-1 and ATG5) results in higher expression of p62, NF-κB activation and release of proinflammatory mediators, silencing of p62 blocks NF-κB activation and the production of IL-6 and TNF[32]. These studies therefore suggest that autophagy may also act to limit TLR-mediated production of proinflammatory cytokines[31,32]. Our finding that ES-62-mediated suppression of the hyperinflammatory responses associated with septic shock reflected triggering of ATG5- and ATG7-dependent autophagosome formation and efficient p62-LC3–mediated autophagic flux and lysosomal degradation of TLR4 and MyD88 resonates with the latter proposal and suggests that manipulating autophagy induction could represent a potent anti-inflammatory strategy.

Collectively, our data indicate that ES-62 seems to act to suppress pathological proinflammatory TLR signaling by inducing autophagy to downregulate the expression of TLR4 and MyD88. In this way, ES-62 acts to lower serum concentrations of inflammatory mediators resulting from TLR signaling to concentrations that do not induce shock and death but remain sufficient to promote bacteria clearance. Thus, in contrast to the situation generally observed after treatment with other anti-inflammatory agents, including nitric oxide synthase inhibitors and monoclonal antibodies to TNF, which increase shock lethality due to suppression of the host's ability to fight infection[24,25], ES-62 protects against polymicrobial peritonitis and sepsis and also prevents multiorgan damage resulting from catastrophic inflammatory responses. The ability of ES-62 to enhance the host's ability to clear infection might reflect inhibition of neutropenia and, relating to this, a possible contributing factor is that whereas ES-62 dampens TLR-mediated production of proinflammatory cytokines, it leaves nitric oxide responses intact[33]. This is a notable finding, as studies have suggested that both of these responses show MyD88 dependence[34]. However, this apparent discrepancy may now be reconciled by our present findings showing that whereas ES-62 acted to downregulate MyD88, it left Mal unaffected, which perhaps suggests that TLR4-Mal interactions might be sufficient for the induction of nitric oxide, as has been described before for some cellular responses, including activation of NF-κB and mitogen-activated protein kinase and some cytokine production[35,36]. Consistent with that, the *Mycobacterium tuberculosis*–derived lipomannan tri-acyl-LM, which is an agonist of TLR2-TLR1, has been reported to promote the production of TNF, IL-12p40 and nitric oxide through MyD88 and/or Mal[37]. Also notable, given the potential bactericidal role of nitric oxide, there is evidence that the Mal variant 180L protects against septic shock[38] by producing enhanced TLR2-TLR4 responses and resistance to infection. Therefore, it is possible that infection with helminths that secrete products such as ES-62 that can downregulate MyD88 may have promoted selection of this variant.

Finally, ES-62 provided protective effects even when administered therapeutically several hours after CLP. In this context, research efforts have been directed toward finding a mechanism for limiting the excessive inflammation triggered by TLRs as a way of potentially providing new therapies to treat sepsis and other inflammatory disorders[39–41]. Thus, administration of ES-62 or, perhaps more likely, synthetic small-molecule derivatives, alone or in combination with antibiotics, after the initiation of sepsis might offer a suitable new therapeutic tool for the treatment of septic shock as well as other microbe-mediated diseases in humans, in whom out-of-control inflammation can often lead to a fatal outcome.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS
P.P., M.A.M., H.K.T., L.A.-R. and J.R. did experiments; S.M.M. supplied reagents; A.J.M. conceived of the study; A.J.M., M.M.H., S.P. and W.H. planned the experiments, supervised the study and wrote the paper; and all authors analyzed data.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Patients. Prospectively enrolled patients were admitted and treated for sepsis in the intensive care unit of the University Hospital, Faculty of Medicine of the University of Zulia-Venezuela from January 2008 to April 2009. All patients presented clinical and/or laboratory variables that fulfilled the criteria for sepsis[5,42]. The study was approved by the Human Subjects Institutional Committee of the Faculty of Medicine and University Hospital of the University of Zulia-Venezuela, and written informed consent was obtained from patients and volunteers.

Human cells. Neutrophils and macrophages were obtained from peripheral blood of healthy volunteers and from the peritoneal lavage and peripheral blood of patients diagnosed with sepsis as described[5,42].

ES-62. Highly purified, endotoxin-free ES-62 was prepared from the rodent filarial nematode *Acanthocheilonema viteae*, and fluorescein isothiocyanate–labeled ES-62 was prepared as described[17,43].

Flow cytometry. Human cells were incubated for various times with or without ES-62 (2 μg/ml). After being stained with mouse immunoglobulin G2a (IgG2a) monoclonal antibody to human TLR4 (HTA125; BD Pharmingen), followed by tetramethylrhodamine isothiocyanate–conjugated rabbit polyclonal antibody to mouse immunoglobulin (81-6714; Invitrogen), Alexa Fluor 488–conjugated mouse IgG1 monoclonal antibody to mouse and/or human TLR2 (T2.5; Santa Cruz Biotechnology) or the appropriate mouse isotype-matched control antibody, cells were analyzed by flow cytometry. For mouse cells, surface expression of TLR4 was analyzed with biotin-labeled rat IgG2a monoclonal antibody to TLR4-MD2 (MTS510; eBioscience) and biotin-labeled rat IgG2a isotype-matched control antibody, followed by streptavidin-conjugated allophycocyanin (60663; AnaSpec).

Coimmunoprecipitation and immunoblot analysis. These procedures were done as described[17]. Cell lysates were prepared from control and ES-62-treated cells for measurement of MyD88 expression, effects of siRNA-mediated gene silencing and effects of various inhibitors on ES-62 activity. Inhibitors were as follows: chloroquine (100 μM), NH_4Cl (50 μM), lactacystin (10 μM) or E64D and pepstatin A (both 10 μg/ml) were from Sigma Aldrich. Antibodies were as follows: mouse monoclonal anti-MyD88 (sc-136970; B-1; Santa Cruz Biotechnology), and rabbit polyclonal anti-ATG5 (ab78073; Abcam), anti-ATG7 (ab53255; Abcam), anti-LC3 (ab58610; Abcam) and anti-p62 (ab91526; Abcam). Blots were also probed with mouse monoclonal anti-α-tubulin (MAB5566; Upstate/Millipore) or anti-β-tubulin (MAB380; Upstate/Millipore) as a loading control. The appropriate horseradish peroxidase–conjugated secondary antibodies were used, and bands were visualized with the ECL Western Blotting Detection System (GE Healthcare).

Confocal and laser-scanning microscopy. For human colocalization studies, cells were treated with LPS or fluorescein isothiocyanate–ES-62, then were fixed and made permeable before being incubated with primary antibodies and then washed and stained with secondary antibodies. Antibodies were as follows: mouse monoclonal antibody to human TLR4 (HTA-125; BD Pharmingen) followed by tetramethylrhodamine isothiocyanate–conjugated rabbit antibody to mouse immunoglobulin (81-6714; Invitrogen); primary mouse monoclonal antibody to MyD88 (B-1; Santa Cruz Biotechnology) followed by tetramethylrhodamine isothiocyanate–conjugated secondary rabbit antibody to mouse IgG (81-6714; Invitrogen); primary rabbit polyclonal antibody to EEA-1 (sc-33585; Santa Cruz Biotechnology) or LAMP1 (sc-5570; Santa Cruz Biotechnology) followed by Alexa Fluor 594–conjugated secondary goat antibody to rabbit IgG (A-11007; Invitrogen); or primary mouse monoclonal antibody to LC3 (5F10; nanoTools Antikoerpertechnik) followed by fluorescein isothiocyanate–conjugated secondary rabbit antibody to mouse IgG (81-6711; Invitrogen). Cells were analyzed with a Zeiss LSM510 confocal microscope[17].

For mouse studies, bone marrow-derived macrophages[5] were fixed and made permeable, then were stained with a rabbit polyclonal anti-MyD88 (ab2064; Abcam) followed by fluorescein isothiocyanate–conjugated goat antibody to rabbit IgG (ab6717; Abcam). Laser Scanning Cytometry (Compucyte) was used for quantitative analysis of 800 cells from each group as described[44,45].

Gene silencing by siRNA. Designed and tested siRNA specific for mouse ATG5 (SI00230664) or ATG7 (SI00900515) and negative control siRNA (SI03650318) were from Qiagen UK. Cells were transfected with HiPerFect transfection reagent (301702; Qiagen) containing siRNA (500 ng/ml), followed by incubation for 48 h. For *in vivo* siRNA-mediated silencing, a published protocol was used[29].

Electron microscopy. Cells were fixed overnight in 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, before being post-fixed for 1 h in 1% (vol/vol) OsO4. Next, cells were dehydrated through an ascending ethanol series and were embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were imaged with a JEOL JEM-1230 transmission electron microscope.

Macrophage and neutrophil degranulation assays. Macrophages or neutrophils (5×10^6 cells per sample) were pretreated or not for 2 or 6 h with ES-62, then were stimulated by the addition of LPS or BLP for 30 or 60 min. Degranulation was assessed by measurement of β-hexosaminidase (macrophages) or β-glucorinidase (neutrophils) as described[46,47].

NF-κB activity. NF-κB was analyzed with the Mercury TransFactor Profiling Kit–Inflammation according to the manufacturer's instructions (BD). Enzymatic products were analyzed in a standard plate reader as described[17].

Cytokine production by human macrophages. Cells (2×10^6) were pretreated or not for 2 or 6 h with ES-62, then were stimulated for 24 h by the addition of LPS (100 ng/ml) or BLP (100 ng/ml). Enzyme-linked immunosorbent assays were used to measure human cytokines and chemokines in culture supernatants (R&D Systems) and HMGB1 (326054329; Shino-Test).

Serum cytokines. Enzyme-linked immunosorbent assays were used to measure cytokines and chemokines in mouse serum (R&D Systems) and HMGB1 (326054329; Shino-Test).

Measurement of nitric oxide. Products of nitric oxide in plasma and bronchoalveolar lavage fluid were measured by fluorometric assay (K252-200) according to the manufacturer's instructions (BioVision).

Mice. Male wild-type, *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ mice 8–10 weeks of age were bred at the National University of Singapore and were maintained on a C57BL/6 genetic background. Studies were done according to the guidelines of the National University of Singapore for animal experimentation and according to protocols and project licenses approved by the National University of Singapore. A standard pellet diet (Glen Forrest) and water were provided *ad libitum*. Animals were maintained under constant light-dark cycles of 12 h and an environmental temperature of 21–23 °C.

LPS-induced endotoxic shock and CLP. These procedures have been described[8]. In prophylactic studies, ES-62 at the appropriate dose was administered by intravenous injection 2 h before LPS administration or induction of CLP and then daily for 3 consecutive days. In therapeutic studies, ES-62, vehicle and/or amoxicillin–clavulanic acid were administered intravenously at the appropriate dose and time after CLP and then daily for 3 consecutive days. Viability and inflammatory parameters and histology were analyzed after mice were killed as described[29].

Histology. Lungs and livers were dissected and then were immersed for 1 d in 10% (vol/vol) formalin fixative. Histological samples were analyzed as described[29].

Bacteria count. Mice were killed after CLP, and peritoneal lavage fluid and blood were collected. Bacterial loads were analyzed as described[29].

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Statistical analysis. Statistical significance among experimental groups was analyzed by the unpaired Student's *t*-test.

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