LETTER

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The TLR4 antagonist Eritoran protects mice from lethal influenza infection

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There is a pressing need to develop alternatives to annual influenza vaccines and antiviral agents licensed for mitigating influenza infection. Previous studies reported that acute lung injury caused by chemical or microbial insults is secondary to the generation of host-derived, oxidized phospholipid that potently stimulates Toll-like receptor 4 (TLR4)-dependent inflammation[1]. Subsequently, we reported that *Tlr4*$^{-/-}$ mice are highly refractory to influenza-induced lethality[2], and proposed that therapeutic antagonism of TLR4 signalling would protect against influenza-induced acute lung injury. Here we report that therapeutic administration of Eritoran (also known as E5564)—a potent, well-tolerated, synthetic TLR4 antagonist[3,4]—blocks influenza-induced lethality in mice, as well as lung pathology, clinical symptoms, cytokine and oxidized phospholipid expression, and decreases viral titres. CD14 and TLR2 are also required for Eritoran-mediated protection, and CD14 directly binds Eritoran and inhibits ligand binding to MD2. Thus, Eritoran blockade of TLR signalling represents a novel therapeutic approach for inflammation associated with influenza, and possibly other infections.

Influenza continues to evolve, and new antigenic variants emerge annually, giving rise to seasonal outbreaks. During annual influenza epidemics, 5–15% of the population is affected with upper respiratory tract infections, with hospitalization and death occurring mainly in the elderly and chronically ill. Annual influenza epidemics are estimated to result in 3–5 million cases of severe illness and 250,000–500,000 deaths yearly worldwide[5,6]. In addition, strains to which humans have no prior immunity may appear suddenly, and the resulting pandemics can be catastrophic, as illustrated by the 1918 'Spanish flu' that killed millions[7,8]. Logistical problems related to the prediction of future immunogenic epitopes, and production and distribution issues, often limit efficacy and/or vaccine availability. Moreover, increasing resistance to existing antiviral therapy, coupled with the need to administer these agents within 2–3 days after infection, limits their usefulness[9–11]. Thus, there is a critical need for a safe and effective therapeutic adjunct and/or alternative to influenza vaccines and antiviral agents.

The prototype TLR4 agonist, Gram-negative lipopolysaccharide (LPS), is a highly potent inflammatory stimulus that has been strongly implicated in Gram-negative septic shock, including acute respiratory distress syndrome caused by endothelial leak[12]. In 2008, a highly provocative paper[1] proposed that induction of acute lung injury, induced by acid aspiration, infection by respiratory viruses and bacteria, or exposure to their products (for example, inactivated H5N1 influenza), was mediated by a common signalling pathway: NADPH oxidase-dependent production of reactive oxygen species (ROS) generated oxidized host phospholipids—for example, oxidized 1-palmitoyl-2-arachidonoyl-phosphaticylcholine (OxPAPC)—that, in turn, potently stimulated TLR4. The authors proposed that the ensuing cytokine storm, and in particular, interleukin-6 (IL-6), mediated acute lung injury. Our subsequent findings that *Tlr4*$^{-/-}$ mice were protected from lethality caused by mouse-adapted influenza virus (A/PR/8/34, known as PR8)[2], led us to propose that blocking TLR4 therapeutically would protect against influenza infection. Here we provide compelling data showing that Eritoran (E5564; Eisai), a potent TLR4 antagonist[3,4], is highly protective when administered therapeutically to mice infected with a lethal dose of influenza.

Figure 1a illustrates our initial protocol. On 'day 0', 6–8-week old, female C57BL/6J mice were infected intranasally (i.n.) with a dose of PR8 that was determined to kill ~90% of mice (~LD_{90}). Two days later, the TLR4 signalling antagonist Eritoran (E5564) was administered daily for 5 consecutive days (200 μg per mouse; days 2–6) intravenously (i.v.). Each mouse was weighed and clinical symptoms (for example, lethargy, piloerection, ruffled fur, hunched posture, rapid shallow breathing, audible

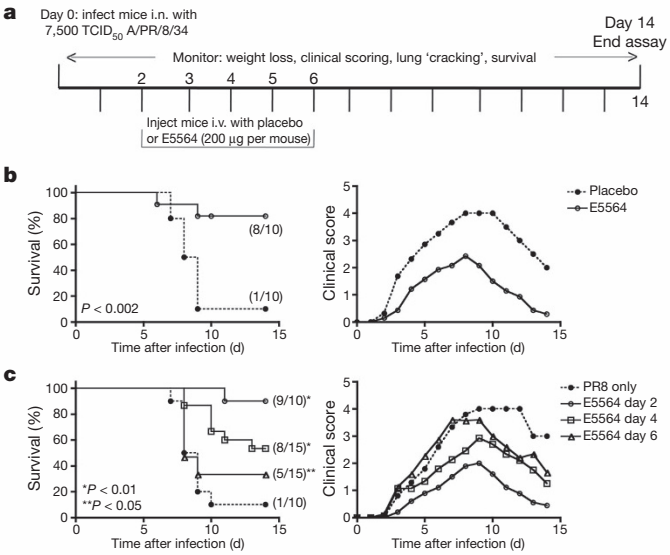

Figure 1 | Eritoran treatment protects mice from lethal influenza challenge. **a**, Basic experimental protocol used to test Eritoran in mice infected with influenza. C57BL/6J mice were infected with mouse-adapted influenza, strain PR8 (~7,500 $TCID_{50}$, i.n.; ~LD_{90}). Two days later, mice received placebo (vehicle only) or E5564 (Eritoran; 200 μg per mouse i.v.) once daily for 5 successive days (days 2 to 6). **b**, Mice were treated as shown in **a**. Left, survival was monitored daily ($P < 0.002$). Right, clinical scores (see Methods) were also measured daily. Each graph represents the combined results of 2 separate experiments, each with 5 mice per treatment group per experiment. **c**, Mice were infected as described in **a**, but treated with Eritoran starting on days 2, 4 or 6 after infection. Left, survival (day 2 and day 4, $P < 0.01$; day 6, $P \leq 0.05$); right, clinical scores. Results are combined results from 2–3 separate experiments, with 5 mice per treatment group per experiment.

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rattling) were scored daily for 2 weeks. Eritoran-treated mice were significantly protected from influenza-induced lethality, whereas 90% of mice administered placebo (vehicle only) died (Fig. 1b, left). This was paralleled by more severe clinical scores (Fig. 1b, right) and weight loss (Supplementary Fig. 1A) in control mice versus Eritoran-treated mice. Treatment of mice with tenfold less Eritoran (20 μg per mouse) decreased survival to 40% (4/10 mice survived; data not shown). The efficacy of Eritoran treatment decreased from 90% survival in mice infected with 7,500 50% tissue culture infective dose ($TCID_{50}$) to 60% and 25% survival in mice infected with 10,000 and 20,000 $TCID_{50}$, respectively (Supplementary Fig. 1B). Treatment with Eritoran also protected mice infected with a lethal dose of the non-adapted 2009 human pandemic influenza strain A/California/07/2009 H1N1 (Supplementary Fig. 1C).

Additional experiments were performed in which Eritoran treatment was started on day 2, day 4 or day 6 after infection. Figure 1c (left) illustrates that 90% of PR8-infected mice that did not receive Eritoran treatment died, whereas mice that received Eritoran starting on days 2, 4 or 6 had statistically significant survival rates of 90%, 53% and 33%, respectively. The clinical scores for these same mice (Fig. 1c, right) illustrate that the later mice began Eritoran treatment, the more severe their clinical scores. Mice that did not receive Eritoran showed the most severe symptoms and highest clinical scores. Similarly, weight loss was progressively greater the later treatment was initiated after infection (data not shown). Thus, in contrast to current antivirals that must be administered soon after infection, Eritoran treatment significantly enhanced survival, even when started as late as 6 days after infection.

Eritoran treatment also led to a statistically significant reduction in influenza-induced lung pathology. Groups of mice received saline only ('mock'), PR8 only ('PR8 untreated'), or were infected with PR8 and treated with Eritoran (PR8 + E5564) starting 2 days after infection (Fig. 1a), and were killed 7 days after infection. In mice infected with PR8 only, extensive lung damage (that is, highly basophilic lining epithelium in the bronchioles, focal degenerating cells undergoing apoptosis or necrosis, and extensive cellular infiltrates (neutrophils, monocytes and lymphocytes)) were observed; however, in infected, Eritoran-treated mice, ~80% of each lung section examined exhibited nearly normal lung architecture (similar to 'mock'), whereas in ~20% of each section, inflammatory infiltrates could be observed, although to a lesser extent than in the lungs of mice infected with PR8 only (Fig. 2a). These observations are supported by blinded histological scoring (Fig. 2b). By day

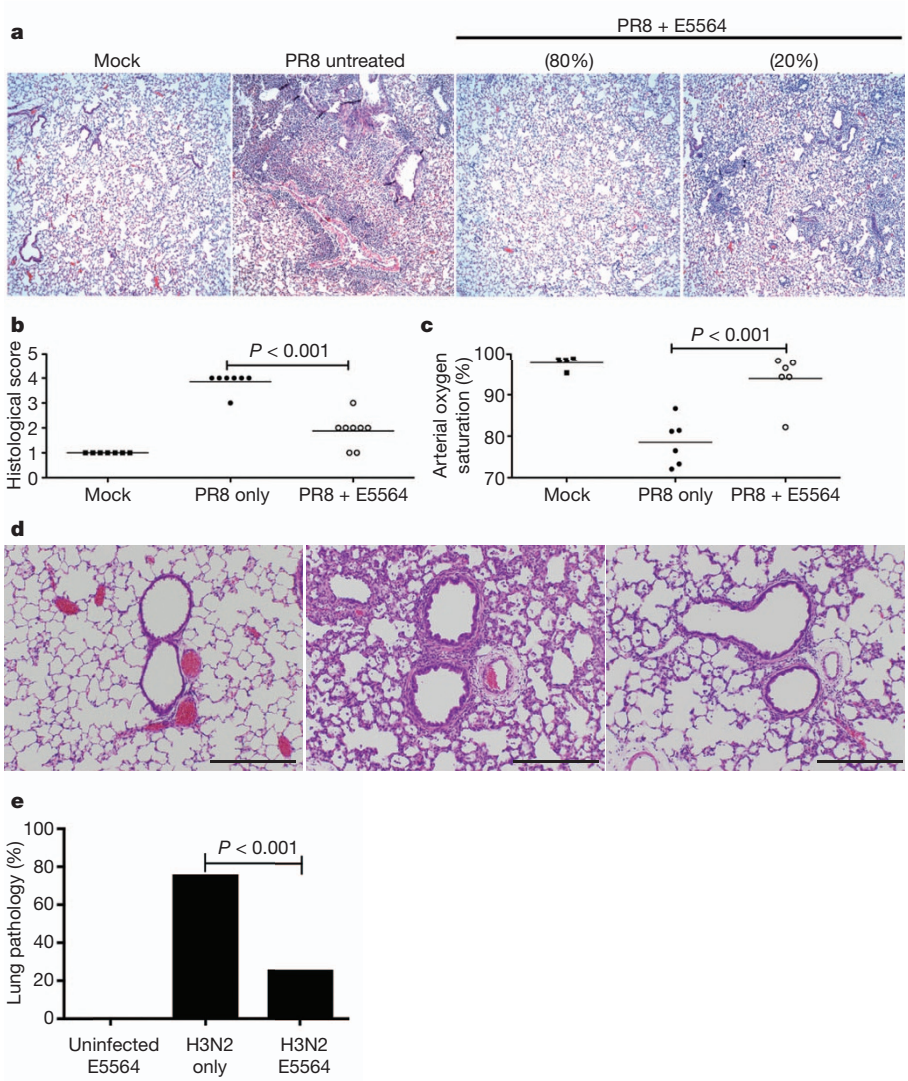

Figure 2 | Eritoran treatment inhibits influenza-induced lung pathology and lung function. **a**, Eritoran treatment improves lung pathology induced by PR8 infection. Representative haematoxylin and eosin (H&E)-stained sections were derived from mice treated as described in the text (4–5 mice per treatment group) killed on day 7 after infection. Original magnification, ×400. **b**, Histological scoring for PR8-infected mice with or without Eritoran treatment ± standard error of the mean (s.e.m.). **c**, Pulse oximetry data collected on day 6 after infection ± s.e.m. ($P < 0.001$, comparing PR8 only with mock-infected or PR8 plus E5564; 4–6 mice per treatment group). **d**, Eritoran treatment improves lung pathology of H3N2 infection of cotton rats. Scale bars, 500 μm. **e**, Histological scoring for H3N2-infected cotton rats with or without Eritoran treatment ($n = 5$ rats per treatment group; data shows mean ± s.e.m.).

14 after infection, Eritoran-treated mice showed normal lung architecture (data not shown). Previously, a direct correlation between pulse-oximetry measurements of oxygen saturation and lung pathology, which reflected the morbidity and survival of mice infected with PR8, was reported[13]. By day 6 after infection, the oxygen saturation levels observed in mock-infected and PR8-infected, Eritoran-treated mice were not significantly different. By contrast, PR8-infected mice demonstrated a significant oxyhaemoglobin desaturation to 78%, suggesting a functional consequence of the alveolar injury demonstrated histologically (Fig. 2c).

To determine whether the therapeutic effect of Eritoran extends to other animal models of human influenza infection, we performed experiments in cotton rats (*Sigmodon hispidus*)[14,15]. A/Wuhan/359/95 (H3N2), a human non-adapted strain of influenza, replicates in lungs of cotton rats on day 1 and produces peak lung pathology on day 4 after infection (Fig. 2d, middle; and Fig. 2e, H3N2 only). Animals treated with Eritoran after H3N2 challenge showed significant reductions in lung pathology on day 4 compared with animals treated with vehicle (Fig. 2d, right; and Fig. 2e, H3N2/E5564).

Figure 3a shows that by day 6 after infection, Eritoran treatment resulted in a statistically significant decrease in lung viral titres that was further decreased by day 7 (Fig. 3b) and day 8 (data not shown). This was not a direct effect of Eritoran on virus replication (Supplementary Table 1). By day 14, all surviving mice had completely cleared the virus, regardless of treatment. Because the effects of influenza infection are systemic[16], levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in sera. Although PR8 infection resulted in significant increases in ALT and AST levels, these were significantly lower in Eritoran-treated mice 6 days after infection (Supplementary Fig. 2).

Influenza infection potently induces expression of both pro- and anti-inflammatory genes *in vivo* that contribute to the inflammatory response[1,17]. To determine whether Eritoran also mitigates PR8-induced gene expression, groups of mice were infected on day 0, treated with Eritoran starting on day 2, and killed on day 2 (3 h after Eritoran treatment) and on days 4 and 6. Total RNA was extracted from lung homogenates and gene expression was measured by quantitative polymerase chain reaction with quantitative real-time reverse transcription (qRT–PCR). Eritoran-treated mice showed significantly blunted pro- and anti-inflammatory gene expression at each time point (Fig. 4a and Supplementary Fig. 3A; $P < 0.001$), as early as 3 h after treatment on day 2. Supplementary Fig. 3B shows that influenza induced type I, II and III interferon (IFN); however, only *Ifnb* and *Ifng* messenger RNA expression were blunted by Eritoran treatment. In addition, cotton rats infected with the non-adapted human Wuhan H3N2 strain and treated with Eritoran showed decreased lung expression of IL-6 and IL-10 (Fig. 4b).

Ifnb$^{-/-}$ mice are highly susceptible to PR8 and are not protected by the potent IFN-β inducer, 5,6-dimethylxanthenone-4-acetic acid[18]. In addition, endogenous IFN-β is required for normal basal and LPS-inducible expression of many proinflammatory cytokines in macrophages[19]. PR8-infected *Ifnb*$^{-/-}$ mice were not protected by Eritoran (Supplementary Fig. 4). This indicates that although both pro-inflammatory and *Ifnb* gene expression are blunted in PR8-infected, Eritoran-treated wild-type mice (Fig. 4a, b), the elaboration of some IFN-β is required for antiviral protection in the absence of significant priming.

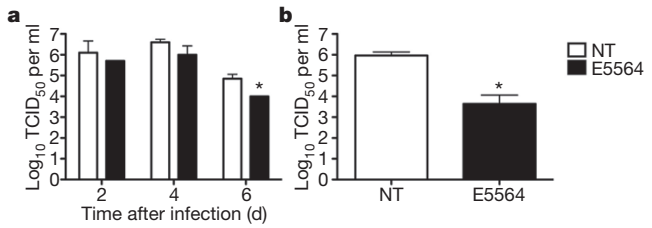

Figure 3 | Treatment with Eritoran reduces lung viral titres. **a**, **b**, Mice were infected with PR8 and then either left untreated (NT) or were treated with Eritoran (E5564) as described in Fig. 1a, and were killed on days 2, 4, 6 (**a**) and 7 (**b**) after infection. **a**, Results represent the combined results of 2 separate experiments (5 mice per treatment group per experiment); *$P < 0.05$. **b**, Day 7 after infection (7 mice per treatment group); *$P < 0.001$.

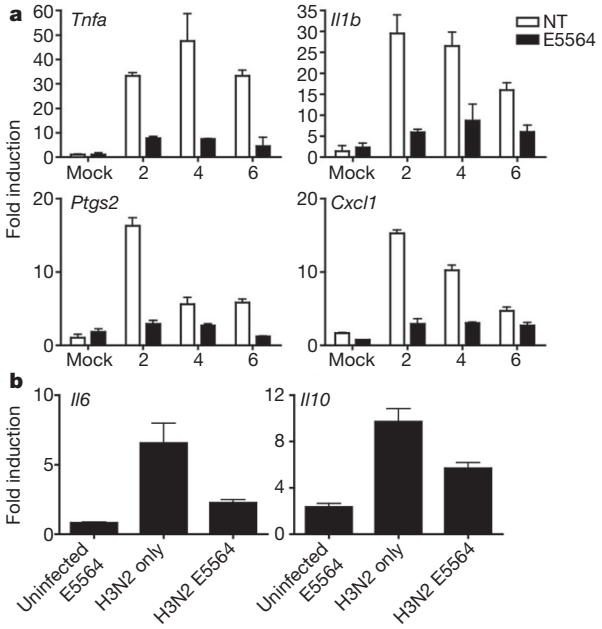

Figure 4 | Eritoran treatment suppresses influenza-induced cytokine gene expression. **a**, **b**, Mice (**a**) and cotton rats (**b**) were treated as described in Fig. 1a and killed on days 2 (3 h after treatment), 4 and 6 after infection (4 mice per treatment group per experiment; data are mean ± s.e.m. from 2 separate experiments; $P < 0.01$ at each time point). Lungs were processed for total RNA and subjected to qRT–PCR for detection of specific gene expression. NT, not treated.

Most potent activation of TLR4 by LPS depends on LPS binding protein (LBP)-catalysed extraction and transfer of individual LPS molecules from aggregated LPS to CD14 and then from CD14 to MD2, followed by engagement and dimerization of TLR4. Crystallographic analysis revealed that Eritoran, like LPS lipid A monomer, binds in the deep hydrophobic pocket of MD2; however, in contrast to lipid A, Eritoran fails to induce TLR4 dimerization and activation, thus acting as a TLR4 antagonist[20].

We confirmed the finding[2] that PR8-induced lethality in mice was TLR4 dependent (Fig. 5a), but also found, surprisingly, that influenza mortality was not CD14 dependent, as indicated by the similar mortality resulting from PR8 infection in wild-type and *Cd14*$^{-/-}$ mice versus the resistance of *Tlr4*$^{-/-}$ mice (Fig. 5a). These findings suggest that TLR4-dependent influenza mortality is mediated by influenza-induced TLR4 agonists that, unlike LPS, do not depend on CD14 for engagement and activation of TLR4/MD2. By contrast, the protection induced in wild-type mice when Eritoran treatment is started on day 2 after infection is absent in *Cd14*$^{-/-}$ mice, indicating that the protective effect of Eritoran in this influenza infection model is CD14 dependent (Fig. 5a).

One possible explanation for the CD14 dependence of Eritoran-mediated protection is that engagement by Eritoran of MD2 and antagonism of TLR4 depends on CD14. To test this possibility, we assayed *in vitro* the capacity of Eritoran to bind CD14 and MD2 by measuring inhibition by Eritoran of LBP-dependent transfer of tritiated lipooligosaccharide ([^{3}H]LOS; the LPS of *Neisseria*) to CD14 (Fig. 5b, left), as well as the transfer of [^{3}H]LOS from CD14 to MD2 (Fig. 5b, right). As the inhibitory effect of Eritoran on the transfer of [^{3}H]LOS to MD2 is 100 times more potent when LBP and soluble CD14 are present, this indicates that the interaction of Eritoran with MD2 occurs most efficiently after LBP-dependent interaction of Eritoran with CD14.

Although MD1, a homologue of MD2 that also binds lipid A[21], was considered as an alternative target for Eritoran, this possibility was

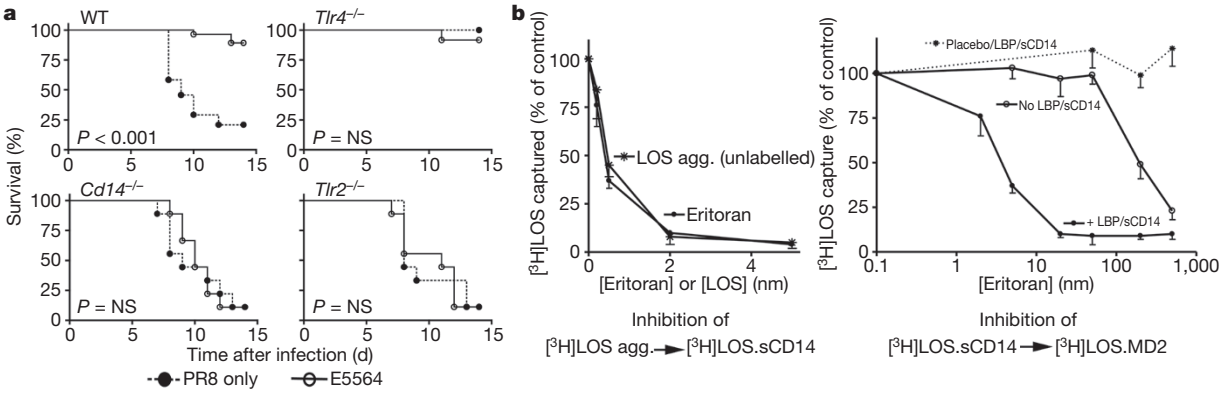

Figure 5 | Molecular requirements of Eritoran-induced protection. **a**, Wild-type (WT), *Tlr4*$^{-/-}$, *Cd14*$^{-/-}$, *Tlr2*$^{-/-}$ mice were either untreated (filled circles), or treated with E5564 (open circles) 2 days after infection, for 5 successive days. Wild-type data were combined from 5 separate experiments (5–6 mice per treatment group per experiment), *Tlr4*$^{-/-}$ data from 3 separate experiments (5–6 mice per treatment group per experiment), *Cd14*$^{-/-}$ mice (2 separate experiments; 5 mice per treatment group per experiment), and *Tlr2*$^{-/-}$ mice (2 separate experiments; 4–5 mice per treatment group per experiment). Wild-type: untreated versus E5564 treatment ($P < 0.0001$); *Tlr4*$^{-/-}$: untreated versus E5564 treatment (P = non-significant (NS)); *Cd14*$^{-/-}$: untreated versus E5564 treatment (P = NS); *Tlr2*$^{-/-}$: untreated versus E5564 treatment (P = NS). **b**, Dose-dependent Eritoran inhibition of the transfer and binding of monomeric [^{3}H]LOS from aggregated (agg.) [^{3}H]LOS to soluble CD14 (sCD14), and from [^{3}H]LOS.sCD14 to MD2. Samples containing [^{3}H]LOS aggregates (0.2 nM), His_6–sCD14 (~0.5 nM), and increasing concentrations as indicated of Eritoran or unlabelled LOS (left) or 2 nM [^{3}H]LOS.sCD14, approximately 2 nM His_6–MD2, and increasing concentrations of Eritoran (or placebo) ± LBP (50 pM) and sCD14 (2 nM) (right) were incubated for 30 min at 37 °C, followed by addition and incubation with NiFF Sepharose beads to capture His-tagged proteins. Formation of complexes of [^{3}H]LOS with His_6–sCD14 (left) or MD2 (right) was assayed by measuring co-capture of [^{3}H]LOS by NiFF Sepharose as previously described[25]. Data are expressed as per cent of co-capture of [^{3}H]LOS observed in the absence of added Eritoran. Data show mean ± s.e.m. of 3 separate experiments with duplicate samples for each dose.

dismissed because MD1 failed to substitute for MD2 to enable TLR4 signalling (Supplementary Fig. 5).

Diacylated lipids can act as TLR2 agonists. Hence, if oxidized host phospholipids (for example, OxPAPC) have a role in TLR4-dependent influenza, it is conceivable that these diacylated lipids could also engage TLR2-containing receptor complexes and that influenza-induced mortality might also be TLR2 dependent. However, *Tlr2*$^{-/-}$ mice showed a similar susceptibility to PR8 infection as wild-type mice (Fig. 5a), arguing against an important role for TLR2 in influenza-induced mortality. Remarkably, however, protection induced by Eritoran treatment beginning at day 2 after infection was absent in *Tlr2*$^{-/-}$ mice, suggesting a novel role for TLR2 in Eritoran action.

If the PR8-induced acute lung injury and mortality we have observed is due substantially to influenza-induced oxidized host phospholipids, as previously proposed[1], these compounds should show preferential activation of TLR4 (versus TLR2) and be inhibited by Eritoran. Commercially obtained OxPAPC comparably activated *Il6* gene expression in wild-type and *Tlr2*$^{-/-}$ peritoneal macrophages, but was inhibited by ~84% in *Tlr4*$^{-/-}$ macrophages when compared to the wild-type response. Cellular activation by OxPAPC was also significantly inhibited by Eritoran, including the low level of TLR4-independent production of *Il6* mRNA observed in *Tlr4*$^{-/-}$ macrophages (Supplementary Fig. 6). This indicates that the response to OxPAPC is predominantly TLR4 dependent and Eritoran sensitive.

To assess the effect of Eritoran on production of oxidized phospholipids during infection, matrix-assisted laser desorption ionization-imaging mass spectrometry (MALDI-IMS) was used to identify alterations in the lipid composition of mouse lungs after PR8 infection, with or without Eritoran treatment. Predicted oxidation products (mass to charge ratio (m/z) 650, 830) of 1-palmitoyl-2-arachidonayl-sn-glycero-3-phosphocholine (PAPC), or other polyunsaturated phosphatidylcholines, as described previously[1], were detected in greater abundance in PR8-infected versus mock-infected or infected and Eritoran-treated lungs (Supplementary Fig. 7). These results were confirmed by electrospray ionization–mass spectrometry (data not shown). Together, these results indicate that oxidized phosphatidylcholines are present in influenza-infected lungs and show that treatment with Eritoran reduces the relative abundance of these characteristic ions. Thus, Eritoran treatment of influenza-infected mice not only blocks the cytokine storm exacerbated by influenza-induced endogenous TLR4 agonists, but also inhibits the production and accumulation of certain oxidized phospholipids in infected lungs, including OxPAPC and possibly others.

Our findings provide further support for a prominent role of influenza-induced oxidized host phospholipids in TLR4-dependent acute lung injury and mortality, and demonstrate a novel therapeutic potential of Eritoran towards this and, potentially, other respiratory infections. In the hypothetical model shown in Supplementary Fig. 8, we would predict, based our data, that Eritoran blocks oxidized-phospholipid-induced TLR4 signalling, thereby mitigating the cytokine storm and subsequent potentiation of phospholipid oxidation induced by ROS. Our failure to detect commensal bacteria or LPS in lung homogenates from PR8-infected mice suggests that the disease manifestations are not the result of secondary bacterial infection (data not shown). The TLR2 and CD14 independence of influenza mortality is consistent with the selective agonist properties of at least certain oxidized host phospholipids that we have observed and suggests that these compounds, unlike most LPS species, can engage MD2/TLR4 in the absence of CD14, perhaps by interacting with an alternative lipid acceptor/donor such as albumin[22,23]. By contrast, the CD14 and TLR2 dependence of the therapeutic action of Eritoran may reflect CD14-dependent engagement of MD2/TLR4 by Eritoran, precluding the action of endogenous TLR4 agonists generated after initiation of infection and early tissue alterations, as well as uncharacterized interactions of Eritoran with TLR2-containing receptors. This possibility is supported by the observation that mycobacterial lipoarabinomannan requires CD14 for TLR2 activation[24]. That influenza infection modulates TLR2 expression in many cell types[25,26], and synthetic TLR2 ligands provide prophylaxis against lethal influenza infection[27], strengthens the notion that TLR2-containing receptors may provide another target for Eritoran. These findings underscore the novel therapeutic utility of Eritoran, either used as a monotherapy or, more likely, as an adjunct to antiviral agents. The utility of Eritoran is further enhanced by its well-documented safety profile[28].

Many drugs targeting endotoxin or the endotoxin-induced cytokine storm were originally developed for use in sepsis, yet none have emerged as effective therapeutics for sepsis. Moreover, whereas many of these drugs failed to provide therapeutic protection in animal models of

endotoxicity or in clinical trials in septic humans, they were found to be of great benefit in other, more localized models of inflammation (such as rheumatoid arthritis and inflammatory bowel disease). Our data suggest that Eritoran might follow a similar development pathway. Eritoran demonstrated remarkable activity *in vitro* and *in vivo*[4,28] in endotoxin challenge studies when administered prophylactically or very early in the 'sepsis cascade'. A non-statistically significant improvement in mortality was also observed in a small phase 2 severe sepsis study[29]. The phase 2 study design and statistical power calculations were expanded to perform a single dose, placebo-controlled phase 3 severe sepsis study (the ACCESS study) in which 1,984 patients were treated in a 2:1 ratio of drug and placebo. The results of this study demonstrated that new treatment regimens including aggressive fluid resuscitation and early goal-directed therapy markedly reduced mortality in the placebo group; however, further benefit to septic patients was not observed with Eritoran treatment[30]. Thus, it is notable that in this study, Eritoran prevented mortality when administered up to 6 days after influenza infection of mice. This more practical timing of treatment may enable effective clinical treatment of influenza and, perhaps, other infectious agents.

METHODS SUMMARY

***In vivo* measurements.** Mice and cotton rats were infected i.n. as indicated with influenza virus, followed by administration of control or Eritoran i.v. Survival, clinical score, weight loss, cytokine gene expression, viral titres, OxPAPC abundance and ALT/AST levels were measured.

Cell culture measurements. Primary murine macrophage cultures were treated with TLR agonists or OxPAPC with or without Eritoran, and *Il6* mRNA was measured by qRT–PCR.

Biochemical measurements. The ability of Eritoran to inhibit binding of LPS to CD14 or the transfer of LPS from CD14 to MD2 was carried out as reported[22,23].

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.N.V. (svogel@som.umaryland.edu).

METHODS

Reagents. Eritoran (E5564) and its corresponding placebo (vehicle only) were provided by Eisai, Inc. Eritoran was prepared at 2.33 mg ml^{-1} in sterile, endotoxin-free water and diluted for injection in sodium-bicarbonate-buffered 5% dextrose water. OxPAPC was purchased from Hycult Biotech. *Escherichia coli* K235 LPS was prepared as previously described[1]. Pam3Cys was purchased from EMC Microcollections.

Mice and cotton rats. Six- to eight-week old wild-type C57BL/6J, *Tlr2*$^{-/-}$ and *Cd14*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Tlr4*$^{-/-}$ mice (provided by S. Akira) on a C57BL/6 background, were bred in a University of Maryland, Baltimore accredited facility. Inbred young adult (4–8 weeks old) cotton rats (*S. hispidus*) were bred at Sigmovir Biosystems. All animal experiments were conducted with Institutional Animal Care and Use Committee approval from the University of Maryland, Baltimore and Sigmovir Biosystems.

Virus. Mouse-adapted H1N1 influenza A/PR/8/34 virus (PR8; ATCC) was grown in the allantoic fluid of 10-day-old embryonated chicken eggs as described[2], and was provided by D. Farber. Non-adapted human influenza virus strain A/Wuhan/359/95 (H3N2), and A/Brisbane/59/07 (H1N1) was obtained and grown as previous described[3,4]. Non-adapted human influenza A/California/07/2009 strain (human pandemic H1N1) was provided by T. Ross.

Virus challenge and treatments. C57BL/6J wild-type, *Tlr4*$^{-/-}$, *Tlr2*$^{-/-}$ and *Cd14*$^{-/-}$ mice were infected with mouse-adapted influenza virus, strain A/PR/8/34 (PR8; ~7,500 $TCID_{50}$, i.n., 25 µl per nares) or the non-adapted human pandemic H1N1 strain, A/California/07/2009 (~10^7 $TCID_{50}$, i.n.). This dose was found in preliminary experiments to kill ~90% (PR8) or ~75% (human H1N1) of infected mice. Two days after infection, mice received either placebo or Eritoran (E5564; 200 µg per mouse in 100 µl, i.v.) daily (day 2 to day 6). In some experiments, some groups of mice were treated with Eritoran starting at day 4 or day 6 after infection and treated for 5 or 3 consecutive days, respectively. In some experiments, some mice were infected with ~10,000 or 20,000 $TCID_{50}$ PR8. Mice were monitored daily for survival, weight loss, and clinical signs of illness (for example, lethargy, piloerection, ruffled fur, hunched posture, rapid shallow breathing, audible crackling) for 14 days. A clinical score ranging from 0 (no symptoms) to 5 (moribund) was ascribed to each mouse daily[5]. In some experiments, mice were killed at the indicated times after infection to harvest serum for liver enzyme levels or lungs for analysis of gene expression, lung pathology, or viral titres.

Cotton rats were infected with non-adapted human influenza strain A/Wuhan/359/95 (H3N2; ~ 10^7 $TCID_{50}$ or p.f.u., i.n., 50 µl per nares). Two hours after infection, animals received E5564 (200 µg per rat in volume, retro-orbital) daily for 5 days (days 0–4). Animals were killed at day 4 post-infection to determine lung pathology and total RNA for measuring gene expression by real-time PCR.

Histopathology. Lungs were inflated and perfused, and fixed with 4% PFA. Fixed sections (8 µm) of paraffin-embedded lungs were stained with H&E. Slides were randomized, read blindly, and examined for tissue damage, necrosis, apoptosis and inflammatory cellular infiltration.

Pulse oximetry measurements. At 6 days after infection, per cent arterial oxygen saturation was measured on individual mice that were lightly anaesthetized with nebutol. An extra small collar sensor and a Small Animal Oximetry Restraint Device (Starr-Gate) was used as per the manufacturer's instructions.

Viral titration. Virus titres were obtained from supernatants of lung homogenates of PR8-infected mice that were either left untreated or treated with Eritoran and harvested on days 2, 4, 6 and 7 after infection, and expressed as $TCID_{50}$ per ml as described previously[6].

qRT–PCR. Total RNA isolation and qRT–PCR were performed as previously described[7]. Levels of mRNA for specific genes are reported as relative gene expression normalized to mock-infected lungs.

Liver enzyme levels. Serum was collected on day 7 from C57BL/6J wild-type mice that were either mock-infected with saline or infected with PR8, and were either left untreated or treated with Eritoran (E5564) starting on day 2 after infection. ALT and AST levels were measured using a Dimension Vista System Flex Reagent cartridge (Siemens Healthcare Diagnostics).

Macrophage cell cultures and treatment. Thioglycollate-elicited peritoneal macrophages from wild-type, *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ mice were enriched as described[8] after plating in 12-well (2×10^6 cells perwell) tissue culture plates. Macrophages were pre-treated with E5564 (10 ng ml^{-1}) for 1 h and then stimulated with LPS (10 ng ml^{-1}), P3C (300 ng ml^{-1}) or OxPAPC (20 µg ml^{-1}) for 8 h.

MALDI-IMS. Mouse lung tissue was snap frozen by floating in an aluminium foil raft on liquid nitrogen. Tissues were cryosectioned (unfixed and unembedded) in 12 µm sections, each section was transferred to a conductive MALDI-IMS glass slide, heat-fixed until visibly dry (approximately 30–60 s), and desiccated for at least 1 h. A 12.5 mg ml^{-1} solution of norharman MALDI matrix solvated in chloroform:methanol:water (1:2:0.8, v:v:v) was spray coated onto slides using the Bruker ImagePrep device. MS data was collected in positive mode (detection range: m/z 400–900), raster width 150 µm, 500 shots per raster, on a Bruker Daltonics AutoFlex Speed Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight Mass Spectrometer (MALDI-TOF/TOF MS; source) using flexControl software. Subsequently, the data were analysed using flexImaging software. After IMS analysis, the matrix was stripped in 70% methanol and tissue sections were restained by H&E for gross histological reference using standard protocols. All MALDI-IMS specific materials, equipment, instruments and software were obtained from Bruker Daltonics. Unless otherwise specified, all reagents were purchased from Sigma-Aldrich.

Statistics. Statistical differences between two groups were determined using an unpaired, two-tailed Student's t-test with significance set at $P < 0.05$. For comparisons between ≥3 groups, analysis was done by one-way ANOVA followed by a Tukey's multiple comparison test with significance determined at $P < 0.05$. For survival studies, a Log-Rank (Mantel-Cox) test was used.