



Published in final edited form as:

*Mucosal Immunol.* 2016 September ; 9(5): 1173–1182. doi:10.1038/mi.2015.141.

## Novel Strategies for Targeting Innate Immune Responses to Influenza

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### Abstract

We previously reported that TLR4<sup>-/-</sup> mice are refractory to mouse-adapted A/PR/8/34 (PR8) influenza-induced lethality and that therapeutic administration of the TLR4 antagonist, Eritoran, blocked PR8-induced lethality and acute lung injury (ALI) when given starting 2 days post-infection. Herein, we extend these findings: anti-TLR4- or TLR2-specific IgG therapy also conferred significant protection of wild-type (WT) mice from lethal PR8 infection. If treatment is initiated 3 h prior to PR8 infection and continued daily for 4 days, Eritoran failed to protect WT and TLR4<sup>-/-</sup> mice, implying that Eritoran must block a virus-induced, non-TLR4 signal that is required for protection. Mechanistically, we determined that (i) Eritoran blocks HMGB1-mediated, TLR4-dependent signaling *in vitro* and circulating HMGB1 *in vivo*, and an HMGB1 inhibitor protects against PR8; (ii) Eritoran inhibits pulmonary lung edema associated with ALI, (iii) IL-1 $\beta$  contributes significantly to PR8-induced lethality, as evidenced by partial protection by IL-1 receptor antagonist (IL-1Ra) therapy. Synergistic protection against PR8-induced lethality

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**Conflict of Interest:** With the exception of FG, none of the authors have a competing interest. FG is an employee of Eisai Inc.

**Author Contributions:** KAS and SNV carried out the study design, with advice from JCGB, AF, CD, TR, KJT, AY, and EKJ. KAS, WL, MCP, LMP, CP, and EKJ performed experiments. FG, TR, TC, AB, AG, CD, KJT, AY, and EKJ provided crucial reagents and advice for this study. KAS and SNV prepared the manuscript with input and approval of all other co-authors.

was achieved when Eritoran and the anti-viral drug, oseltamivir, were administered starting 4 days post-infection. Eritoran treatment does not prevent development of an adaptive immune response to subsequent PR8 challenge. Overall, our data support the potential of a host-targeted therapeutic approach to influenza infection.

## Introduction

Influenza continues to evolve with new antigenic variants emerging annually, as exemplified by the last several influenza seasons in which the recommended vaccine was considerably less efficacious than predicted<sup>1-4</sup>. Therefore, there remains a pressing need to develop alternatives to the annual influenza vaccines and anti-viral agents currently used to mitigate the effects of influenza infection. Multiple Pattern Recognition Receptors (PRRs), including TLR3, TLR4, TLR7, TLR8, TLR10, and the intra-cytosolic sensor, RIG-I, have been implicated in influenza-induced disease, although TLR10 is not functional in mice<sup>5-12</sup>. CD14 is required for influenza-induced cytokine production by mouse macrophages, independent of TLR2 and TLR4<sup>13</sup>. In addition, influenza-infected MyD88<sup>-/-</sup> and MyD88/TRIF doubly deficient mice show a dramatic reduction of pulmonary cytokine production when compared to WT mice<sup>5,11,12</sup>, indicating the important role of these TLR signaling pathways in disease.

Imai et al.<sup>14</sup> proposed that chemical or microbial insults trigger NADPH-dependent reactive oxygen species that generate a host-derived oxidized phospholipid, oxidized 1-palmitoyl-2-arachidonyl-phosphatidylcholine (OxPAPC), in lungs. They concluded that regardless of the initial sensing involved in pathogen recognition, OxPAPC initiates a common TLR4-, TRIF-, and IL-6-dependent pathway in macrophages that leads to ALI. We showed that treatment of influenza-infected mice with Eritoran, the most potent, synthetic lipid A analog known<sup>15</sup>, blocked influenza-induced lethality and ALI. When administered daily to WT mice for 5 days, starting on days 2, 4, or 6 post-infection, Eritoran treatment significantly improved survival and clinical symptoms, while decreasing ALI, OxPAPC accumulation, the cytokine storm, and systemic inflammation<sup>16</sup>. Herein, we extend our previous findings by characterizing the role of TLR4 and TLR2 in influenza-induced lethality and ALI and provide new insights into the molecular mechanisms underlying protection by modulators of these important TLR-mediated inflammatory pathways.

## Results

### Eritoran in Influenza models

We previously reported that TLR4<sup>-/-</sup> mice were refractory to mouse-adapted influenza, PR8<sup>16,17</sup>, and have now confirmed that TLR4<sup>-/-</sup> mice are also refractory to a more pathogenic, mouse-adapted pandemic H1N1 strain, ma.Ca/04<sup>18</sup> (Supplementary Figure 1A). We also showed previously that therapeutic treatment of PR8-infected, WT mice therapeutically with Eritoran, significantly protected against lethality, and attenuated ALI, findings now reproduced in C57BL/6 mice infected with the ma.Ca/04 strain (Supplementary Figure 1B and 1C). Eritoran also protected PR8-infected BALB/c mice (data not shown). We found that an additional daily dose of Eritoran (*i.e.*, administered once

vs. twice daily), starting four days post-infection, failed to improve the protection achieved with a single dose daily (data not shown). Together, these data expand our previous observations that therapeutic treatment of mice with Eritoran protects against lethality with additional influenza strains and newly explored conditions of infection. The following experiments were designed to provide mechanistic insights into Eritoran-mediated protection and to identify the pathways that contribute to influenza-induced disease that are affected by Eritoran treatment.

### Elucidation of signaling requirements underlying influenza-induced lethality and protection by Eritoran

Eritoran blocks TLR4 signaling by binding in the deep hydrophobic pocket of its co-receptor, MD2, thereby blocking ligand-induced dimerization<sup>19</sup>. To validate the role of TLR4 in PR8-induced lethality, mice were treated with a highly specific anti-TLR4 antibody<sup>20</sup> (Figure 1A). Anti-TLR4 IgG, but not an isotype-matched control IgG, administered i.v. on day 2 only or on days 2 and 4 post-PR8 infection, protected mice against lethal infection (Figure 1B) and elicited significantly improved clinical scores ( $p < 0.004$ ; Figure 1C). This result confirms that TLR4 signaling is, indeed, central to influenza-induced lethality and clinical symptoms.

TLR4 activates both the MyD88- and TRIF-dependent signaling pathways<sup>8</sup>. One of the central conclusions of Imai et al.<sup>14</sup> was that TLR4-mediated ALI induced by inactivated H5N1 influenza or the host-derived oxidized phospholipid, OxPAPC, is entirely TRIF-dependent. However, MyD88 has been implicated in the host response to influenza<sup>9,12</sup>. IRAK4, the first enzyme recruited to MyD88, initiates signaling leading to IKK $\alpha/\beta/\gamma$  complex activation, I $\kappa$ B $\alpha$  phosphorylation, and ultimately, NF- $\kappa$ B activation. The TRIF pathway drives IRF3 activation and results in delayed NF- $\kappa$ B activation, independent of IRAK4<sup>21</sup>. To delineate the downstream pathway(s) underlying the host response to influenza and the protective mechanisms of Eritoran, we compared PR8-induced lethality and the efficacy of Eritoran in IRAK4 kinase dead knock-in (IRAK4<sup>KDKI</sup>) mice that have a catalytically inactive form of IRAK4 that blocks MyD88-dependent signaling, vs. TRIF<sup>-/-</sup> mice. IRAK4<sup>KDKI</sup> mice exhibited a slightly delayed mean time to death compared to WT mice, and Eritoran therapy resulted in ~60% survival compared to ~90% in WT mice (Figure 2A). Interestingly, TRIF<sup>-/-</sup> mice were more resistant to PR8 infection than WT or IRAK4<sup>KDKI</sup> mice (~50% survival), but not as refractory as TLR4<sup>-/-</sup> mice<sup>16,17</sup>. However, treatment with Eritoran significantly improved the survival of TRIF<sup>-/-</sup> mice to WT levels ( $p < 0.001$ ; Figure 2B). VIPER is peptide TLR4-inhibitory peptide derived from the A46 protein of vaccinia virus that has been shown to inhibit both MyD88- and TRIF-dependent TLR4 signaling by binding to and targeting the sorting adaptors TIRAP and TRAM<sup>22</sup>. When WT mice were infected with PR8 and treated therapeutically with either a cell-permeating VIPER peptide, 9R-VIPER, or Eritoran, 9R-VIPER treatment resulted in partial protection (~50%), consistent with a role for TIRAP and/or TRAM in protection (Supplemental Figure 2). Thus altogether, both MyD88- and TRIF-dependent pathways contribute to influenza-mediated disease and Eritoran-induced protection.

We reported previously that TLR2<sup>-/-</sup> mice were similarly sensitive to WT mice for PR8-induced lethality. However, unlike WT mice, Eritoran therapy failed to protect TLR2<sup>-/-</sup> mice; thus, TLR2 was presumed to be a direct or indirect target for Eritoran<sup>16</sup>. To confirm the role of TLR2 in influenza-induced disease, we used a monoclonal antibody (mAb) directed against TLR2 (clone T2.5) that blocks TLR2-mediated signaling *in vivo*<sup>23</sup>. Groups of mice were either treated with anti-TLR2 or with an isotype control antibody 3 h prior and 1 day post-PR8 infection, while two other groups of mice received anti-TLR2 or control antibody on days 2 and 4 post-PR8 infection (Figure 3A). Similar to the protection achieved with anti-TLR4 IgG (Figure 1), treatment of PR8-infected WT mice with anti-TLR2 antibody significantly protected against lethality when administered on days 2 and 4 post-infection ( $p < 0.001$ ; Figure 3B); however, anti-TLR2 treatment was not effective when administered earlier. These results suggest the presence of a TLR2 agonist released late after PR8 infection contributes to lethality.

To extend these findings, WT, TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, and TLR2/4 double knockout mice were infected with a sub-lethal dose (LD<sub>10</sub>) of PR8 and monitored for 14 days. The TLR2/4 double knockout mice were much more susceptible than the WT or individual knockout mice (Supplementary Figure 3A). ALI was significantly worse in TLR2/4 double-knockout mice than in WT, with inflammatory infiltrates throughout the parenchyma and alveolar spaces (composed of neutrophils and lymphocytes) (Supplementary Figure 3B). These findings suggest that a TLR2 agonist induced early during virus infection is necessary for the resistance of TLR4<sup>-/-</sup> mice to lethal PR8 infection.

### Timing of Eritoran treatment is critical for protection

Neither differential influenza replication (Figure 5A, left panel) nor the levels of inducible IFN- $\beta$  mRNA (Figure 4A, right panel) accounted for the resistance of the TLR4<sup>-/-</sup> mice to PR8 infection. Eritoran therapy protected PR8-infected WT mice (Figure 4B and 4C, open circle, left panel), but did not affect the resistance of TLR4<sup>-/-</sup> mice (Figure 4B and 4D; open circle, right panel), as we reported previously<sup>16</sup>. However, when Eritoran treatment was initiated prophylactically (3 h prior to PR8 infection) and continued daily for an additional 4 days (Figure 4B, pretreatment/early regimen; closed squares), WT mice were not protected from lethality (Figure 4C, closed square, left panel). This finding implies that an early influenza-inducible, but late-acting mediator of lethality and ALI must be the target of Eritoran in WT mice. Surprisingly, this identical regimen rendered TLR4<sup>-/-</sup> mice susceptible to PR8 infection (Figure 4B and 4D, closed square, right panel), indicating that a non-TLR4 target of Eritoran is necessary for the resistance of TLR4<sup>-/-</sup> mice to PR8 infection, consistent with our data obtained with the TLR2/4 double knockout mice. Pre-/early treatment with Eritoran had no effect on the susceptibility of CD14<sup>-/-</sup> or TLR2<sup>-/-</sup> mice (data not shown).

**P5779, an HMGB1 antagonist, blocks influenza-mediated lethality**—We previously reported that lungs of Eritoran-treated, PR8-infected mice (as shown in Figure 1A) showed blunted cytokine induction as well as accumulation of OxPAPC<sup>16</sup>, a danger associated molecular pattern (DAMP) shown by Imai et al. to mediate ALI by its action on macrophages through TLR4<sup>14</sup>. High Mobility Group B1 (HMGB1), a DAMP first implicated in endotoxicity and Gram negative sepsis<sup>24</sup>, has been reported to be released

during severe influenza infection<sup>25</sup> and to activate TLR4 by binding to the TLR4 co-receptor, MD2<sup>26</sup>. HMGB1-stimulated WT murine macrophages induced MyD88- (TNF $\alpha$ ) and TRIF- (IFN- $\beta$ ) dependent gene expression that was inhibited by Eritoran *in vitro* (Figure 5A). Mice infected with PR8 (data not shown) and cotton rats infected with a non-adapted human influenza pdH1N1 strain, exhibited increased circulating HMGB1 that was inhibited by Eritoran treatment *in vivo* (Figure 5B). Thus, HMGB1, like OxPAPC, may represent a DAMP that is released relatively late after infection that contributes to influenza-induced ALI through TLR4 activation. P5779 is a small molecule inhibitor of HMGB1 that was shown recently to prevent MD-2/HMGB1 interaction and block HMGB1-induced TLR4 signaling, while not interfering with LPS-induced cytokine/chemokine induction<sup>27</sup>. P5779 protected mice against hepatic ischemia/reperfusion injury, APAP chemical toxicity, and sepsis<sup>27</sup>. To assess the efficacy of P5779 in influenza infection, WT C57BL/6J mice were infected with PR8 and, two days later mice were treated with either Eritoran (E5564), an inactive control peptide, or with P5779 for 5 consecutive days. Both Eritoran- and P5779-treated mice showed significant survival and lowered clinical scores, while mice treated with the control inhibitor showed higher clinical scores and succumbed to infection (Figure 5C and 5D).

**A PAR<sub>2</sub> antagonist blocks influenza-induced lethality and lung leak**—A host-derived protein, originally termed zonulin (now known to be pre-haptoglobin 2)<sup>28</sup>, was found to increase intestinal permeability by phosphorylation of tight junction proteins<sup>29</sup>. Signaling was dependent upon protease-activated receptor 2 (PAR<sub>2</sub>)<sup>28</sup>, a signaling protein that we have shown previously to interact physically and functionally with TLR4<sup>30</sup>. A zonulin analog peptide antagonist, AT-1001 (larazotide acetate), is well-tolerated in humans and attenuates gut inflammation associated with celiac disease<sup>31</sup>. Recently, AT-1001 was also reported to attenuate ALI in mice induced by intrapulmonary deposition of IgG immune complexes or LPS by inhibiting phosphorylation of the tight junction protein, ZO-1, reducing the number of leukocytes and myeloperoxidase activity in bronchoalveolar lavage fluid (BALF)<sup>32</sup>. Since both PAR<sub>2</sub><sup>-/-</sup> and TLR4<sup>-/-</sup> mice are comparably refractory to lethal PR8 infection<sup>17</sup>, we compared the efficacy of AT-1001 vs. Eritoran therapy during a lethal influenza challenge. WT mice were infected with PR8 and treated with vehicle (saline), Eritoran, or AT-1001 for 5 consecutive days starting on day 2 post-infection. Treatment of mice with AT-1001 resulted in significant protection and lowered clinical scores, comparably to Eritoran treatment (Figures 6A, B). Since AT-1001 reduced the pulmonary edema associated with LPS- or immune complex-induced ALI<sup>32</sup>, we tested whether Eritoran and AT-1001 mediated a decrease in lung edema caused by PR8 infection as measured by wet-to-dry (W/D) weight ratio. Mice infected with PR8 and treated with vehicle showed significantly higher W/D ratios than mice infected with PR8 and treated with either Eritoran or AT-1001 (Figure 6C), suggesting that an additional protective effect of Eritoran during influenza infection is to attenuate ALI by blunting pulmonary edema.

**Contribution of IL-1 to influenza-induced lethality**—We previously showed that IL-1 $\beta$  mRNA is strongly inhibited in the lungs of PR8-infected, Eritoran-treated mice<sup>16</sup>. Subsequently, Teijero et al. reported that influenza-infected IL-1R<sup>-/-</sup> mice exhibited diminished cytokine levels in the BALF 2 days p.i.<sup>12</sup>, but neither ALI nor lethality were

evaluated. rIL-1 receptor antagonist (IL-1Ra; generic anakinra) is used clinically to treat highly inflammatory diseases (*e.g.*, rheumatoid arthritis, cryopyrin-associated periodic syndromes and Macrophage Activation Syndrome)<sup>33</sup>. PR8-infected C57BL/6J WT mice treated therapeutically with rIL-1Ra showed significant, but intermediate, survival (Figure 7A), and clinical scores (Figure 7B), in contrast to the protection afforded by Eritoran. This suggests that while IL-1 $\beta$  participates in mediating influenza-induced disease, other mediators, whose action is inhibited by Eritoran, are likely involved.

### **Eritoran treatment improves the efficacy of oseltamivir therapy against influenza**

The efficacy of Eritoran administered alone or combined with the approved neuraminidase inhibitor antiviral therapy, Tamiflu™ (oseltamivir), was assessed. To be effective, oseltamivir treatment is recommended within 2 days of the onset of symptoms<sup>34,35</sup>. Since, in our model, mice show clinical symptoms starting at day 3 post-infection<sup>16</sup>, we compared mice treated with vehicle, Eritoran alone, oseltamivir alone, or both Eritoran and oseltamivir starting on days 2, 4, or 6 post-infection for 5 consecutive days. When treatment was initiated on day 2 post-infection, both agents were highly protective, with no benefit when administered together (Supplementary Figure 4). However, when administration of treatment was delayed until 4 or 6 days post-infection, treatment with oseltamivir alone showed little protective effect (particularly when administered starting at Day 6), while Eritoran still elicited a significant degree of protection from lethality, as we reported previously<sup>16</sup>. Importantly, when initiated on day 4, the combined Eritoran/oseltamivir treatment resulted in a significant improvement in survival ( $p < 0.05$ ; Supplemental Figure 4A), as well as significant reduction of viral titers ( $p < 0.05$ ; Supplemental Figure 4B). This is consistent with Zheng et al. who showed co-administration of celecoxib, a COX-2 inhibitor, and zanamivir improved survival of influenza-infected mice better than treatment with zanamivir alone<sup>36</sup>. Our previous study showed that Eritoran blunts COX-2 induction during influenza infection<sup>16</sup>.

### **Survivors of PR8 infection after Eritoran treatment are protected from lethal re-infection**

Importantly, mice that were protected from PR8 by Eritoran survived secondary PR8 challenge 4 weeks after initial infection, without additional Eritoran therapy (Supplementary Figure 5A). The half-life of Eritoran is approximately 5 h in rodents<sup>37</sup>, so it would not be expected to be involved in the protective effect seen when survivors were re-infected 28 days after the first PR8 infection. To test this hypothesis, mice were treated with Eritoran for five successive days. Five days after the last Eritoran treatment, mice were infected with PR8. A control group was treated with Eritoran starting 2 days post-infection for 5 consecutive days (days 2-6). As shown in Supplementary Figure 5B, the lethality seen in the mice given Eritoran early is equivalent to mice that were given vehicle (saline), supporting the idea that the half-life of Eritoran is too short to mediate protection observed upon re-challenge of survivors (Supplementary Figure 5A). To confirm that the Eritoran-treated, PR8-infected surviving mice develop an adaptive memory response, we repeated the assay from Supplementary Figure 5A and carried out hemagglutination inhibition assays using the sera from mice infected and treated with Eritoran. In mice that were PR8-infected, followed by Eritoran treatment (days 2-6), strong hemagglutination inhibition titers were observed four weeks after infection (ranging from 640 to 2560;  $n=5$ ). Thus, the anti-inflammatory effect

of Eritoran during primary infection does not prevent development of an adaptive immune response against influenza.

## Discussion

Influenza is a major health concern globally. The virus mutates rapidly, leading to anti-viral resistance or altered expression of immunogenic epitopes such that extant vaccines are rendered ineffective. Based on our previous studies<sup>16</sup>, we demonstrated that Eritoran (E5564), a well-tolerated, synthetic TLR4 antagonist<sup>38,39</sup>, represents a novel therapeutic approach to ameliorate influenza-induced ALI by blocking TLR-mediated signaling in response to host-derived DAMPs. Herein, we delineate further the cellular and molecular underpinnings for both induction of ALI and its abatement by Eritoran therapy.

Our data show that Eritoran, administered after infection, does not alter the refractoriness of TLR4<sup>-/-</sup> mice to influenza infection<sup>16</sup>; both Eritoran and anti-TLR4 antibody therapy protected WT mice, and, Eritoran binds to both CD14 and MD2 *in vitro*<sup>16</sup>, both of which are required for TLR4 signaling<sup>19</sup>. In addition to 9R-VIPER (Suppl. Fig. 2), the cell-permeating TLR2 TIR decoy peptide, 2R9, that blocks TLR4, 7, and 9 signaling by blocking recruitment of TIRAP to the TLR TIR domain<sup>40</sup>, protected WT mice from PR8-induced lethality comparably to Eritoran. Together, these findings strongly implicate TLR4 in both influenza-induced disease and as a target for Eritoran-mediated protection. Since Eritoran pre/early treatment renders TLR4<sup>-/-</sup> mice susceptible to PR8 infection, Eritoran must also interact with a non-TLR4 PRR that is required early for induction of resistance. We postulate that Eritoran, administered by the pre/early treatment, binds CD14 and inhibits transfer of specific PAMPs or DAMPs to TLR2<sup>41</sup>, TLR3<sup>42</sup>, TLR7, and/or TLR9<sup>43</sup> since CD14 has been shown to act as a co-receptor for each of these PRRs, and since the latter three have been implicated in the host response to influenza<sup>5-12</sup> and are capable of inducing IFN- $\beta$ <sup>21</sup>. This hypothesis is supported by our observation that WT and TLR4<sup>-/-</sup> mice, when treated with Eritoran by the early regimen, are as susceptible as IFN- $\beta$ <sup>-/-</sup> mice to PR8 infection<sup>16</sup>, and that CD14<sup>-/-</sup> mice could not be protected by Eritoran pre/early or therapeutic regimens<sup>16</sup>, presumably because they cannot produce cytokines as suggested by Pauligk *et al*<sup>13</sup>. Our previous work showed that 5,6-dimethylxanthene-4-acetic acid (DMXAA), a potent inducer of IFN- $\beta$ , protected WT, but not IFN- $\beta$ <sup>-/-</sup> mice, from PR8-induced lethality when administered 3 h prior to and on day 1 post-PR8 infection<sup>44</sup>, again supporting the need for IFN- $\beta$  early in infection for survival.

The role of TLR2 in Eritoran-mediated protection of PR8-infected mice is enigmatic. Despite the fact that we observed that anti-TLR2 mAb, but not control IgG, protected WT mice from PR8 when administered therapeutically, the same anti-TLR2 mAb, when administered -3 h prior to and 1 day after PR8 infection, provided only minimal protection. This observation strongly suggests that TLR2, like TLR4, also plays a damaging role later in infection. OxPAPC<sup>45</sup> and HMGB1<sup>26</sup> have been reported to be both TLR2 and TLR4 agonists that are induced later in infection. These DAMPs may synergize to increase TLR2 expression and/or TLR2-dependent signaling. Our observation that TLR2/4 double knockout mice are highly susceptible to sublethal PR8 infection may suggest that the absence of both TLR2 and TLR4 would leave MyD88 more available for IL-1 and IL-18 signaling.

Alternatively, it is also possible that TLR2 is required to produce something that mediates resistance to PR8 in TLR4<sup>-/-</sup> mice.

Eritoran blunts both the influenza-induced “cytokine storm” and the accumulation of OxPAPC<sup>16</sup>, an oxidized phospholipid TLR4 DAMP<sup>14</sup>. Mechanistically, these findings have now been extended by showing that (i) Eritoran blocked HMGB1-mediated TLR4-dependent signaling *in vitro*, HMGB1 release into serum *in vivo*, and protected comparably to P5779, a highly selective HMGB1 inhibitor; and (ii) inhibited pulmonary lung edema equivalently to AT-1001, an inhibitor of zonulin-induced pulmonary edema<sup>32</sup>. Others have shown that influenza induces necroptosis in lung epithelial cells<sup>46,47</sup>, and that HMGB1 is released from necroptotic cells<sup>48</sup>. Therefore, it is possible that treatment of mice with Eritoran blocks release of HMGB1 by blocking necroptosis as well as the subsequent signaling through the TLR4/MD2 complex. We previously reported that lung sections from placebo-treated mice showed significant pathology, including epithelial necrosis, and that Eritoran-treated mice had intact lung epithelia compared to the placebo group<sup>16</sup>.

Eritoran acts on cells that express TLR4/MD2. At this time, we do not know if stromal cells and/or cells of myeloid lineage are involved in the protective effect. Zhang et al. recently reported that, in addition to its profound effects on the lung, influenza infection affects mucosal epithelium in the intestinal tract<sup>49</sup>, consistent with our report of systemic inflammation induced by PR8 infection<sup>16</sup> and our results showing that administration of AT-1001 (larazotide acetate), an inhibitor of intestinal and lung leakage<sup>28,31,32</sup>, reduced PR8-induced mortality. Thus, it is possible that influenza-mediated intestinal leakage underlies changes in gut microbiota that are required for development of adaptive immunity<sup>50</sup>. In a model of trauma/hemorrhagic shock, Sodhi et al. reported that ALI was induced in WT mice, but not in mice selectively engineered to lack TLR4 on gut epithelial cells<sup>51</sup>. In this model, ALI in WT mice could be blocked by neutralizing HMGB1 or by treatment with a small molecule TLR4 inhibitor<sup>51</sup>. Additional experiments will be required to test the hypothesis that DAMP release by influenza-induced necrosis of lung epithelial cells acts locally to induce ALI, as well systemically to cause gut leak and an altered microbiota<sup>50</sup>.

Additionally, the partial protection achieved by treatment of PR8-infected mice supports a role for IL-1 signaling in PR8-induced lethality, as suggested by Tejeiro *et al.*<sup>12</sup>; however, the data also suggest a role for additional inflammatory mediators in PR8-induced disease since the protection is partial. Nonetheless, the IL-1 $\alpha$  precursor (pro-IL-1 $\alpha$ ) is expressed in the nuclei of most tissues bound to chromatin<sup>52</sup>. During an acute ischemic event, the loss of oxygenation and increased acidosis triggers release of the (pro-IL-1 $\alpha$  into the cytosol and then into the milieu upon cellular necrosis. Pro-IL-1 $\alpha$  binds to the IL-1R1 and is biologically active<sup>53</sup>, causing the induction of chemokines that mediate neutrophilic infiltration. Thus, the partial protection elicited by IL-1Ra (Figure 7) may be attributable to blockade of the interaction of pro-IL-1 $\alpha$  with the IL-1R1, in addition to the down-regulation of IL-1 $\beta$  mRNA induced in PR8-infected, Eritoran-treated mice.

Our observation that Eritoran-treated mice that survived initial infection and exhibited blunting of the cytokine storm were protected from secondary influenza infection suggests



that Eritoran does not prevent development of an adaptive immune response. During primary influenza infection, antibody levels are detectable by day 10 post-infection and persist above baseline for weeks<sup>54</sup>. We observed that significant levels of anti-HA antibodies were induced in mice infected with PR8 followed by Eritoran therapy on days 2-6, even 4 weeks after infection. Thus, Eritoran does not prevent induction of an adaptive immune response to infection. While such mice remain refractory to secondary PR8 infection 28 days after the primary infection, future studies will be required to determine if Eritoran treatment alters the enhanced sensitivity to secondary bacterial infections seen in influenza-infected mice.

Overall, our findings underscore the complex nature of microbe-host inflammatory cell interactions that control the host's ability to respond to invading virus. We provide evidence that multiple receptors on innate immune cells are likely involved; however, it is also likely that unknown interactions among these receptors in response to microbial and host ligands significantly affect, both qualitatively and quantitatively, the host response.

## Methods

### Reagents

Eritoran (E5564) was provided by Eisai Inc. (Andover, MA) and prepared as described previously<sup>16</sup>. *Escherichia coli* K235 LPS was prepared as previously described<sup>55</sup>. The anti-TLR2 IgG and isotype control IgG were purchased from Affymetrix (Santa Clara, CA). Recombinant HMGB1 was provided by Kevin Tracey (Feinstein Institute for Medical Research, Manhasset, NY). 9R-VIPER was synthesized by GenScript (Piscataway, NJ). P5779 and control peptide were provided by Yousef Al-Abed (Feinstein Institute for Medical Research, Manhasset, NY). The anti-TLR4 IgG and isotype control IgG were provided by Thierry Roger and Thierry Calandra (Infectious Diseases Service, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland). AT-1001 was kindly provided by Alessio Fassano (Division of Pediatric Gastroenterology and Nutrition, MGH, Boston, MA). IL-1Ra (anakinra) was obtained from Sweden Orphan BioVitrum (SOBI), Stockholm, Sweden.

### Mice and cotton rats

Six to 8-week old, WT C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice with targeted mutations were bred onto or derived directly from a C57BL/6J background. TLR4<sup>-/-</sup> mice (provided by Shizuo Akira, Osaka, Japan; bred at UMB (Baltimore, MD) and Univ. Massachusetts Medical School, (Worcester, MA)), TRIF<sup>-/-</sup> (bred at U. Mass. Med. School). IRAK4<sup>KDKI</sup> (provided by Lilly Research Laboratories, Indianapolis, IN, bred at UMB), TLR2<sup>-/-</sup> mice (provided by Shizuo Akira; bred at U. Mass. Med. School), and TLR2/TLR4 double knockout mice (bred at U. Mass. Medical School (Worcester, MA)). BALB/cByJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mouse strains were housed and bred in specific pathogen-free conditions. Experiments were conducted in accordance with the guidelines set forth by the University of Maryland, Baltimore and the University of Massachusetts Medical School Department of Animal Medicine and approved by each institute's Institutional Animal Care and Use Committees (IACUC).

Inbred young adult (4-8 weeks old) cotton rats (*Sigmodon hispidus*) were bred at Sigmovir Biosystems, Inc. (Rockville, MD). All cotton rat experiments were conducted with Institutional Animal Care and Use Committee (IACUC) approval from Sigmovir Biosystems, Inc.

## Virus

Mouse-adapted H1N1 influenza A/PR/8/34 virus ("PR8") (ATCC, Manassas, VA) was grown previously described<sup>56</sup> and was kindly provided by Dr. Donna Farber (Columbia University, NY). Mouse-adapted H1N1 influenza maCa.04 was provided by Daniel Perez (University of Georgia, Athens, GA). Non-adapted human influenza virus strain pH1N1 was obtained and grown as previously described<sup>57</sup>.

## Virus challenge and treatments

Mice were infected with mouse-adapted influenza virus, strains A/PR/8/34 intranasally (i.n.) (PR8; ~7500 TCID<sub>50</sub>, 25 µl/nares) or maCa.04 (~2200 TCID<sub>50</sub>, i.n.). Two days after infection, mice received either vehicle or E5564 intravenously (Eritoran; 200 µg/mouse in 100 µl; i.v.), anti-TLR4 IgG (2 mg/mouse, i.v.) or its isotype control IgG, anti-TLR2 (T2.4 clone; 100 µg/mouse, i.v.) or its isotype control IgG, 9R-VIPER (200 nmol; i.p.), P5779 or its control peptide (500 µg/mouse; i.p.), AT-1001 (150 µg/mouse, i.v.), IL-1Ra (150 µg/mouse, i.v.), or Oseltamivir orally (p.o.) (1 mg/mouse) daily (Day 2 to Day 6). In some experiments, groups of mice were treated with Eritoran or Oseltamivir starting at day 4 or day 6 post-infection and treated for 5 successive days. In some experiments, some groups of mice were treated with E5564 (200 µg/mouse, i.v.) 3 h prior to infection with PR8 and then treated for 4 consecutive days starting day 1 post-infection (days 1-5). In some experiments, groups of mice were treated with anti-TLR2 (T2.5 clone, 100 µg/mouse, i.v.) or its control isotype IgG 3 h prior to infection with PR8 and then treated for a second time at day 1 post-infection for a total of two treatments. Mice were monitored daily for survival, weight loss, and clinical signs of illness (*e.g.*, 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<sup>16,58</sup>.

## Histopathology

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

## TLR2/4 double knockout mouse studies

C57BL/6J WT, TLR4<sup>-/-</sup>, TLR2<sup>-/-</sup>, and TLR2/4 double knockout mice were 8-12 weeks of age at the time of infection. Influenza A/PR/8/34 (Charles River Laboratories, Wilmington, MA) virus stocks were diluted in sterile phosphate buffered saline (PBS) and kept on ice prior to use. Mice were anesthetized with isoflurane and infected 40,000 pfu (30 µl; i.n.) per mouse. In two assays, mice were monitored for survival for 14 days post-infection. In another assay, mice were sacrificed on day 5 post-infection and the lungs harvested for

pathology. Lungs were inflated *in situ* with 1 ml of formalin (10% formaldehyde) obtained from the U. Mass. Med. School Morphology Core. The inflated lungs were fixed for 24-48 h, after which they were transferred to PBS. Lungs were bisected vertically and processed for paraffin embedding by the University of Massachusetts Medical School Morphology Core. Slides were prepared and H&E stained for histological analysis.

### **Lung wet-to-dry weight ratio**

The lung wet-to-dry (W/D) weight ratio was used as an index of pulmonary edema after infection with influenza in mice that were untreated or treated with either E5564 or AT-1001. On day 7 post-infection, mice were euthanized and dissected for the total lung, and the lung weight was measured immediately after excision (wet weight). Lung tissue was then air dried for 5-6 days and re-weighed everyday until a dry weight was stable and this acted as the final dry weight. The W/D weight ratio was calculated by dividing the wet by the final dry weight<sup>59</sup>.

### **Quantitative real-time PCR (qRT-PCR)**

Total RNA isolation and qRT-PCR were performed as previously described<sup>16</sup>. Levels of mRNA for specific genes are reported as relative gene expression normalized to mock-infected lungs.

### **Macrophage cell cultures and treatment**

Thioglycollate-elicited peritoneal macrophages from C57BL/6J WT mice were enriched as described<sup>16</sup> after plating in 12-well ( $2 \times 10^6$  cells/well) tissue culture plates. Macrophages were pre-treated with Eritoran (10 ng/ml) for 1 h and then stimulated with LPS (10 ng/ml) or HMGB1 (1  $\mu$ g/ml) for 2 h.

### **HMGB1 serum levels**

Cotton rats were infected with  $9 \times 10^4$  TCID<sub>50</sub> of pH1N1 intranasally on day 0. On day 2 post-infection, animals were treated with saline (mock) or with 37.33 mg/kg of Eritoran i.v., daily. Blood samples were collected on days 0, 4, and 6 post-infection and serum was used for measuring HMGB1 levels using an ELISA kit according to manufacturer's protocol (IBL International, Toronto, Ontario, Canada).

### **Viral titration**

Virus titers were obtained from supernatants of lung homogenates of PR8-infected mice that were either left untreated (NT) or treated with Eritoran only, Oseltamivir only, or both Eritoran and Oseltamivir and harvested on day 7 post-infection and expressed at TCID<sub>50</sub>/ml as described previously<sup>44</sup>.

### **Hemagglutination Inhibition (HAI) assay**

Prior to serological analysis, mice sera were treated with receptor-destroying enzyme (RDE; Denka Seiken, Cat. # 370013). Serum was mixed with RDE and incubated at 37°C for 20 h, followed by heat inactivate at 56°C for 30 min. Finally, 0.6 ml of 1× PBS was added to give a 1:10 dilution of the initial sample. To quantify HAI activity, two fold serial dilutions of

treated sera in 96-well plates were incubated with 4 hemagglutinating units of PR8 virus at room temperature for 15 min. Turkey red blood cells at 0.5% in PBS were added to each dilution and gently mixed. Plates were incubated for 30 min-1 h at room temperature. The HAI titer was reported as the reciprocal of the highest dilution of serum that inhibited hemagglutination.

### 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  $\geq 3$  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.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

This work was supported by NIH grants AI104541 (SNV and JCGB), DK048373 (AF), AI15614 (CD), Swiss National Science Foundation grants 320030\_149511 (TR), 310030\_138488 and 149511 (TC), by Science Foundation Ireland grant 11/PI/1056 (AGB).

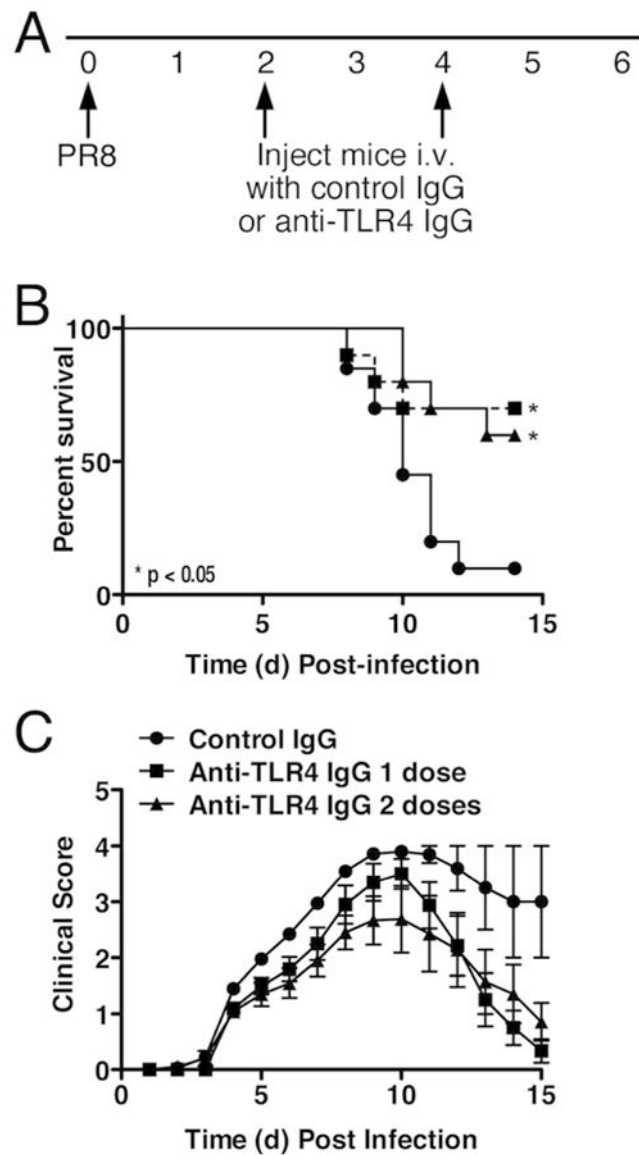
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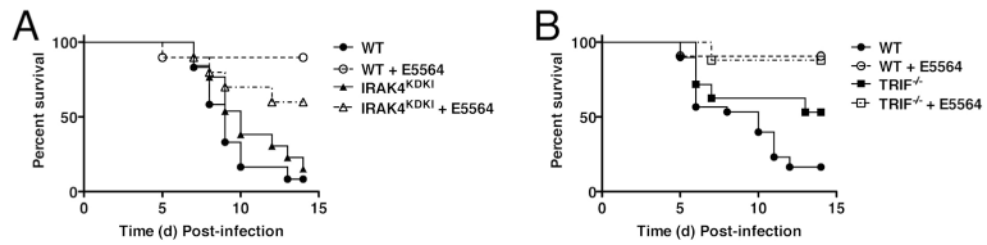
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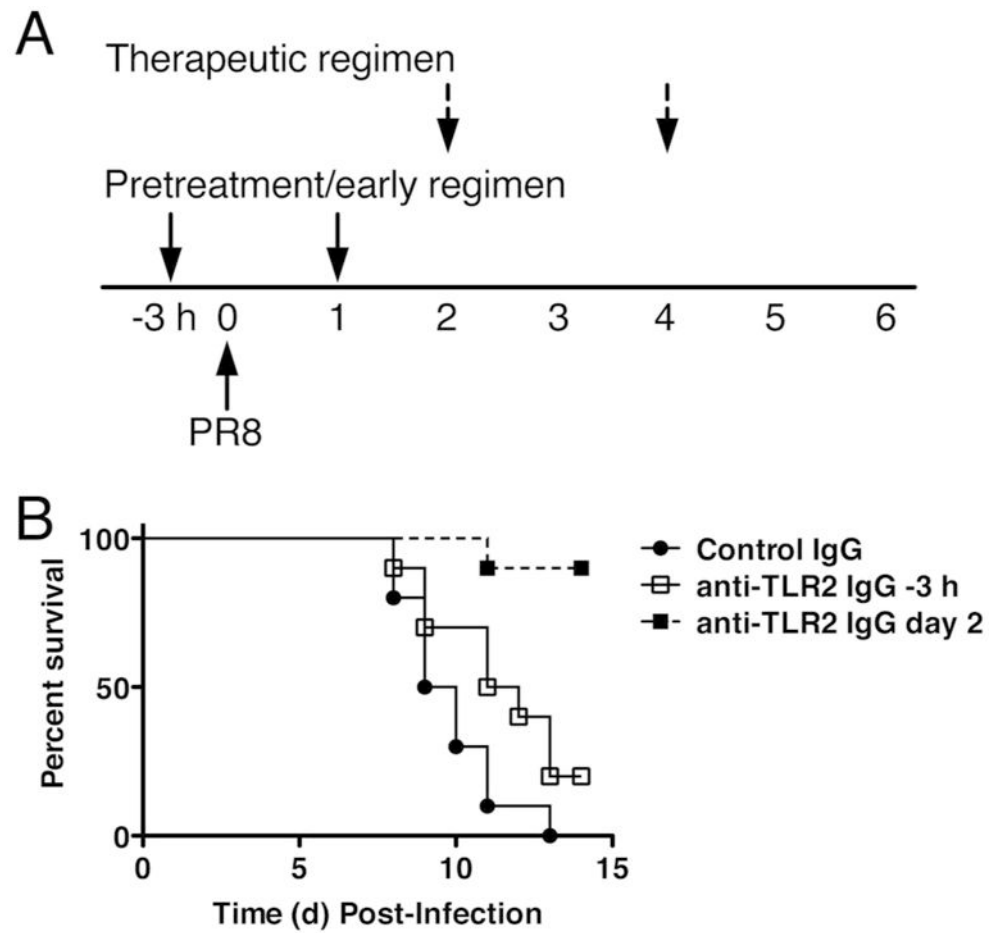
**Figure 1.**

Anti-TLR4 IgG treatment protects mice from lethal influenza challenge. (A) C57BL/6J mice were infected with mouse-adapted influenza strain PR8 (~7500 TCID<sub>50</sub>, i.n.). Mice received either control IgG or a highly specific anti-TLR4 IgG (2 mg/mouse; i.v.) once (day 2 only) or twice (days 2 and 4). Survival (B) and clinical scores (C) were monitored daily. Each graph represents the combined results of 2 separate experiments (5 mice/treatment group/experiment).

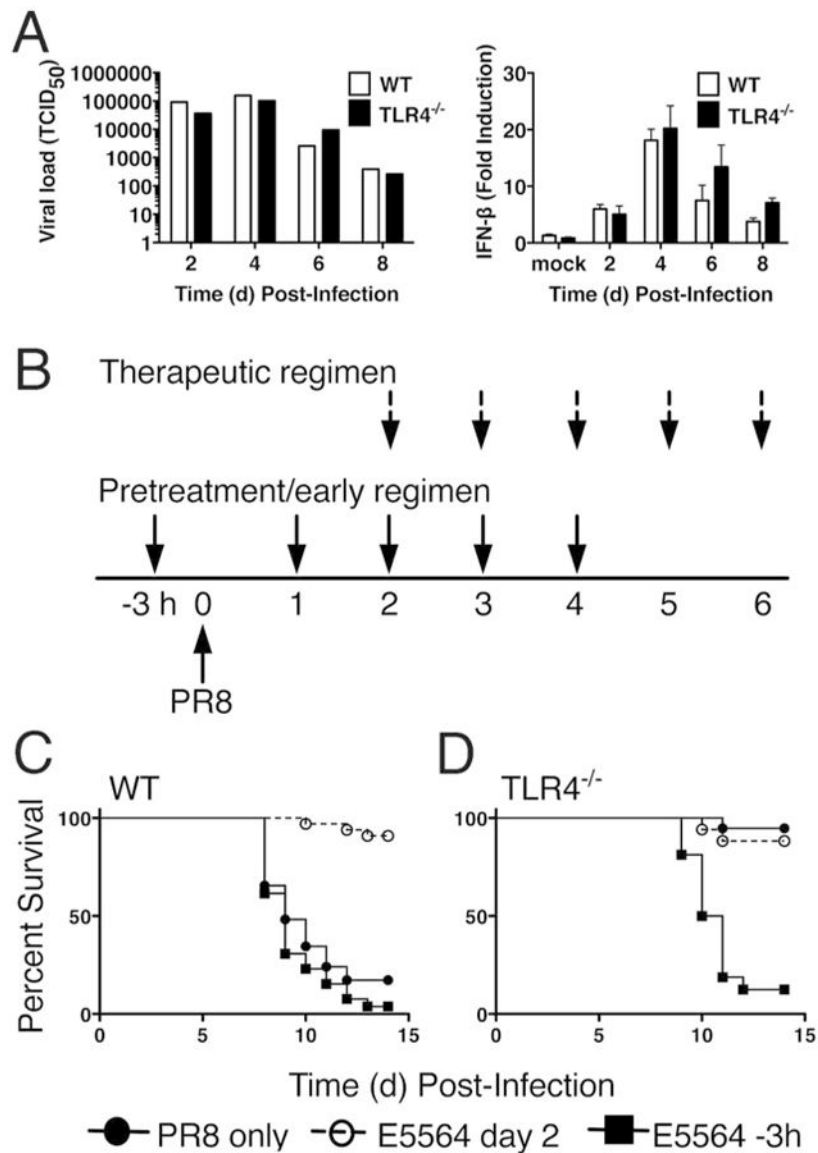


**Figure 2.**

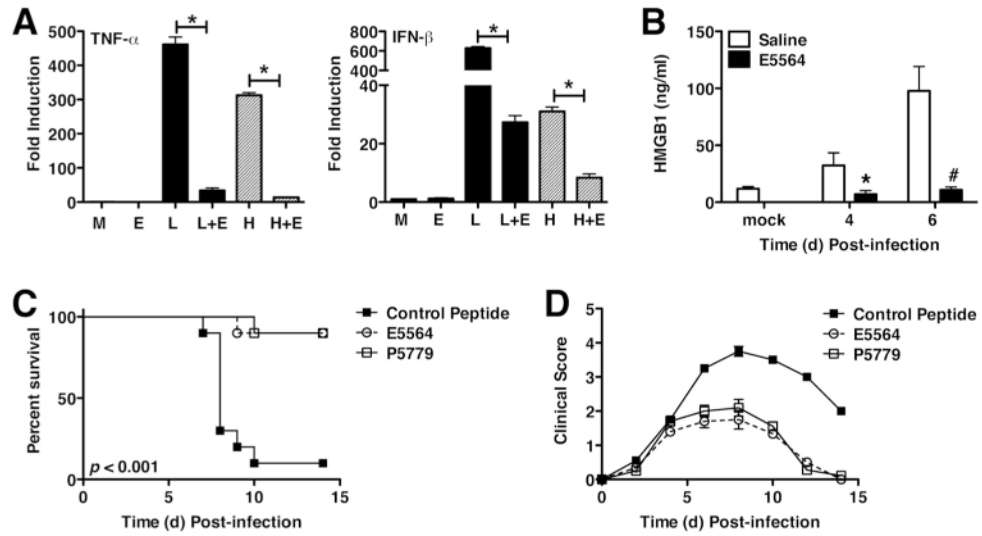
Effect of Eritoran on IRAK4<sup>KDKI</sup> and TRIF<sup>-/-</sup> mice. WT C57BL/6J (A and B), IRAK4<sup>KDKI</sup> (A) and TRIF<sup>-/-</sup> (B) mice were infected with mouse-adapted influenza strain PR8 (~7500 TCID<sub>50</sub>, i.n.). Mice received vehicle (saline; i.v.) or Eritoran (E5564; 200 µg/mouse; i.v) daily from day 2 to day 6 post-infection. Survival was monitored for 14 days. Data shown is combined results of 2-3 separate experiments (5-10 mice/treatment group/experiment).



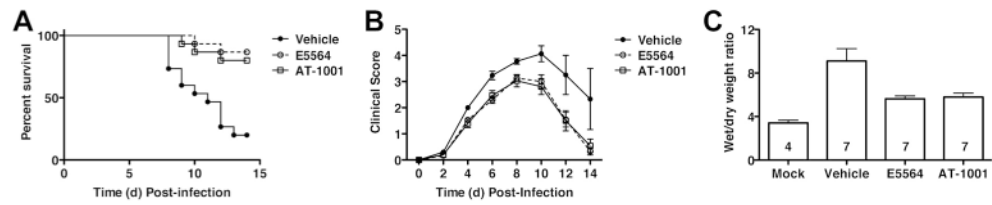
**Figure 3.** Anti-TLR2 IgG treatment protects mice from lethal influenza challenge. (A) Experimental protocol. C57BL/6J mice were either treated with isotype control IgG or anti-TLR2 (T2.5; 100  $\mu$ g/ms; i.v.) 3 h prior to and 1 day post-infection or on days 2 and 4 post-infection. Survival (B) was monitored daily. Data shown is combined results of 2 separate experiments (5 mice/treatment group/experiment).



**Figure 4.** Pretreatment of WT or TLR4<sup>-/-</sup> mice with Eritoran does not protect from lethal influenza challenge. (A) WT and TLR4<sup>-/-</sup> mice were infected with mouse-adapted influenza strain PR8 (~7500 TCID<sub>50</sub>, i.n.) and sacrificed on days 2, 4, 6 and 8 post-infection. Lungs were harvested and assayed for viral titers and IFN-β mRNA. Data shown is combined results of 2 separate assays (4-5 mice/group/experiment). (B) Basic experimental protocol used to compare pretreatment/early vs. therapeutic treatment with Eritoran in PR8-infected mice. C57BL/6J WT and TLR4<sup>-/-</sup> mice were either untreated (solid circles), or treated with Eritoran starting 3 h prior to infection and on day 1 for 4 successive days (3 h; solid squares; pretreatment/early regimen), or starting 2 days after infection for 5 successive days (open circles; therapeutic regimen). (C and D) Mice were monitored daily for survival. The data represent the combined results of two separate experiments (5-6 mice/treatment/experiment).

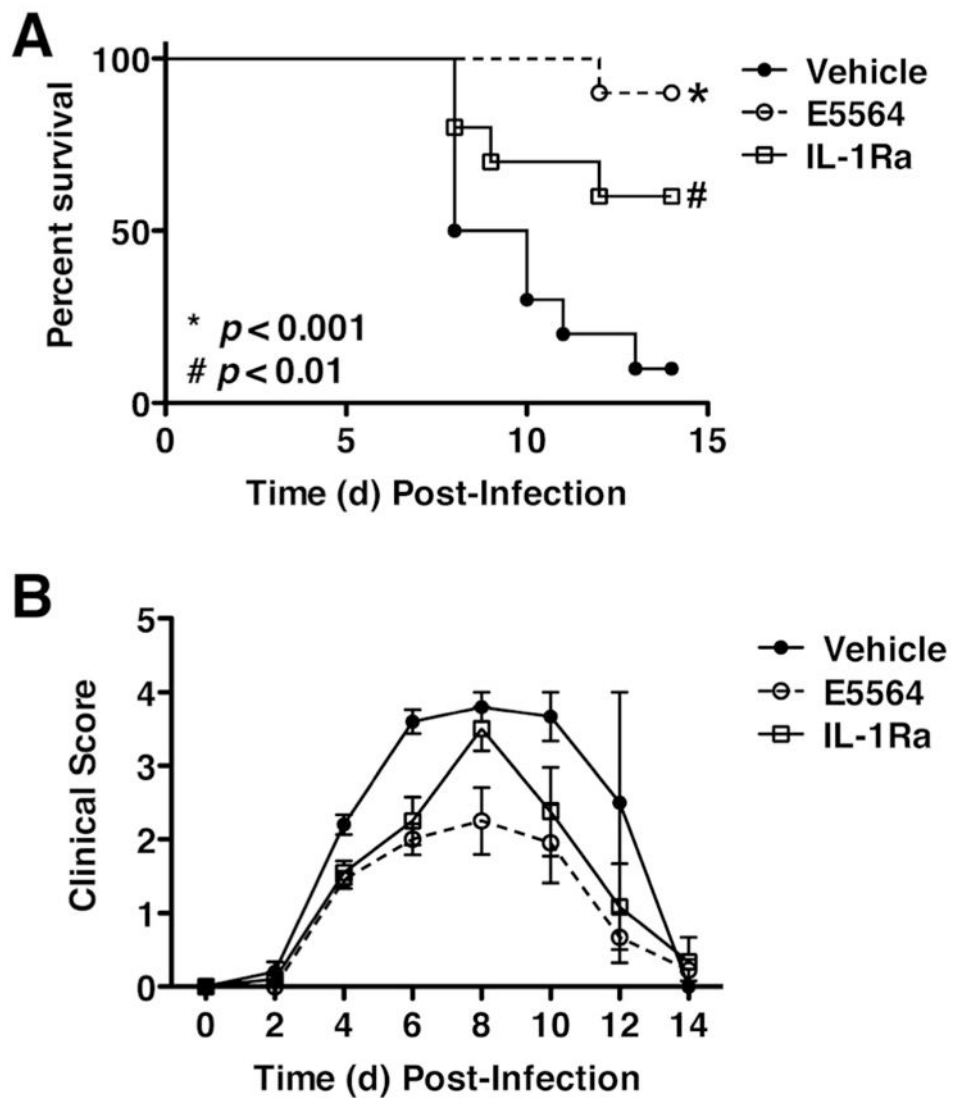


**Figure 5.** Eritoran blunts HMGB1-induced TLR4 signaling *in vitro* and influenza-induced HMGB1 release and lethality *in vivo*. (A) Thioglycollate-elicited C57BL/6J macrophages were treated with medium alone (M) or Eritoran (E; 10 ng/ml) for 1 h, and then with LPS (L; 10 ng/ml) or HMGB1 (H; 1  $\mu$ g/ml) for 2 h. Total RNA was processed and subjected to qRT-PCR for detection of TNF $\alpha$  or IFN- $\beta$  mRNA expression. (B) Cotton rats (6-8/time point/treatment) were infected with pdH1N1 A/California/04/09 ( $9 \times 10^5$  TCID $_{50}$  by i.n.), then treated with Eritoran or saline starting at 2 days post-infection. Serum HMGB1 levels were measured by ELISA (\*  $p < 0.05$ ). (C) C57BL/6J mice were infected with mouse-adapted influenza strain PR8 ( $\sim 7500$  TCID $_{50}$ , i.n.). Mice received control peptide (500  $\mu$ g/mouse; i.p.), Eritoran (E5564; 200  $\mu$ g/mouse; i.v.), or the HMGB1 peptide, P5779 (500  $\mu$ g/mouse; i.p.) from day 2 to day 6 post-infection. Survival (C) and clinical scores (D) were monitored daily for 14 days. Data shown is combined from 2 experiments (5-10 mice/treatment group/experiment).



**Figure 6.**

Effect of AT-1001 against lethal influenza challenge. (A) C57BL/6J mice were infected with mouse-adapted influenza strain PR8 (~7500 TCID<sub>50</sub>, i.n.; ~LD<sub>90</sub>). Mice received vehicle (saline; i.v.), Eritoran (E5564; 200 µg/mouse; i.v.), or AT-1001 (150 µg/mouse; i.v.) from day 2 to day 6 post-infection. Survival (A) and clinical scores (B) were monitored daily for 14 days. Data shown is combined from 3 separate experiments (5 mice/treatment group/experiment). (C) Lung wet-to-dry (W/D) weight ratio as an index for pulmonary edema after infection. C57BL/6J mice were infected and treated as described above. On day 7 post-infection, lungs were harvested and lung weights were measured immediately after excision and recorded as wet weight. Lung tissue was air dried for 5-6 days and re-weighed until a stable dry weight obtained. The W/D weight ratio was calculated by dividing the wet by dry weight. The N for each group is indicated in each bar. Each vertical bar represents the mean ( $\pm$  s.e.m.).



**Figure 7.** Effect of IL-1Ra against lethal influenza challenge. C57BL/6J mice were infected with mouse-adapted influenza strain PR8 (~7500 TCID<sub>50</sub>, i.n.). Mice received vehicle (saline; i.v.), Eritoran (E5564; 200 µg/mouse; i.v.), or the IL-1Ra (150 µg/mouse; i.v.) from day 2 to day 6 post-infection. Survival (top panel) and clinical scores (bottom panel) were monitored daily for 14 days. Data shown is combined from 2 separate experiments (5 mice/treatment group/experiment).