EGFR kinase activity is required for TLR4 signaling and the septic shock response

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Abstract

Mammalian Toll-like receptors (TLR) recognize microbial products and elicit transient immune responses that protect the infected host from disease. TLR4—which signals from both plasma and endosomal membranes—is activated by bacterial lipopolysaccharides (LPS) and induces many cytokine genes, the prolonged expression of which causes septic shock in mice. We report here that the expression of some TLR4-induced genes in myeloid cells requires the protein kinase activity of the epidermal growth factor receptor (EGFR). EGFR inhibition affects TLR4-induced responses differently depending on the target gene. The induction of interferon- β (IFN- β) and IFN-inducible genes is strongly inhibited, whereas TNF- α induction is enhanced. Inhibition is specific to the IFN-regulatory factor (IRF)-driven genes because EGFR is required for IRF activation downstream of TLR—as is IRF co-activator β -catenin—through the PI3 kinase/AKT pathway. Administration of an EGFR inhibitor to mice protects them from LPS-induced septic shock and death by selectively blocking the IFN branch of TLR4 signaling. These results demonstrate a selective regulation of TLR4 signaling by EGFR and highlight the potential use of EGFR inhibitors to treat septic shock syndrome.

Keywords AKT; β -catenin; EGFR; IRF; PI3 Kinase; septic shock; TLR

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction; Signal Transduction

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Introduction

Toll-like receptors (TLRs) represent a specialized class of innate immune sensors that play a key role in detecting invading pathogens [1–4]. The microbial components and their replication products, known as pathogen-associated molecular patterns (PAMPs), which include nucleic acids, lipids and proteins of microbial origin, can be directly recognized by the TLRs. TLRs are type I transmembrane proteins, with long ectodomains, containing leucine-rich

repeats that recognize the PAMPs, and short cytoplasmic domains, containing Toll/IL-1 receptor (TIR) domain, required for the intracellular signaling events. Till date, thirteen TLRs have been identified in mammals; some are expressed on the plasma membrane and others on the endosomal membrane. Upon interaction with the PAMPs in the respective location, the TLRs trigger the signaling process which, via a series of protein–protein interactions, phosphorylation and ubiquitination reactions, activates the latent transcription factors, NF- κ B and IRF-3 [3,5–7]. The activated transcription factors translocate to the nucleus and induce transcription of cytokines and other antimicrobial genes [8].

Unlike other TLRs, TLR4 is expressed on and signals from both the cell surface and the endosomal membrane. It detects lipopolysaccharides (LPS) from Gram-negative bacteria, by a coordinated action of two additional proteins, CD14 and MD-2 [9,10]. LPS-activated TLR4 triggers two distinct signaling branches through the recruitment of the two TIR-containing adaptor proteins, MyD88 and TRIF, respectively [11]. MyD88 is recruited to the cell surface TLR4 by an additional adaptor, TIRAP, whereas TRIF is recruited to the endosomal TLR4 by an additional adaptor, TRAM [12,13]. The MyD88-dependent TLR4 signaling branch primarily activates NF- κ B, which triggers the induction of inflammatory cytokines. The TRIF-dependent TLR4 signaling branch activates both NF- κ B and IRF-3, the combined action of which is responsible for the induction of the IFN- β gene [13]. In contrast to the protective antimicrobial function, excessive TLR4 signaling can be detrimental to the host by causing bacterial sepsis, an inflammatory response, caused by dysregulated TLR4 signaling [14]. Experimentally, a septic response can be elicited in mice by LPS administration; pulmonary functions and the structural integrity of the lung are severely compromised during this response [15,16].

TLR4/TRIF-dependent induction of IFN- β , via transcriptional activation of IRF-3, contributes to the promotion of bacterial sepsis [17,18]. We have shown that HDAC-dependent deacetylation of β -catenin is required for the complete activation of IRF-3 and IFN- β induction, by TLR4 and TLR3 signaling [19]. Consequently, mice deficient in TRIF, HDAC6 or IRF-3 are protected from LPS-induced sepsis and viral pathogenesis. We have also shown that TLR3, another endosomal TLR, which senses dsRNA and signals exclusively through TRIF to activate NF- κ B and IRF-3, requires tyrosine

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phosphorylation of its TIR domain for signaling. The TIR domain of TLR3 has two critical tyrosine residues, Tyr759 and Tyr858, the phosphorylation of which is essential for the recruitment of TRIF to TLR3 [20]. The protein tyrosine kinase, EGFR, is recruited to the dsRNA-activated TLR3 and initiates the signaling process by phosphorylating Tyr858 of TLR3 [21]. A role of EGFR in the induction of cytokines, such as IL-6, has been noted before [22–24]. The most well-known cellular function of EGFR is its ability to signal from the plasma membrane; upon binding EGF or other ligands, it dimerizes triggering the tyrosine kinase activity. But recent studies suggest that EGFR can also be activated without ligands [25], it can function in intracellular membranes [21], and it functions as a protein kinase in the nucleus [26]. Because TLR4 is tyrosine-phosphorylated upon LPS stimulation [27] and its endosomal branch signals through TRIF, we investigated whether, like TLR3, TLR4 also requires the EGFR kinase for transcriptional signaling. Our results demonstrate that the EGFR activity is required for some, but not all, branches of TLR4 signaling.

Results

In myeloid cells, EGFR is required for TLR4 signaling by the IRF, but not the NF- κ B, pathway

Our earlier observation that the protein tyrosine kinase, EGFR, is required for TLR3 signaling [21], which uses TRIF as the exclusive adaptor, prompted us to examine the need of EGFR for the TRIF-dependent signaling pathway used by TLR4. In this study, we have used human and mouse myeloid cells, all of which expressed, as expected, various levels of endogenous EGFR (Appendix Fig S1A). To block the EGFR kinase activity, we used gefitinib, a clinically used EGFR-specific inhibitor, which completely abolished the EGF-induced phosphorylation of EGFR (Appendix Fig S1B). Because IFN- β induction by TLR4 requires TRIF, we used it as the readout. In the mouse macrophage cell line RAW264.7, the induction of *Irfb1* mRNA by LPS, the TLR4 ligand, peaked at 6 h and it was almost completely inhibited by gefitinib (Fig 1A). As expected, the same was true for IFN- β protein synthesis and secretion (Fig 1B). The TRIF branch of TLR4 signaling activates the IRF family of transcription factors, which are required for IFN induction. Because IRFs can also induce many ISGs, LPS treatment induced the ISG, *Ifit1*, and this induction was completely blocked by not only gefitinib, but also erlotinib and AG1478, two other inhibitors of EGFR (Fig 1C). This phenomenon was true for other myeloid cells as well. In bone marrow-derived dendritic cells (BMDCs), induction of both *Irfb1* mRNA and *Ifit1* mRNA was inhibited by gefitinib (Appendix Fig S2A) and the *IFIT1* mRNA induction was inhibited in the human monocytic line, THP1 (Appendix Fig S2B). Similarly, in primary mouse bone marrow-derived macrophages (BMDMs), the induction of *Irfb1* and *Ifit1* mRNAs was inhibited by gefitinib (Fig 1D and Appendix Fig S2C). In BMDMs from *IFNAR* KO mouse, which do not respond to type I IFN, *Irfb1* mRNA was induced by LPS and the induction was inhibited by gefitinib (Fig 1D); however, there was little induction of *Ifit1* mRNA in these cells treated with only LPS, indicating that its induction was primarily mediated by LPS-induced IFN (Appendix Fig S2C). We observed similar inhibition of the induction of IFN- β mRNA, when expression of EGFR (ErbB1) was

knocked down in RAW264.7 cells by the corresponding shRNA, demonstrating that the ErbB1 isoform of EGFR is important here (Fig 1E). These results established that the induction of the IRF-driven genes by TLR4 required EGFR and its protein kinase activity.

To extend our analysis to those TLR4-induced genes whose transcription is driven by NF- κ B, the other major transcription factor activated by LPS, we chose TNF- α as the prototype. Surprisingly, the induction of *Tnf* mRNA by LPS in RAW264.7 cells was not inhibited by gefitinib; this was true for different time points (Fig 2A) and different doses of LPS treatment (Appendix Fig S3A). The amounts of TNF- α secreted by gefitinib-treated and untreated cells were also similar (Fig 2B). Similarly, gefitinib did not inhibit *TNF* mRNA induction in the human monocytic THP1 cells (Appendix Fig S3B). In mouse BMDMs, it strongly enhanced *Tnf* mRNA induction (Appendix Fig S3C); such enhancement, although to a much lesser degree, was also observed in RAW264.7 cells (Fig 2A and Appendix Fig S3A).

The above observations led us to determine the need of EGFR for the induction of all genes by TLR4. For this purpose, we performed mRNA expression profiling of RAW264.7 cells that had been LPS-treated in the presence or the absence of gefitinib. Three biological replicates were analyzed and those mRNAs, which were induced by LPS at least twofold, were selected for our analysis. The ratio of the fold induction in the absence of the inhibitor and that in its presence (E/F) gave us the index of inhibition (examples in Fig 2C, upper table); the reciprocal ratio (F/E) provided the enhancement index (examples in Fig 2C, lower table). Among the 312 qualified mRNAs, the levels of induction of a few genes were essentially unchanged (Appendix Table S1). However, the induction of 278 mRNAs was inhibited by gefitinib to various extents (red dots, Fig 2D upper panel), whereas the induction of 34 mRNAs was enhanced in the presence of gefitinib (green dots, Fig 2D lower panel). Among the highly inhibited mRNAs was the *Irfb1* mRNA, and among the more enhanced mRNAs was the *Tnf* mRNA. The levels of a few examples of both classes of mRNAs were verified by qRT-PCR (Appendix Fig S3D). These results demonstrated that the induction of genes by TLR4 signaling does not globally require EGFR kinase activity; some genes are impervious, others require it, whereas a few are induced better in its absence.

Next, we tested whether the dichotomy observed for the need of EGFR could be attributed to different properties of the two signaling branches that use MyD88 or TRIF, respectively, as adaptors. For this purpose, we tested the nature of gefitinib sensitivity of TLR4 signaling in MyD88 KO macrophages, in which only the TRIF branch is functional. Like the MyD88 branch, the TRIF branch of TLR4 signaling activates NF- κ B as well [11]; however, in MyD88 KO cells, the response is reduced, as compared to the response in Wt cells, in which both branches of NF- κ B activation operate (Appendix Fig S4). We observed again that *Tnf* mRNA induction was barely inhibited by gefitinib (Fig 3A); in contrast, *Irfb1* mRNA induction was strongly inhibited by gefitinib (Fig 3B). The above conclusions were confirmed when we measured the levels of the corresponding cytokines in the culture media of the MyD88 KO cells; gefitinib slightly enhanced TNF- α secretion (Fig 3C) but strongly inhibited the secretion of IFN- β (Fig 3D). Finally, the need of EGFR in TLR4 signaling was confirmed by a functional assay. Recently, it was reported that the TRIF branch of TLR4 signaling cooperates with stress signals generated by tunicamycin, an inducer of the

Figure 1. EGFR activity is required for IFN-β and ISG induction by TLR4 signaling.

A RAW264.7 cells, untreated or pre-treated with gefitinib (Gf, 10 μM) for 1 h, were stimulated with LPS (1 μg/ml) for the indicated times in the absence or the presence of Gf. *Ifnb1* mRNA induction was analyzed by qRT-PCR. ND: not detectable.

B RAW264.7 cells, untreated or pre-treated with Gf, were stimulated with LPS for 6 h in the absence or the presence of Gf; IFN-β secretion in the supernatants was quantified by ELISA.

C RAW264.7 cells, untreated or pre-treated with various EGFR inhibitors (gefitinib: 10 μM, erlotinib: 10 μM, AG1478: 10 μM) for 1 h, were stimulated with LPS for 6 h in the absence or the presence of the inhibitors. The cell lysates were analyzed for the induction of *Ifit1* by Western blot; actin was used as a loading control.

D Wt or *IFNAR* KO primary BMDMs, untreated or pre-treated with Gf, were stimulated with LPS for 6 h in the absence or the presence of Gf; *Ifnb1* mRNA induction was quantified by qRT-PCR.

E RAW264.7 cells, stably expressing shRNA against EGFR or non-targeting (NT) control, were stimulated with LPS for 6 h, when the *Ifnb1* mRNA induction was quantified by qRT-PCR (upper panel). EGFR-knockdown levels were analyzed by Western analyses of the cell lysates in which actin was used as a loading control (lower panel).

Data information: Error bars were calculated as SEM from three biological replicates, and the data are representative of at least three independent experiments. Wherever applicable, the *P*-values were calculated using two-tailed unpaired Student's *t*-test; **P* < 0.05, ***P* < 0.005, ****P* < 0.0001. Source data are available online for this figure.

cellular unfolded protein response; together, they activate the inflammasome, causing the secretion of IL-1β [28]. As expected, we observed that, in LPS-treated BMDMs, there was a basal level of inflammasome activation, as measured by IL-1β secretion, which was substantially boosted by tunicamycin treatment. Gefitinib inhibited both the basal and the enhanced levels of IL-1β secretion (Fig 3E), demonstrating that EGFR was needed for the proper biological functioning of the TRIF branch of TLR4 signaling.

Mechanism of selective action of EGFR on TLR4 signaling

We wanted to investigate the biochemical basis of the observed selective effects of EGFR on gene induction by TLR4 signaling. TLR4 internalization upon LPS treatment is a critical step for the activation of the endosomal TRIF branch. We tested whether EGFR activity was required for this step. Gefitinib treatment did not inhibit LPS-induced TLR4 internalization (Appendix Fig S5). We have previously

Figure 2. EGFR activity differentially regulates TLR4-induced genes.

A RAW264.7 cells, untreated or pre-treated with Gf, were stimulated with LPS for the indicated times in the absence or the presence of Gf. *Tnf* mRNA induction was analyzed by qRT-PCR.

B RAW264.7 cells, untreated or pre-treated with Gf, were stimulated with LPS for 6 h in the absence or the presence of Gf; TNF- α secretion in the supernatants was quantified by ELISA.

C RAW264.7 cells, untreated or pre-treated with Gf, were stimulated with LPS for 6 h in the absence or the presence of Gf. Microarray analyses were performed for the TLR4-induced genes from the biological triplicates of each group (as indicated in the table, A: NT, no treatment; B: LPS treatment; C: Gf treatment; D: LPS+Gf treatment). Examples of five genes, whose induction was either inhibited (upper table) or enhanced (lower table) by Gf, are shown. E/F represents inhibition index and F/E represents enhancement index for each TLR4-induced gene.

D Graphical presentation of all TLR4-stimulated genes (which were induced >2-fold by LPS) on the basis of their inhibition index (E/F > 1, red dots, upper panel) or enhancement index (F/E > 1, green dots, lower panel) by Gf. *Ifnb1* represents a Gf-inhibited and *Tnf* represents a Gf-enhanced, TLR4-inducible gene.

Data information: Error bars were calculated as SEM from three biological replicates, and the data are representative of at least three independent experiments. Wherever applicable, the *P*-values were calculated using two-tailed unpaired Student's *t*-test; **P* < 0.05.

demonstrated ligand-induced association of TLR3 and EGFR in TLR3-expressing HEK293 cells [21]; we used a similar HEK293 cell line, expressing CD14, MD2 and TLR4, to test TLR4-EGFR interaction. Although not of myeloid origin, the 293-TLR4 cell line signals normally and it has been extensively used by others to study

cytokine induction by LPS. We failed to detect any physical association of TLR4 and EGFR, using co-immunoprecipitation assays, under conditions at which TLR3 interacted with EGFR (Fig 4A). We, therefore, focused on the downstream events of TLR4 signaling. LPS-triggered TLR4 signaling causes rapid activation of several protein

Figure 3. EGFR activity is selectively required for IRF-dependent TLR4-mediated gene induction.

- A MyD88 KO primary BMDMs, untreated or pre-treated with Gf, were stimulated with LPS for 6 h in the absence or the presence of Gf; *Tnf* mRNA induction was quantified by qRT-PCR.
- B In the same samples as in (A), *Ifnb1* mRNA induction was quantified by qRT-PCR.
- C MyD88 KO primary BMDMs, untreated or pre-treated with Gf, were stimulated with LPS for 6 h in the absence or the presence of Gf; TNF- α secretion in the supernatants was quantified by ELISA.
- D In the same samples as in (C), IFN- β secretion in the supernatants was quantified by ELISA.
- E MyD88 KO primary BMDMs, pre-treated with tunicamycin (Tm) or vehicle (DMSO) for 4 h, were stimulated with LPS for 18 h in the absence or the presence of Gf. IL-1 β secretion in the supernatants was quantified by ELISA.

Data information: Error bars were calculated as SEM from three biological replicates, and the data are representative of at least three independent experiments. Wherever applicable, the *P*-values were calculated using two-tailed unpaired Student's *t*-test; ***P* < 0.005.

kinases; we inquired whether these effects required EGFR kinase activity. For this purpose, RAW264.7 cells were pre-treated with the EGFR inhibitor, gefitinib, and the kinetics of LPS-induced phosphorylation of different protein kinases or their substrates were measured in gefitinib-treated and untreated cells. EGFR activity was not required for LPS-induced phosphorylation of I κ B α and the p65 subunit of NF- κ B (Fig 4B, upper panels). Similarly, the rapid activation of ERK1, ERK2, JNK1 and JNK2 kinases by TLR4 was unaltered by gefitinib treatment (Fig 4B, middle panels). In contrast, the activation of AKT by TLR4 signaling was completely inhibited by gefitinib (Fig 4C). The requirement of EGFR for AKT activation by TLR4 was further confirmed in EGFR-knockdown cells (Fig 4D). AKT activation is primarily mediated by the PI3 kinase (PI3K)

pathway, and as expected, the PI3K inhibitor, LY294002 (LY), blocked LPS-induced AKT phosphorylation (Fig 4E). The above results demonstrated that EGFR was required for the TLR4 activation of the PI3K/AKT pathway but not the other pathways activated by IKKs, MAPKs and SAPKs. These observations were in tune with the lack of requirement of EGFR for the induction of many NF- κ B-driven genes in response to TLR4 signaling. To investigate the need of EGFR in the IFN branch of TLR4 signaling, we examined the status of IRF-3 activation, by TLR4 signaling, as measured by its specific phosphorylation (on Ser388) and nuclear translocation. In RAW264.7 cells, there was a transient phosphorylation and nuclear translocation of IRF-3 in response to TLR4 activation; gefitinib inhibited IRF-3 activation, as measured by both properties of IRF-3 (Fig 5A). The same

conclusion was true for IRF-3 activation in primary BMDMs (Fig 5B). Moreover, IRF-3 activation, as measured by its phosphorylation (Fig 5C) or its nuclear translocation (Fig 5D), was impaired in EGFR-knockdown cells as well. Another IRF, IRF-7, is also activated by TLR4 signaling, and nuclear translocation of IRF-7 was impaired in EGFR-knockdown cells (Fig 5E).

As discussed above, we observed that EGFR was required for the activation of the PI3K/AKT pathway by TLR4. We next examined whether the loss of activation of this pathway could account for the differential need of EGFR for the induction of the NF- κ B- and

IRF-driven genes. Indeed, when LY, a potent inhibitor of PI3K, was tested on TLR4-mediated gene induction, the IRF-induced genes, *Ifit1* and *Ifnb1* were hardly induced in the presence of a low or a high dose of LY (Fig 6A). In contrast, the PI3K inhibitor had no effect on *Tnf* induction by LPS (Fig 6B). However, unlike gefitinib, LY did not inhibit IRF-3 phosphorylation and nuclear translocation in response to TLR4 stimulation (Fig 6C). To further probe the mechanisms behind the failure of nuclear IRF-3 to induce IRF-driven genes, we inquired whether EGFR was required for the activation of β -catenin, an obligatory co-activator of IRF-3 and possibly other IRFs.

Figure 4. EGFR activity is required for TLR4-mediated AKT, but not NF- κ B, ERK and JNK, activation.

- A HEK293.CD14.MD2.TLR4 or HEK293.TLR3 [20] cells were stimulated with LPS or polyI:C for 1 h, and then, the cell lysates were immunoprecipitated with TLR4 antibody or Flag (TLR3) antibody. The immunoprecipitates were analyzed for EGFR by Western blot.
- B RAW264.7 cells, untreated or pre-treated, were stimulated with LPS, in the absence or the presence of Gf, for the indicated times. The cell lysates at various times after LPS stimulation were analyzed for phospho-I κ B α (on Ser³²), phospho-p65 (on Ser⁵³⁶), phospho-ERK1/2 [phospho p44/p42 (Thr²⁰²/Tyr²⁰⁴)] and phospho-JNK1/2 (phospho p54/p46) by Western blot. Tubulin was used as the loading control.
- C RAW264.7 cells, untreated or pre-treated with Gf, were stimulated with LPS, in the absence or the presence of Gf, for the indicated times; the cell lysates at various times after LPS stimulation were analyzed for phospho-AKT (on Ser⁴⁷³) or total AKT by Western blot. Actin was used as the loading control.
- D RAW264.7 cells, expressing shRNA against EGFR or non-targeting (NT) control, were stimulated with LPS for the indicated times; the cell lysates were analyzed for phospho-AKT (on Ser⁴⁷³) or total AKT by Western blot.
- E RAW264.7 cells, untreated or pre-treated with LY294002 (LY, 10 μ M) for 1 h, were stimulated with LPS (1 μ g/ml) in the absence or the presence of LY; the cell lysates were analyzed for phospho-AKT (on Ser⁴⁷³) or total AKT by Western blot.

Source data are available online for this figure.

Figure 5. EGFR activity is required for the activation of IRFs by TLR4 signaling.

- A RAW264.7 cells, untreated or pre-treated with Gf, were stimulated with LPS for the indicated times in the absence or the presence of Gf. Nuclear fractions, prepared using a previously described procedure [20], and whole cell lysates at various times after LPS stimulation were analyzed for phospho-IRF-3 (on Ser³⁸⁸) or total IRF-3 by Western blot. HDAC1 was used as a nuclear marker protein and tubulin was used as a cytoplasmic marker protein.
- B Primary BMDMs, untreated or pre-treated with Gf, were stimulated with LPS for the indicated time in the absence or the presence of Gf. Cell lysates or nuclear fractions post LPS stimulation were analyzed for phospho-IRF-3 (on Ser³⁸⁸) or total IRF-3 by Western blot. HDAC1 was used as a nuclear marker protein.
- C RAW264.7 cells, expressing shRNA against EGFR or non-targeting (NT) control, were stimulated with LPS for the indicated periods of time. Cell lysates at various times after LPS stimulation were analyzed for phospho-IRF-3 (on Ser³⁸⁸) or total IRF-3 by Western blot. Actin was used as a loading control.
- D RAW264.7 cells, expressing shRNA against EGFR or non-targeting (NT) control, were stimulated with LPS for the indicated period of time. Nuclear fractions were analyzed for total IRF-3 by Western blot. HDAC1 was used as a nuclear marker protein.
- E RAW264.7 cells, expressing shRNA against EGFR or non-targeting (NT) control, were stimulated with LPS for the indicated period of time. Nuclear fractions were analyzed for IRF-7 by Western blot. HDAC1 was used as a nuclear marker protein.

Source data are available online for this figure.

This possibility was all the more relevant because the PI3K pathway is known to activate β -catenin by the phosphorylation of Ser552 [29,30]. TLR4 activation caused β -catenin phosphorylation on Ser552 which was inhibited by the PI3K inhibitor, LY; more importantly, the EGFR inhibitor, gefitinib, also inhibited β -catenin activation (Fig 6D). Furthermore, similar impairment of β -catenin activation was observed in cells in which EGFR had been knocked down (Fig 6E). The above results demonstrated that the primary need of EGFR in TLR4 signaling was to activate the PI3K/AKT pathway which was required for β -catenin activation and IRF-driven gene expression.

In vivo relevance of EGFR activity in TLR4 actions

Our observations with cells in culture encouraged us to examine the effect of EGFR inhibition on TLR4 signaling in mice. To inhibit EGFR kinase activity, we administered gefitinib in mice using an established protocol [31]. TLR4 signaling was triggered by the injection

of LPS, which is known to cause septic shock and death in mice. Large amounts of TNF were present in the sera of mice 4 h after LPS injection, and gefitinib pretreatment had no significant effect on TNF induction (Fig 7A). The serum levels of TNF diminished rapidly between 4 and 16 h after LPS injection, and gefitinib had no effect on this decline (Appendix Fig S6A). One of the primary organs affected in septic shock is the lung [15]. We measured the levels of several mRNAs, in the lungs of gefitinib-treated and untreated mice, 16 h after LPS administration. There was no significant difference between the two groups with respect to *Tnf* mRNA induction (Figure 7B). However, the induction of three IRF or IFN-inducible mRNAs, *Ifit1*, *Ifit2* and *Ifit3*, was significantly inhibited in gefitinib-treated mice (Fig 7C and Appendix Fig S6B and C). These results, in general, mirrored our *in vitro* results by displaying a selective sensitivity of the IRF branch of TLR4 signaling to EGFR inhibition. To further investigate the physiological consequence, we examined the histology of lung sections from mice treated with LPS for 16 h.

Figure 6. EGFR and PI3K activities are required for the IRF-3/β-catenin, but not the NF-κB, branch of TLR4 signaling.

A RAW264.7 cells, pre-treated with LY294002 (LY, at various concentrations as indicated) for 1 h, were stimulated with LPS in the absence or the presence of LY. *Ifit1* or *Ifnb1* mRNA induction was analyzed by qRT-PCR, and IFN-β secretion in the supernatants was quantified by ELISA.

B In the same samples as in (A), *Tnf* mRNA induction was analyzed by qRT-PCR and TNF-α secretion in the supernatants was quantified by ELISA.

C RAW264.7 cells, untreated or pre-treated with LY (10 μM) for 1 h, were stimulated with LPS for the indicated time in the absence or the presence of LY. Nuclear fractions or cell lysates were analyzed for phospho-IRF-3 (on Ser³⁸⁸) or total IRF-3 by Western blot. HDAC1 was used as a nuclear marker protein.

D RAW264.7 cells, untreated or pre-treated with LY or Gf, were stimulated with LPS for 2 h, in the absence or the presence of the inhibitors. Nuclear fractions were analyzed for phospho-β-catenin (on Ser⁵⁵²) by Western blot. HDAC1 was used as a nuclear marker protein.

E RAW264.7 cells, expressing shRNA against EGFR or non-targeting (NT) control, were stimulated with LPS for 2 h. Nuclear fractions were analyzed for phospho-β-catenin (on Ser⁵⁵²) by Western blot. HDAC1 was used as a nuclear marker protein.

Data information: Error bars were calculated from three biological replicates, and the data are representative of at least three independent experiments. Wherever applicable, the *P*-values were calculated using two-tailed unpaired Student's *t*-test; **P* < 0.05, ***P* < 0.005; NS, non-significant. Source data are available online for this figure.

Compared to the control mice, the LPS-treated mouse lungs displayed alveolar wall thickening and increased immune cell infiltration; gefitinib treatment alleviated these detrimental effects of LPS (Fig 7D). Finally, the majority of the LPS-treated mice died from septic shock, whereas very few died when they were treated with both LPS and gefitinib (Fig 7E). These results show that EGFR action is required for the induction of LPS/TLR4-mediated septic shock.

Discussion

There are many components of the TLR4 signaling pathways that need Tyr phosphorylation for their action [5]. TLR4 itself is

phosphorylated at Tyr674 and Tyr680 in a ligand-dependent fashion, a required step for its ability to signal [27]; MAL and MyD88, the two adaptors for TLR4 signaling from the plasma membrane, have also been reported to be Tyr-phosphorylated [32,33]. Similarly, caveolin-1, another protein involved in MyD88-mediated TLR4 signaling, is Tyr-phosphorylated in a signal-dependent fashion [34]. Several protein tyrosine kinases (PTKs), but not EGFR, have been implicated in mediating TLR4 signaling by the MyD88 branch; such PTKs include Bruton's tyrosine kinase, Syk and Src family kinase (especially Lyn) [5]. EGFR is the first identified PTK that affects only the IRF branch of TLR4 signaling; even the TRIF-mediated NF-κB signaling branch was relatively resistant to gefitinib treatment (Fig 3A). Unlike TLR3 [21], TLR4 does not appear to be a direct

Figure 7. Gefitinib treatment protects against LPS-induced septic shock without any effect on TNF- α production.

C57BL/6 mice, pre-treated with Gf (120 mg/kg body weight) or vehicle (DMSO) by oral gavage for 7 days, were injected with LPS (40 mg/kg body weight, intra-peritoneal) and analyzed for various parameters at different times after LPS treatment as described below.

- A** Serum TNF- α levels, in mice with or without Gf treatment, were quantified after 4 h of LPS injection by ELISA; each dot represents a mouse. The P-values were calculated using two-tailed unpaired Student's t-test; ns, non-significant.
- B, C** *Tnf* or *Ifit1* mRNA induction, in the lungs of DMSO- or Gf-treated mice, was quantified after 16 h of LPS injection, by qRT-PCR; the P-values were calculated using two-tailed unpaired Student's t-test; ** $P < 0.005$; ns, non-significant.
- D** H&E staining of lung sections; left panel: 16 h of PBS treatment, middle panel: DMSO pre-treatment followed by LPS treatment for 16 h, right panel: Gf pre-treatment followed by LPS treatment. Pictures indicate increased alveolar wall thickening and cell infiltration in LPS-treated mice which were reduced upon Gf pre-treatment (scale bar: 50 μ m).
- E** Percent survival of Gf or DMSO-pre-treated mice at various time points after LPS injection. The P-values were calculated using two-tailed unpaired Student's t-test.
- F** A model of EGFR-mediated regulation of TLR4 signaling and septic shock responses. For TLR4-mediated IFN induction and septic shock response, both TRIF-mediated signaling and intracellular EGFR kinase activity are required to activate IRF-3 and β -catenin.

target of Tyr phosphorylation by EGFR. If that were the case, we would have observed, not such a selective, but a more global effect of EGFR on TLR4 signaling. As anticipated from the above statement, we could not detect any physical interaction between TLR4 and EGFR (Fig 4A). Because activation of TLR4, by LPS, stimulates its internalization from the plasma membrane, it was important to establish that the EGFR kinase activity was not required for this process; cell surface TLR4 was similarly internalized upon LPS treatment in the presence or the absence of gefitinib (Appendix Fig

S5). This is consistent with the observation that TRIF-dependent NF- κ B signaling from the endosomal membrane was unaffected by gefitinib. These observations support the notion that the promotion of the IRF branch of TLR4 signaling is mediated by the kinase activity of intracellular EGFR. When cells were serum-starved, which is known to accumulate EGFR preferentially to the plasma membrane, *Ifnb1* mRNA induction was inhibited, whereas TNF mRNA induction was stimulated (Appendix Fig S7). Moreover, cetuximab, an antibody that, unlike gefitinib, blocks only cell

surface EGFR, could not block *IFNB1* mRNA induction by LPS (Appendix Fig S8A). As expected, cetuximab treatment had no effect on *TNF* induction (Appendix Fig S8B). Intracellular activity of EGFR has been noted before [21], although the relevant ligand is unknown; moreover, ligand-independent kinase activity of Wt EGFR has been reported as well [25]. The functional effect of EGFR on TLR4 signaling seems to be indirectly mediated by its downstream targets. Among the many kinases activated by LPS stimulation of TLR4, only the PI3K/AKT branch required the presence of active EGFR. Although EGFR is well known for activating the PI3K pathway, it was unexpected to observe that TLR4 uses EGFR for this purpose.

Gene induction profiling revealed that the need of EGFR was non-uniform. The induction of IFN and IFN-stimulated genes was strongly dependent on EGFR kinase activity; however, many genes were relatively unaffected, whereas the induction of others, such as *TNF- α* , was enhanced in the absence of EGFR activity. Because activation of NF- κ B, the major TLR4-activated transcription factor, did not require EGFR, the induction of genes whose transcription is primarily driven by NF- κ B was unaffected by the absence of EGFR. However, it should be pointed out that the induction of a few genes, such as *Il6* and *Il1b* (Appendix Fig S3D), which use NF- κ B for their induction, also required EGFR. It is possible that the PI3K pathway indirectly stimulates the induction of these genes by phosphorylating their co-activators. It is also possible that additional transcription factors, which require EGFR activity, may positively regulate their induction. An example of the latter class is the *Ifnb1* gene, whose transcription is also driven by NF- κ B, but in conjunction with IRF-3 and AP1. We have previously shown the need of the PI3K pathway for gene induction by IRF-3 [20], a fact that explains the effects observed here on the IRF branch of TLR4 signaling. Our previous work also established that the PI3K/AKT pathway can affect IRF action in two ways. In the absence of the activity of the pathway, IRF-3 cannot be fully phosphorylated and activated. Additionally, β -catenin, which is an obligatory co-activator for IRF's transcriptional action in the nucleus, is known to be activated and translocated to the nucleus by the phosphorylation of a specific Ser residue by the PI3K/AKT pathway [29,30]. Inhibiting EGFR caused a block in both IRF activation and β -catenin activation. The latter block is probably the critical block because the PI3K inhibitor, LY, blocked IRF-mediated gene induction by blocking only β -catenin nuclear translocation. However, it is apparent that EGFR has an additional effect on TLR4-mediated phosphorylation and nuclear translocation of IRF-3 (Fig 5). The latter effect is unlikely to be mediated by the PI3K/AKT pathway because LY did not have any inhibitory effect on IRF-3 phosphorylation and nuclear translocation (Fig 6C). EGFR may have a post-transcriptional role in TLR4-induced gene expression as well; such an effect can explain the disconnect between *TNF* mRNA induction and *TNF* secretion in certain cases (compare Figs 2A and B; 3A and C). Our observations led us to the formulation of a working model for the involvement of EGFR in TLR4 signaling (Fig 7F). Binding of LPS to TLR4 and EGF to EGFR on the plasma membrane leads to their endocytosis, and from the endosomal membrane, TLR4 signals through TRAM and TRIF to activate several transcription factors. However, the activation of IRF-3 and its co-activator, β -catenin, requires not only TLR4 signaling, but also the kinase activity of intracellular EGFR. If EGFR activity is blocked, the LPS-mediated

induction of IFN and ISGs is impaired and the septic shock response is compromised.

Surprisingly, although the need of EGFR activity was for only the IRF branch of TLR4 signaling, the biological effects of TLR4-mediated actions of LPS were profoundly affected in gefitinib-treated mice. Administration of LPS triggers the septic shock response in mice to which they eventually succumb. The levels of inflammatory cytokines, such as *TNF- α* , in circulation, go up immediately after LPS administration [35,36]; this phase is followed by infiltration of the lung by immune cells and alterations of the lung architecture [35,37]. The importance of IRF-driven IFN induction by LPS in triggering pathogenesis is well documented [17–19]. As anticipated from the results of our cell culture studies, prophylactic administration of gefitinib had no effect on *TNF* induction by LPS *in vivo*. In contrast, the induction of the IRF-driven *Ifit* genes by LPS was strongly inhibited in the absence of EGFR activity. More importantly, gefitinib protected the mice from both LPS-induced lung injury and death (Fig 7). The above findings have important therapeutic implications for the treatment of patients suffering from sepsis induced by microbial infection. Obviously, further investigations with experimental animals will be required for optimizing the efficacy of such therapeutic interventions. However, because many EGFR inhibitors, including gefitinib, and EGFR antibodies are in wide use for treating a variety of cancers, their safety, pharmacological properties and efficacies have been already well established.

Materials and Methods

Cells

Mouse macrophage cell line (RAW264.7) was maintained in DMEM, supplemented with 10% FBS and penicillin–streptomycin; human monocytic cell line (THP-1) was maintained in RPMI 1640, supplemented with 10% FBS, penicillin–streptomycin and β -mercaptoethanol. Primary bone marrow-derived dendritic cells (BMDCs) and macrophages (BMDMs) were isolated and differentiated as described before [21,38]. HEK293.CD14.MD2.TLR4 cells were obtained from Invivogen and maintained as per the manufacturer's instructions. EGFR-knockdown RAW264.7 cells were generated by lentiviral transduction of mouse EGFR-specific shRNA (Sigma, #TRCN0000055222) followed by selection under puromycin; a non-targeting shRNA (#SHC002) was used as a control.

Reagents and treatments

Lipopolysaccharides (LPS, #L2880) from *E. coli* O55:B5 and tunicamycin were obtained from Sigma-Aldrich; EGFR inhibitors gefitinib (#S1025), erlotinib (#sc-202154A) and AG1478 (#658552) were obtained from Selleckchem, Santa Cruz and Calbiochem, respectively, and used as described in the figure legends; PI3 kinase inhibitor LY294002 (#270-038) was obtained from Alexis Biochemicals. Cetuximab (Erbix, Bristol-Myers Squibb) was obtained from Cleveland Clinic pharmacy. When noted, the cells were pretreated with EGFR inhibitors or LY294002 for 1 h, after which the cells were treated with LPS (1 μ g/ml or as indicated) for 6 h in the presence of the inhibitor. For inflammasome activation assay, the cells were

primed with tunicamycin for 4 h before LPS treatment, as described before [28]. Antibody against murine *Ifit1* was raised in our laboratory; EGFR antibody (#06-847) was obtained from Millipore; antibodies against phospho-IRF-3 (#4947), phospho-AKT (#4060), phospho-I κ B α (#2859), phospho-p65 (#3033), phospho-ERK1/2 (#9101), phospho-JNK (#9251), AKT (#4685), phospho- β -catenin (#9566), phospho-EGFR (Tyr1068, #2236) and tubulin were from Cell Signaling; and antibodies against TLR4 (#sc-293072), IRF-3 (#sc-9082), IRF-7 (#sc-9083) and HDAC1 (#sc-7872) were from Santa Cruz.

Mice, LPS-induced septic shock and gefitinib treatment *in vivo*

Wt mice were obtained from Taconic Inc., and *IFNAR* KO mice (all in C57BL/6 genetic background) were maintained in our facility. For inducing septic shock, 8- to 10-week-old Wt mice were intraperitoneally injected with LPS (40 mg/kg body weight) and monitored the survival of mice for 10 days. To investigate the effect of EGFR inhibitor on LPS-induced septic shock, the mice were treated with gefitinib (120 mg/kg body weight, Selleckchem) or vehicle (DMSO) for 7 days by oral gavage prior to intraperitoneal injection of LPS. All mice procedures were approved by IACUC.

RNA isolation and qRT-PCR analyses

After 6 h of LPS treatment, the cells were harvested, total RNA was isolated using RNA isolation kit (Roche), and cDNA was prepared using ImProm-II Reverse Transcription Kit (Promega). For real-time PCR, 0.5 ng of cDNA was applied to 384-well plate using Applied Biosystem's Power SYBR Green PCR mix in Roche LightCycler 480 II. The expression levels of the induced mRNAs were normalized to 18S rRNA. To investigate *in vivo* gene expression, lungs were harvested from mice after 16 h of LPS treatment and quickly frozen in dry ice. Total RNA was isolated from frozen lungs using Trizol extraction, and the cDNAs were prepared using ImProm-II Reverse Transcription Kit and then subjected to qRT-PCR analyses as described above.

Quantification of secreted cytokines by ELISA

ELISA kits for mouse IFN- β (#42400-1), TNF- α (#88-7324-22) and IL-1 β (#MLB00C) were obtained from PBL, eBioscience and R&D, respectively. Culture supernatants from LPS-treated cells (RAW264.7 or primary BMDMs) were used for quantification of the secreted proteins by ELISA using manufacturers' instructions. For measuring the secreted cytokines *in vivo*, blood was collected by cardiac puncture from anesthetized mice after LPS injections into serum separator tubes (BD Biosciences) and sera were isolated following manufacturer's protocol. TNF- α levels were then assessed in serum samples using mouse TNF- α ELISA kit from eBioscience.

Microarray analyses

RAW264.7 cells (in biological triplicate) were pretreated with gefitinib (10 μ M) for 1 h, and stimulated with LPS (1 μ g/ml) for 6 hours, when total RNA was isolated, treated with DNase I and the RNA was further purified by using the RNeasy kit (Qiagen). The purified RNA was then analyzed using an Illumina Mouse Ref-8 gene array, and the data analysis was carried out by using Illumina

Genome Studio V2011.1. We selected the mRNAs, which were induced at least twofold by LPS, and quantified the inhibition index (E/F) and the enhancement index (F/E) (as presented in Fig 2C and D), and graphed using Prism 5.02 software.

Cell fractionation, lysis, Western blot and immunoprecipitation

Nuclear and cytosolic fractions were isolated using previously described procedures [20]. Western blotting was performed using previously described procedures [38]. Briefly, cells were lysed in 50 mM Tris buffer, pH 7.4 containing 150 mM of NaCl, 0.1 % Triton X-100, 1 mM sodium orthovanadate, 10 mM of sodium fluoride, 10 mM of β -glycerophosphate, 5 mM sodium pyrophosphate and protease inhibitors (Roche); the total protein extracts were analyzed by SDS-PAGE followed by Western blot. Immunoprecipitations were carried out using previously described procedures [21].

Tissue histology

Lungs were harvested from mice after 16 h of LPS treatment. The tissues were perfused with PBS before fixing in formalin (Sigma) and then paraffin-embedded. The 5- μ m sections were cut and then stained with hematoxylin and eosin to view lung pathology.

Immunofluorescence

RAW264.7 cells were stimulated with LPS in the absence or the presence of gefitinib. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 and or left non-permeabilized. The endogenous TLR4 was immunostained with anti-TLR4 antibody (1:1,000, Abcam, #Ab22048), followed by Alexa Fluor-conjugated secondary antibody (1:1,000) in 5% goat serum. The objects were mounted with VectaShield/DAPI, analyzed by fluorescence microscopy and processed with Adobe Photoshop software. Relative fluorescence of the cell surface expressing TLR4 was quantified with ImageJ software using previously described procedure [39].

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.02 software. *P*-values were calculated using two-tailed, unpaired Student's *t*-tests, and the statistical significance is shown in all the relevant figures. *P*-values for survival curves were calculated using log-rank test.

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Author contributions

SC designed and performed the experiments, analyzed the data, interpreted the results and participated in manuscript writing. MV, DP, SA and SKB designed and performed the experiments and analyzed the data. VF analyzed

the data. GCS designed the experiments, interpreted the results and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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