Erlotinib protects against LPS-induced Endotoxicity because TLR4 needs EGFR to signal

Sarmishtha Dea,b, Hao Zhouc, David DeSantisd, Colleen M. Cronigerd, Xiaoxia Lied, and George R. Starka,b,1

aDepartment of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195; bDepartment of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195; and cDepartment of Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH 44106

Contributed by George R. Stark, June 24, 2015 (sent for review April 14, 2015; reviewed by Stefanie N. Vogel and Katherine A. Fitzgerald)

Several components of the canonical pathway of response to lipopolysaccharide (LPS) are required for the EGFR-dependent activation of NFκB. Conversely, the ability of Toll-like Receptor 4 (TLR4) to activate NFκB in response to LPS is impaired by down regulating EGFR receptor (EGFR) expression or by using the EGFR inhibitor erlotinib. The LYN proto-oncogene (LYN) is required for signaling in both directions. LYN binds to the EGFR upon LPS stimulation, and erlotinib impairs this association. In mice, erlotinib blocks the LPS-induced expression of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) and ameliorates LPS-induced endotoxicity, revealing that EGFR is essential for LPS-induced signaling in vivo.

Significance

The activation of nuclear factor κB (NFκB) in the normal inflammatory response is rapidly down regulated, whereas constitutive NFκB activation is a hallmark of cancer. We now reveal cross signaling between EGFR receptor (EGFR) and Toll-like receptor 4 (TLR4). NFκB activation in response to EGFR requires, in addition to EGFR, TLR4 and two downstream proteins. Conversely, EGFR is required for TLR4-mediated activation of NFκB in response to lipopolysaccharide (LPS). The LYN proto-oncogene (LYN) is required for NFκB activation in response to either ligand. In mice, the EGFR inhibitor erlotinib greatly reduces both cytokine expression and endotoxicity in response to LPS, suggesting that EGFR inhibitors may find use in treating septic shock.

Author contributions: S.D., X.L., and G.R.S. designed research; S.D., H.Z., and D.D. performed research; S.D., H.Z., C.M.C., X.L., and G.R.S. analyzed data; and S.D. and G.R.S. wrote the paper.

Reviewers: S.N.V., University of Maryland, Baltimore; and K.A.F., University of Massachusetts Medical School.

The authors declare no conflict of interest.

1To whom correspondence may be addressed. Email: starkg@ccf.org or des@ccf.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511794112/-/DCSupplemental.
the TLR4 ligand LPS activates EGFR in several different cell lines and in vivo models (30–34).

Sepsis is a severe inflammatory response to infection, leading to an imbalance between pro- and anti-inflammatory responses (35). LPS induces a systemic inflammation that mimics many of the initial clinical features of sepsis, including increases in proinflammatory cytokines (36). During sepsis the majority of cytokines have multiple intrinsic effects, mediating not only immune defenses but also pathological manifestations. Treatment of LPS-injected animals with neutralizing antibodies against proinflammatory cytokines resulted in improved outcomes (37, 38). However, several clinical trials of antiinflammatory cytokines, including TNFα and anti-IL-1 therapy failed to improve the survival of septic patients (39, 40).

In this study we elucidate the role of TLR4 in the EGF-induced activation of NFκB, which requires a functional interaction between EGFR and TLR4. The EGFR inhibitor erlotinib blocks TLR4-mediated NFκB activation, indicating that the kinase activity of EGFR is necessary. Down-regulating the expression of the SRC family member LYN impairs EGF-mediated NFκB activation. Furthermore, EGFR is required for TLR4 to activate NFκB. LYN binds to both EGFR and TLR4 in response to LPS, and this binding is blocked by erlotinib. Importantly, erlotinib also inhibits NFκB-dependent cytokine production in mice and protects mice from LPS-induced lethality. These in vivo findings reveal a potential therapeutic role for erlotinib in protection against septic shock.

**Results**

**EGFR-Mediated NFκB Activation Requires MYD88 and TAK1.** Our recent study elucidated the important role of EGF in mediating NFκB activation (4). Signaling to NFκB might depend solely on EGFR, or might also involve another receptor. TLRs activate NFκB (13), and MYD88, a universal adaptor protein, is crucial for the ability of all TLR/IL-1R family members, except TLR3, to induce NFκB activation (41). Therefore, it was logical to determine whether MYD88 is required for EGF-dependent NFκB activation. Before we could study MYD88 expression in human mammary epithelial (HME) cells, we needed to prevent apoptosis by expressing a high level of the antiapoptotic protein BCL2. Increased IKK phosphorylation and IκB phosphorylation, degradation, and resynthesis were observed in control HME-BCL2 cells treated with EGF but not in MYD88 knockdown cells (Fig. S1A). Knockdown of MYD88 diminished EGF-induced ERK phosphorylation as well (Fig. S1A). TGF-β-activated kinase 1 (TAK1) phosphorylates and activates IKK in TLR/IL-1 pathways, leading to the phosphorylation of IκB and activation of NFκB (42). To test the involvement of TAK1 in EGF-dependent NFκB activation, stable pools of HME cells expressing shRNAs against TAK1 or scrambled shRNA were generated. Down-regulation of TAK1 impaired EGF- or IL-1-stimulated phosphorylation of IKK, IκB, and ERK, and also impaired the degradation and resynthesis of IκB (Fig. S1B). We conclude that MYD88 and TAK1, which are essential for TLR/IL-1-mediated NFκB activation, are also required for NFκB activation in response to EGF.

**TLR4 Silencing Impairs EGF-Induced NFκB Activation.** Next we investigated the role of individual TLRs in this pathway. Because we observed rapid activation of NFκB in response to EGF, we reasoned that a cell surface TLR was most likely to be involved. We began by focusing on TLR4. In HME cells, decreasing the expression of TLR4 inhibited the EGF-dependent phosphorylation of IKK and IκB (Fig. 1A). TLR4 down-regulation in nonsmall cell lung carcinoma (NSCLC) A549 cells also inhibited EGF-dependent phosphorylation of IKK and IκB, as well as the subsequent degradation and resynthesis of IκB (Fig. S24). Because A549 cells already have high constitutive levels of activated NFκB, the ability of EGF to drive a further increase in IκB phosphorylation is limited. The EGF-induced phosphorylation of EGFR was similar in control and TLR4-deficient cells (Fig. 1A and Fig. S24). These results indicate that TLR4 is necessary for EGF-dependent NFκB activation in both nonmalignant and malignant human cells.

**TLR4 Is Phosphorylated in Response to EGF.** Tyrosine phosphorylation of the cytosolic Toll/interleukin-1 receptor (TIR) domain of TLR4 is required for NFκB activation in response to LPS (43, 44).

---

**Fig. 1.** TLR4 is required for EGF-induced NFκB activation and EGF-stimulated phosphorylation of TLR4 is inhibited by erlotinib. (A, Left) HME cells were transfected with nontargeted siRNA (NTsiRNA) or with an siRNA against TLR4. After 48 h, the TLR4 mRNA expression level was determined by RT-PCR. (Right) EGF-starved cells were treated with EGF for 5 min and the levels of phosphorylated EGFR, IKK, and IκB were analyzed by the Western method. β-actin was used as a loading control. (B) EGF-starved HME cells were stimulated with EGF, and phosphorylated and total TLR4 levels were detected by the Western method. (C) EGF-starved HME cells were pretreated with erlotinib (50 μM) for 1 h or left untreated. The cells were then stimulated with EGF and immunoblotted for phosphorylated and total TLR4. Each experiment in A–C was carried out two or three times independently, with results similar to the representative examples that are shown. (D) A diagram showing that, upon EGF stimulation, activated EGFR phosphorylates TLR4, leading to NFκB activation through MYD88 and TAK1. Erlotinib inhibits the kinase activity of EGFR and suppresses TLR4 phosphorylation.
Because TLR4 is essential for EGF-induced NFκB activation, we investigated whether EGF causes TLR4 phosphorylation. Using an antibody that recognizes phosphorylated tyrosine residue 674 (44), we observed a substantial increase in TLR4 phosphorylation in HME cells and A549 cells stimulated with EGF (Fig. 1B and Fig. S2B). Pretreatment with erlotinib for 1 h blocked the EGF-dependent phosphorylation of TLR4 (Fig. 1C), indicating that the kinase activity of EGFR is required for TLR4 phosphorylation in response to EGF. Our mechanistic findings are summarized in Fig. 1D.

**EGFR Is Essential for LPS-Induced Activation of NFκB.** Because HME cells die following knockdown of EGFR, we used HME-BCL2 cells to study the role of EGFR in the response to LPS. The substantial increases in the phosphorylation of EGFR, IKK, and IκB and the degradation and resynthesis of IκB in response to LPS were impaired when EGFR was down-regulated (Fig. 2A). The phosphorylation of v-akt murine thymoma viral oncogene homolog (AKT) and ERK was increased upon LPS stimulation in control cells, but not in EGFR-knockdown cells (Fig. 2A), indicating that EGFR is required for LPS-mediated AKT and ERK phosphorylation. EGFR also plays a role in TLR4-dependent signaling in cancer cells, because the ability of LPS to activate NFκB was impaired when EGFR expression was down-regulated in A549 and OVCAR3 cells (Fig. 2B and Fig. S3A). To determine whether the kinase activity of EGFR is required for LPS-dependent signaling to NFκB, we treated HME cells with erlotinib for 1 h before stimulating them with LPS. Erlotinib blocked the LPS-dependent phosphorylation of IKK and IκB, and the degradation and resynthesis of IκB (Fig. 2C). Inhibition of EGFR kinase activity by erlotinib also diminished LPS-induced TLR4 phosphorylation in A549 cells (Fig. 2D), impaired NFκB activation in A549 and OVCAR3 cells, and blocked ERK and AKT phosphorylation (Figs. 2E and S3B).

**Kinases in the SRC Family Are Involved in EGFR-TLR4 Signaling to NFκB.** Surprisingly, we were not able to observe binding of EGFR and TLR4 to each other in response to EGF or LPS using confocal microscopy or coimmunoprecipitation (Fig. S4 A and B). These negative results make it unlikely that EGFR phosphorylates TLR4 directly but do not rule it out completely. This finding is distinct from the results of Yamashita et al. (20), with a different TLR family member. These workers showed that TLR3 binds to EGFR in response to dsRNA. For EGFR-TLR4 signaling to NFκB we assumed that one or more additional kinases are required. We began by investigating the SRC family of kinases, because SRC is well known to mediate EGFR phosphorylation (45, 46) and a SRC family member is known to be involved in LPS-dependent NFκB activation (43), and also in TLR3-dependent signaling (20). In response to LPS TLR4 can be activated by SRC family members through the phosphorylation of Y674 (44). The EGF-dependent phosphorylation of IKK and IκB was substantially inhibited by prior exposure of HME or A549 cells to the SRC family inhibitor PP2, which also greatly diminished EGF-induced EGFR phosphorylation and eliminated downstream AKT and ERK phosphorylation (Figs. S4 and S5). This result suggests an important role for one or more SRC family members in EGF-dependent NFκB activation. LPS-induced NFκB activation in A549 cells was also inhibited by pretreatment with PP2 (Fig. S5C), suggesting that

![Fig. 2.](image-url)
The involvement of TLR4 in EGFR-dependent NFκB activation was previously reported (43). To elucidate whether LYN is also involved in the activation of NFκB in response to EGF, we knocked its expression down in HME cells. Reduction of LYN expression attenuated the EGF-dependent phosphorylation of IKK and the degradation and resynthesis of IκBα (Fig. 3A). Down regulation of LYN also impaired EGF-mediated IKK and IκBα phosphorylations in A549 cells (Fig. S6). An association of LYN with constitutively activated EGFR in lung adenocarcinoma cells has been reported by Sutton et al. (46). Coimmunoprecipitation experiments in HME cells demonstrated that, upon stimulation with LPS, LYN is recruited to both EGFR and TLR4 (Fig. 3B), consistent with the previous finding of Medvedev et al. (43) for TLR4. The LPS-stimulated increase in the association of LYN with EGFR or TLR4 in A549 cells was mediated by TAK1 (48) and kinase-inactive TAK1 impairs NFκB activation in response to LPS (49). Consistently, TAK1 is also necessary for EGF-dependent NFκB activation (Fig. S1B). To the best of our knowledge, this is the first report showing that EGF-induced NFκB activation requires both TLR4 and two downstream components in the canonical TLR4-dependent pathway. Tyrosine phosphorylation of TLR4 is essential for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20). We observed that down-regulation of HME cells and expression of wild-type EGFR is required for this activation. This result indicates that the kinase activity of SRC is also required for signaling, and the TIR domain TLR4 mutants Y674A and Y680A are defective in the LPS-dependent activation of NFκB (43). Although a number of protein tyrosine kinases have been implicated in TLR4-dependent signaling, it is not clear how these key residues of TLR4 become phosphorylated. However, it has been shown recently that TLR3 activation requires two tyrosine kinases, EGFR and SRC (20).
Helicobacter pylori secretory protein HP0175 in human gastric epithelial cells. However, the interaction was observed only after 60 min, and we have found that EGF- or LPS-mediated NFκB activation is strongly induced within 5–10 min. Furthermore, we were not able to observe any interaction between EGFR and TLR4 in response to EGF or LPS (Fig. S4), suggesting that additional kinases are likely to mediate an indirect interaction between these two receptors. The SRC family inhibitor PP2 blocks LPS-mediated NFκB activation (43) and, consistent with this report, we now show that PP2 blocks NFκB activity upon LPS stimulation in A549 cells.

LPS stimulation leads to the recruitment of LYN, a SRC family member, to TLR4 in HEK293TLR4/MD-2 stable transfectants (43). Consistent with this earlier report, we now show that LPS stimulation leads to the recruitment of LYN to TLR4 in HME and A549 cells. We also demonstrate that stimulation with LPS leads to the recruitment of LYN to EGFR, and this association is blocked by erlotinib, indicating that the kinase activity of EGFR is required. Erlotinib also blocks the LPS-stimulated recruitment of LYN to TLR4, revealing that the kinase activity of EGFR is also necessary for this association. The involvement of LYN in an LPS mediated pathway was reported earlier (43, 47, 57). In this study, we observed the involvement of LYN specifically in the activation of EGFR in response to LPS and also in the activation of TLR4 in response to EGF, and conclude that LYN is a key kinase in establishing cross talk between EGFR and TLR4, leading to downstream signaling (Fig. 3C). Additional studies are necessary to explore further details of how LYN functions in response to LPS in mediating TLR4-dependent signaling. In particular, because EGFR and TLR4 do not bind to each other in response to LPS, and because LYN can be activated by oligomerization of TLR4 alone, it is not clear why EGFR is needed in order for LPS to activate NFκB.

EGFR kinase activity by erlotinib impaired LPS-stimulated NFκB activation in nontumorigenic HME cells as well as in cancer cells. Earlier studies have shown that the transactivation of EGFR is required for LPS-induced COX-2 activation (32, 33, 55) or NRAS activation (34). LPS-induced increase in human beta-defensin-3 expression requires EGFR activation in oral squamous cell carcinoma cells (31). However, it has not been established that EGFR is essential for the LPS-dependent activation of NFκB. We now show in addition that EGFR activation is required for LPS-induced NFκB activation.

Basu et al. (56) reported that EGFR binds to TLR4 in response to the Helicobacter pylori secretory protein HP0175 in human gastric epithelial cells. However, the interaction was observed only after 60 min, and we have found that EGF- or LPS-mediated NFκB activation is strongly induced within 5–10 min. Furthermore, we were not able to observe any interaction between EGFR and TLR4 in response to EGF or LPS (Fig. S4), suggesting that additional kinases are likely to mediate an indirect interaction between these two receptors. The SRC family inhibitor PP2 blocks LPS-mediated NFκB activation (43) and, consistent with this report, we now show that PP2 blocks NFκB activity upon LPS stimulation in A549 cells.

LPS stimulation leads to the recruitment of LYN, a SRC family member, to TLR4 in HEK293TLR4/MD-2 stable transfectants (43). Consistent with this earlier report, we now show that LPS stimulation leads to the recruitment of LYN to TLR4 in HME and A549 cells. We also demonstrate that stimulation with LPS leads to the recruitment of LYN to EGFR, and this association is blocked by erlotinib, indicating that the kinase activity of EGFR is required. Erlotinib also blocks the LPS-stimulated recruitment of LYN to TLR4, revealing that the kinase activity of EGFR is also necessary for this association. The involvement of LYN in an LPS mediated pathway was reported earlier (43, 47, 57). In this study, we observed the involvement of LYN specifically in the activation of EGFR in response to LPS and also in the activation of TLR4 in response to EGF, and conclude that LYN is a key kinase in establishing cross talk between EGFR and TLR4, leading to downstream signaling (Fig. 3C). Additional studies are necessary to explore further details of how LYN functions in response to LPS in mediating TLR4-dependent signaling. In particular, because EGFR and TLR4 do not bind to each other in response to LPS, and because LYN can be activated by oligomerization of TLR4 alone, it is not clear why EGFR is needed in order for LPS to activate NFκB.

LPS, a potent immunostimulatory component of Gram-negative bacteria, can induce systemic inflammation and sepsis (58) by triggering the release of many cytokines, including TNFα, IL-1β, and IL-6 (59). We now demonstrate that treatment of mice with erlotinib inhibits the production of inflammatory cytokines following LPS administration. It is noteworthy that erlotinib, a well known drug used extensively in cancer treatment, is also beneficial in suppressing the inflammatory signal triggered by LPS. Importantly, we also show that erlotinib protects mice from LPS-mediated lethality. Because too much activation of LPS/TLR4 signaling can lead to acute endotoxicity and chronic inflammatory disorders, our findings highlight the potential utility of erlotinib in inhibiting these devastating responses to infection. Septic shock is a complex disease for which preventive and therapeutic strategies are unfortunately lacking. Developing a better understanding of its pathophysiology underpins the development of more efficacious management regimes. Therefore, further investigation of the use of erlotinib, or other EGFR inhibitors, to modulate LPS-mediated endotoxicity may contribute to the development of a novel strategy for therapeutic intervention to ameliorate septic shock in the future.

Materials and Methods

The human mammary epithelial cell line hTERT-HME1, from Clontech, and the human breast cancer cell line A549, from American Tissue Culture Collection, were
used to show that knockdown of TL4 prevented activation of NF-kB in response to LPS and that TL4 was phosphorylated by EGFR. HME1 cells expressing BCL2 were used to show that activation of NF-kB by LPS requires the kinase activity of EGFR. Inhibitors and knockdown experiments showed that LYN is required for signaling in both directions. C57BL/6J mice from the Jackson Laboratory were used to show that inhibiting EGFR blocked IL-6 and TNFα expression in response to LPS, using ELISA assays, and that pretreatment with an EGFR inhibitor protected the mice from LPS-mediated endotoxicity. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic. Detailed materials and methods are provided in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Maoji Yang for excellent technical assistance, Yuxin Wang for help with the manuscript and Judy Drabza of the Image Core of the Lerner Research Institute, Cleveland Clinic for help with Western microscopy. This work was funded by National Institutes of Health Grant PO1 CA62220 (to G.R. and X.L.).