Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-β (TRIF)-Mediated Signaling Contributes to Innate Immune Responses in the Lung during Escherichia coli Pneumonia

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Bacterial pneumonia remains a serious disease and is associated with neutrophil recruitment. Innate immunity is pivotal for the elimination of bacteria, and TLRs are essential in this process. Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) is an adaptor for TLR3 and TLR4, and is associated with the MyD88-independent cascade. However, the importance of TRIF in immune responses against pulmonary bacterial pathogens is not well understood. We investigated the involvement of TRIF in a murine model of Escherichia coli pneumonia. TRIF−/− mice infected with E. coli display attenuated neutrophil migration; NF-κB activation; and TNF-α, IL-6, and LPS-induced C-X-C chemokine production in the lungs. In addition, E. coli-induced phosphorylation of JNK, ERK, and p38 MAPK was detected in bone marrow-derived macrophages (BMMs) of TRIF−/− mice, but attenuated in BMMs of TRIF+/− mice. Furthermore, E. coli-induced TNF-α and IL-6 production was attenuated in BMMs of TRIF−/− mice. E. coli LPS-induced late MAPK activation, and TNF-α and IL-6 production were abolished in BMMs of TRIF−/− mice. Moreover, TRIF is not required for LPS-induced neutrophil influx, and keratinocyte cell-derived chemokine, MIP-2, and LPS-induced C-X-C chemokine production in the lungs. Using TLR3−/− mice, we ruled out the role of TLR3-mediated TRIF-dependent neutrophil influx during E. coli pneumonia. A TLR4-blocking Ab inhibited E. coli-induced TNF-α and IL-6 in BMMs of both TRIF−/− and TRIF+/− mice, suggesting that TRIF-mediated signaling involves TLR4. We also found that TRIF is critical to control E. coli burden in the lungs and E. coli dissemination. Thus, rapid activation of TRIF-dependent TRIF-mediated signaling cascade serves to augment pulmonary host defense against a Gram-negative pathogen.

Previous investigations have demonstrated the important roles of adapter proteins in the MyD88-dependent cascade of TLR4 signaling, such as TIRAP and MyD88, in host defense in the lung. For example, MyD88 is shown to be important for host response in the lung against *Pseudomonas aeruginosa* (17, 18), nonmotile *Haemophilus influenzae* (19), and *Klebsiella pneumoniae* (20, 21). We recently reported that TIRAP plays a critical role in host response in the lung during pneumonia induced by *Escherichia coli* (22) and *K. pneumoniae* (23), but not *Pseudomonas aeruginosa* (23). In contrast, the role of adaptors in the MyD88-independent cascade, such as TRIF and TRAM against pulmonary bacterial pathogens, is not well understood.

In this study, we sought to define the role of TRIF in pulmonary host defense against a Gram-negative pathogen, *E. coli*. We also used a bacterial product from *E. coli*, endotoxin (LPS), a canonical TLR4 agonist, to demonstrate whether TRIF is important to induce lung inflammation. We observed that activation of the TLR4-mediated TRIF-dependent signaling cascade seems to be important for an effective innate immune response in the lung against *E. coli* despite the fact that TLR4-TRIF signaling does not seem to be required to induce innate pulmonary immune response against *E. coli* LPS. Our results provide new insights into the pulmonary innate immune mechanisms by which Gram-negative pathogens induce severe pneumonia.

### Materials and Methods

#### Mouse strains

TRIF gene-disrupted mice (TRIF⁻/⁻) were on a C57BL/6 background and, therefore, C57BL/6 were used as controls. Mouse infection protocols were adapted from the whole lung to collect cells in the airspace and to obtain proteins for biochemical studies. Whole lungs were homogenized in 10 ml of sterile saline for 30 s, and the homogenates were plated in serial 10-fold dilutions on MacConkey and trypticase soy agar (TSA) plates.

#### Mouse infection protocol

Bacteria were prepared for mouse inoculation, as described in previous studies (22). *E. coli* (American Type Culture Collection 25922) were grown in trypticase soy broth at 37°C overnight under constant agitation. Bacteria were harvested by centrifugation, washed twice in sterile isotonic saline, and resuspended in sterile 0.9% saline at a concentration of 20 × 10⁶ CFU/ml. Mouse strains were anesthetized with i.p. avertin (250 mg/kg), followed by intratracheal (i.t.) inoculation of 50 μl of bacteria (10⁶ CFU/mouse). The neck incision was closed with sterile staples under aseptic conditions. Control mice were i.t. inoculated in a similar manner with 50 μl of saline. The initial mouse inoculums were confirmed by plating serial 10-fold dilutions on MacConkey and tryptic soy agar (TSA) plates.

#### Mouse LPS challenge protocol

The induction of lung inflammation in a murine model by LPS aerosolization has been described in our previous studies (25–28). Briefly, isolated mouse band 3 neutrophils from the bone marrow were incubated with 5 μl of culture on MacConkey and TSA plates.

#### Bronchoalveolar lavage fluid (BALF) collection

At the indicated time points after *E. coli* or LPS challenge, mice were euthanized by CO₂ asphyxia and exsanguinated by cardiac puncture. A micropipette was used to open the thoracic cavity, and the trachea was isolated and cannulated with a 20-gauge catheter. BALF was obtained from the whole lung to collect cells in the airspace and to obtain proteins for cytokine and chemokine detection, as described previously (25–28). A total of 3.0 ml of BALF was retrieved from each mouse, and 0.5 ml of BALF was centrifuged and placed on glass cytopsin slides, which were then stained by modified Wright-Giemsa staining (Diff-Quick; Fisher Scientific) to determine leukocyte subtypes based on their cellular and nuclear morphology. A total of 500 cells was counted in this respect. Leukocytes in BALF were determined using a hemocytometer. For determination of keratinocyte cell-derived chemokine (KC), MIP-2, LPS-derived C-X-C chemokine (LIX), TNF-α, and IL-6 by ELISA, 2 ml of the undiluted cell-free BALF was centrifuged, passed via a 0.22-μm filter, and used immediately or kept at −20°C.

#### NF-κB activation

Translocation of the p65 subunit of NF-κB into the nucleus of lung cells was detected using an ELISA-based assay using oligonucleotides to specifically recognize the 5′-GGAGCTTCCC-3′ nucleotide sequence of Rel/NF-κB family (Active Motif), as described in our previous publications (22, 29). A total of 20 μg of nuclear extract obtained from each lung sample after 2 h post-*E. coli* or post-LPS challenge was added to the NF-κB-specific oligonucleotide-coated 96-well plate and incubated for 1 h at room temperature. After washing the wells three times, a primary Ab specific for p65 subunit of Rel/NF-κB was added and incubated for 1 h at room temperature. The wells were washed three times to remove excess *Ab*, an anti-HPB conjugate was added, and the color development was monitored continuously. Color development was measured at the OD of 450 nM. This assay system has no cross-reactivity with any other transcription factors because of the immobilized specific nucleotide sequence and the specific Ab for Rel/NF-κB.

#### Cytokine and chemokine determination in BALF

Cytokine and chemokine levels were assessed in BALF or culture supernatants of bone marrow-derived macrophage (BMM) cultures using a cytokine- or chemokine-specific sandwich ELISA, as described in our earlier publications (25–28). The minimum detection limit is 2 pg/ml cytokine or chemokine protein.

#### BMM culture

BMMs were differentiated using DMEM containing 10% FBS and L-929 cell supernatant, as described (30). BMMs were grown in 12-well plates for 6 days, and cells were then stimulated for the indicated times with *E. coli* LPS (100 ng/ml), flagellin (100 ng/ml), or viable *E. coli* (10⁵ or 10⁶/ml), and processed in SDS-loading buffer before being separated on SDS polyacrylamide gels. Gels were transferred onto nitrocellulose membranes, and proteins were detected using the indicated Ab specific for phosphorylated or total MAPKs, such as JNK, ERK, and p38. The Western blots were quantitated and expressed as fold increase in response to *E. coli* after background (unstimulated) subtraction. In another set of experiments, BMMs were pretreated with a TRIF4-blocking Ab (27) or isotype *Ab*. Supernatants were harvested from BMMs at 18 h after stimulation with the same agonists for TNF-α and IL-6 by ELISA.

#### Bacterial enumeration in the lungs and spleens

Whole lungs were homogenized in 10 ml of sterile saline for 30 s, and resulting 20 μl of homogenates was plated in serial 10-fold dilutions on MacConkey and TSA plates. Cytokine and chemokine levels were assessed in BALF or culture supernatants of bone marrow-derived neutrophils or alveolar macrophages (AMs) from TRIF⁻/⁻ mice have impaired ability to uptake and/or kill *E. coli*, an uptake/bactericidal assay was performed, as described (22, 28). *E. coli* obtained after two washings with sterile 0.9% saline was used at a concentration of 10⁶ CFU/ml, was mixed with 10⁶/ml murine bone marrow-derived neutrophils or AMs in RPMI 1640 medium containing 10% FBS in a microfuge tube, and rotated at 50 rpm at 37°C for 2 h. To demonstrate *E. coli* dissemination, spleens were homogenized in 5 ml of culture on MacConkey and TSA plates.

#### Actin polymerization measurement

To determine whether neutrophils from TRIF⁻/⁻ mice have impaired actin assembly in response to inflammatory mediators, cells were exposed to KC, MIP-2, and TNF-α, as described previously (31, 32). Briefly, isolated mouse band 3 neutrophils from the bone marrow were incubated with 5 ng/ml MIP-2 or KC for 15 min. 1 μg/ml TNF-α for 1 h, or kept unstimulated for 1 h at 37°C. At the end of incubation, neutrophils were fixed and actin cytoskeleton changes were recorded using a flow cytometer.

#### Data analysis

All data are expressed as means ± SE. Data were analyzed with Student’s *t* test (between two groups) or with the one-way ANOVA (>2 groups).
We found that TLR4 sought to ascertain the role of TLR4 in Gram-negative pneumonia. The MyD88-independent cascade also plays a role. We next mate controls (MyD88-dependent cascade is critical to induce lung defense against A), demonstrating that the MyD88-at 24 h postinfection (Fig. 1). In sharp contrast with the involvement of TLR4-dependent and TLR4-independent cascades. Although reduced, MyD88 mice have impaired lung inflammatory response after E. coli infection. Reduced neutrophil accumulation in the airspaces of TRIF mice after E. coli, but not after E. coli LPS challenge. BALF were collected from TRIF and TRIF mice at 6 and 24 h after bacterial (A), at 8 and 24 h after LPS (B), or at 24-h saline administration (B). Data are expressed as mean ± SE of five to seven mice from three separate experiments at each time point. * Significant differences between TRIF and TRIF mice (p < 0.05).

Results

**MyD88-dependent and MyD88-independent cascades contribute to lung inflammation in response to E. coli**

First, we examined the role of MyD88 in Gram-negative bacterial pneumonia. Mice deficient in MyD88 (MyD88−/−) or its littermate controls (MyD88+/+) were challenged with E. coli through the i.t. route, and neutrophils were counted as an index of lung inflammation. In MyD88−/− mice, neutrophil counts in the BALF were substantially reduced in response to 10⁶ CFU/mouse E. coli at 24 h postinfection (Fig. 1A), demonstrating that the MyD88-dependent cascade is critical to induce lung defense against E. coli. Although reduced, MyD88−/− mice still had significant neutrophil influx in response to 10⁶ CFU/mouse E. coli (Fig. 1A), indicating that the MyD88-independent cascade also plays a role. We next sought to ascertain the role of TLR4 in Gram-negative pneumonia. We found that TLR4−/− mice had attenuated neutrophil influx in response to the same dose of E. coli (Fig. 1A), demonstrating the involvement of TLR4-dependent and TLR4-independent cascades. In sharp contrast with the E. coli data, MyD88- and TLR4-dependent cascade, but not TLR4-independent cascade, is required for E. coli LPS-induced inflammation in the lung (Fig. 1B). *TRIF mice have impaired lung inflammatory response after E. coli infection*

Because neutrophil recruitment in MyD88−/− mice was attenuated, but not abolished, in response to E. coli (Fig. 1A), we investigated whether TRIF in the MyD88-independent signaling cascade is required for E. coli-induced lung inflammation. When TRIF−/− and TRIF+/+ mice were i.t. inoculated with E. coli, reduced neutrophil migration was observed in BALF of TRIF mice as compared with their wild-type controls at both 6 and 24 h after postinfection (Fig. 2A). In contrast, no apparent difference in neutrophil influx in response to E. coli LPS was noted between TRIF−/− and TRIF+/+ mice at 8 and 24 h after saline challenge (Fig. 2B). These findings conclude that TRIF contributes to E. coli-, but not LPS-induced lung inflammation, including neutrophil recruitment.

Actin assembly of neutrophils to chemokines and cytokines was investigated to determine whether defective actin cytoskeleton changes contribute to attenuated neutrophil accumulation in the lungs of TRIF−/− mice in response to E. coli infection (27). In this regard, our results demonstrate that TRIF−/− neutrophils are equally effective as the TRIF+/+ neutrophils to act in cytoskeleton changes in response to cytokines and chemokines (Fig. 2C), ruling

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** Reduced neutrophil accumulation in the airspaces of MyD88−/− and TLR4−/− mice after E. coli or E. coli LPS administration. The MyD88−/−, TLR4−/−, and C57BL/6 animals underwent BALF collection at 24 h after bacterial (A), LPS (B), or saline (A and B) challenge. Neutrophils were counted in BALF. Data expressed as mean ± SE of five to six animals from three separate experiments in each group at each time point. * Significant differences between TRIF−/− and TRIF+/+ mice (p < 0.05).

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** Decreased neutrophil recruitment in the airspaces of TRIF−/− mice after E. coli, but not after E. coli LPS challenge. BALF were collected from TRIF−/− and TRIF+/+ mice at 6 and 24 h after bacterial (A), at 8 and 24 h after LPS (B), or at 24-h saline administration (B). Data are expressed as mean ± SE of five to seven mice from three separate experiments at each time point. Significant differences between TRIF−/− and TRIF+/+ are indicated by *, p < 0.05. C, Effect of TIRAP deficiency in actin cytoskeleton changes. Bone marrow-derived band 3 cells were isolated and stimulated with TNF-α, KC, or LPS at designated time points. Cells were then labeled with nitrobenzoxadiazole-phallacidin, and actin assembly changes were measured by a flow cytometer. Four animals from two separate experiments were used in each group, and the data were normalized to unstimulated controls giving a relative fluorescence index of 1.
out this as a mechanism for decreased neutrophil accumulation in the lungs of TRIF−/− mice after *E. coli* infection.

**TLR3 deficiency does not influence lung inflammation after *E. coli* challenge**

Because the TLR3 signaling cascade is also associated with TRIF (14), we next examined the role of TLR3-mediated TRIF signaling in *E. coli*-induced neutrophil accumulation in the lungs in response to *E. coli* challenge. As shown in Fig. 3, there were no differences in neutrophil influx in the lungs between the TLR3−/− and TLR3+/+ mice observed after *E. coli* challenge (Fig. 3). These data demonstrate that TLR3-mediated TRIF-dependent signaling does not play a significant role in the induction of pulmonary inflammation following *E. coli* infection. Because TRIF is an adaptor molecule for both TLR3 and TLR4, these observations support that TRIF-dependent signaling triggered by *E. coli* is initiated by TLR4.

**TRIF deficiency impairs NF-κB activation in the lung against *E. coli***

We next sought to determine whether the absence of functional TRIF influenced NF-κB translocation in the lung in response to *E. coli* infection. We measured active NF-κB in the nuclear fraction of whole lung homogenates after *E. coli* and *E. coli* LPS challenge. Our observations demonstrate that TRIF-dependent signaling mediates NF-κB activation in lung cells following *E. coli* challenge (Fig. 4). Although NF-κB translocated in the lungs of TRIF−/− and TRIF+/+ after LPS challenge, a modest, but significative reduction in NF-κB translocation was observed in the lungs of TRIF−/− mice as compared with their littermate controls (TRIF+/+) after LPS challenge (Fig. 4).

**TRIF−/− mice have impaired cytokine and chemokine production in response to *E. coli***

BALF studies were performed following challenge with *E. coli* or *E. coli* LPS. Although production of KC and MIP-2 was not decreased in response to *E. coli* (Fig. 5, B and C), TNF-α, IL-6, and LIX production in the airspaces of TRIF−/− mice after *E. coli* infection was decreased in TRIF−/− or TRIF+/+ mice at 6 or 24 h after bacterial challenge, and total white blood cells and neutrophils were enumerated. Data are the mean ± SEM of five to seven mice per group at each time point (p < 0.05). F–J, Attenuated TNF-α, but not KC, MIP-2, IL-6, and LIX production in the airspaces of TRIF−/− mice after *E. coli* LPS challenge. Data are expressed as mean ± SEM of six mice per group at each time point. Statistical significance was calculated as p < 0.05.
BMMs from TRIF−/− mice have attenuated activation of MAPKs and TNF-α and IL-6 production in response to E. coli and E. coli LPS

Next, we determined the effects of TRIF deficiency on MAPK signaling. When TRIF−/− macrophages were stimulated with E. coli, reduced activation of JNK, ERK, and p38 was observed only at late time point, i.e., at 60 min (Fig. 6, A and B). In addition, late MAPK activation was abolished in macrophages obtained from TRIF−/− mice in response to flagellin, a canonical TLR5 agonist (Fig. 6A). These data suggest that TRIF is important for E. coli and E. coli LPS-induced late activation of MAPKs in BMMs.

We also examined whether TRIF plays a role in the expression of cytokines and chemokines by BMMs in response to E. coli or LPS derived from E. coli culture supernatants obtained from TRIF−/− macrophages demonstrated attenuated production of TNF-α and IL-6 by TRIF−/− BMMs to E. coli (Fig. 6C). In a similar manner, TNF-α and IL-6 production by TRIF−/− BMMs was abolished after E. coli LPS stimulation (Fig. 6D). To demonstrate whether TRIF-dependent cascade involves TLR4 in E. coli-induced effects in BMMs of TRIF−/− mice, we used a TLR4-blocking Ab. Our results demonstrate that TLR4 Ab blocks E. coli-induced TNF-α and IL-6 responses in BMMs obtained from both TRIF−/− and TRIF+/+ mice (Fig. 6E), suggesting that TLR4-dependent TRIF-mediated (MyD88-independent)
signaling cascade is essential for cytokine responses against *E. coli*.

**TRIF contributes to *E. coli* clearance in the lung and bacterial dissemination**

Having established that TRIF contributes to efficient innate immune response in the lungs against *E. coli*, we next investigated whether TRIF is important for host defense against this pathogen. The lungs of TRIF<sup>−/−</sup> mice had more *E. coli* burden in the lungs as compared with its littermate controls at 24 h postinfection (Fig. 7A). More bacterial dissemination was noted in TRIF<sup>−/−</sup> mice at 6 and 24 h post-*E. coli* challenge (Fig. 7B). Furthermore, survival did not differ between TRIF<sup>−/−</sup> and TRIF<sup>+/+</sup> mice after *E. coli* challenge (10<sup>6</sup>/mouse) (Fig. 7C). Taken together, these findings demonstrate that TRIF is essential in limiting bacterial colonization in the lungs and bacterial dissemination in the bloodstream despite the fact that TRIF is not important for survival.

An alternative mechanism for higher CFUs observed in the lungs of TRIF<sup>−/−</sup> mice after *E. coli* challenge is impairment of uptake/bactericidal capacity of neutrophils or resident AMs in the lungs. To examine this possibility, we conducted an in vitro uptake/killing assay of *E. coli* by neutrophils and AMs. *E. coli* was cultured in the presence of neutrophils from TRIF<sup>−/−</sup> or TRIF<sup>+/+</sup> mice in vitro, and the bacterial CFUs in the supernatant at 2 h were calculated. No differences between CFUs in the presence of neutrophils (Fig. 7D) or AMs (Fig. 7E) obtained from TRIF<sup>−/−</sup> or TRIF<sup>+/+</sup> mice were observed at 2 h, suggesting that TRIF<sup>−/−</sup> cells have an intact ability to uptake/kill as similar to TRIF<sup>+/+</sup> neutrophils or AMs.

**Discussion**

Bacterial pneumonia continues to be a major illness associated with significant morbidity and mortality in the United States (1–4). Gram-negative microbes are major causative agents for acute pneumonia (2, 3). The high prevalence of pneumonia and the emergence of antibiotic resistant bacterial strains demand designing better therapeutic options to control this disease.

Innate immune response plays a critical role in bacterial clearance both locally and systematically. TLRs are important in the induction of innate immunity to bacterial pathogens (6). TLRs, particularly TLR4, induce at least two divergent signaling cascades that involve at least four adaptor molecules. The MyD88-dependent cascade involves TIRAP and MyD88 (11–13), whereas the MyD88-independent cascades involve TRIF and TRAM (14, 15). Although there is abundant evidence from in vivo studies that MyD88-dependent signaling cascades contribute to lung defense against bacterial pathogens (17–23), the role of the MyD88-independent cascade in the induction of host defense in the lung has not been well studied. We attempted to define the role of TRIF, an adaptor in the MyD88-independent cascade, in host defense in the lung against a Gram-negative bacterium *E. coli*. The present investigation clearly established that TRIF contributes to lung immune response and limits bacterial growth in the lungs and bacterial dissemination during *E. coli* pneumonia. Furthermore, our results demonstrate that genetic deletion of TRIF did not affect LPS-induced pulmonary inflammation.

Neutrophil accumulation in the lungs is a pathological hallmark of bacterial pneumonia (33–35). In our earlier studies, we have established that TIRAP is important for neutrophil recruitment into the lungs during *E. coli* pneumonia (22). In those investigations, the importance of MyD88 or TLR4 in neutrophil recruitment was not examined. In the current investigation, we have observed that MyD88 and TLR4 are required for neutrophil accumulation in the lungs during *E. coli* pneumonia. Our findings are consistent with a previous report, indicating that TLR4 is important for neutrophil influx during *E. coli* pneumonia (36). Our observations also demonstrate that TRIF is important for neutrophil recruitment during *E. coli* pneumonia. It has been well documented that TRIF is an adaptor molecule for TLR3- and TLR4-mediated signaling cascades (14, 15). The observation of unimpaired neutrophil accumulation in TLR3<sup>−/−</sup> mice during *E. coli* pneumonia (Fig. 3), together with the finding that anti-TLR4 Ab blocks *E. coli*-induced TNF-α and IL-6 expression in BMMS of TRIF<sup>−/−</sup> and TRIF<sup>+/+</sup> mice (Fig. 6), point toward the involvement of TRIF in the TLR4 signaling cascade. In this regard, the sole previous investigation determined the role of TRIF to a bacterial pathogen and concluded that TLR4-mediated TRIF-dependent cascade does not play a role in neutrophil recruitment in the lungs after nontypeable *H. influenzae* infection. However, two earlier investigations (19, 37) determined the role of TLR4 in pulmonary defense against *H. influenzae* in vivo.
using TLR4-mutant mice and reported an enhanced susceptibility in mutant mice. Therefore, our study is the first one demonstrating the role of TRIF associated with TLR4 in the induction of neutrophil recruitment in the lung against a bacterial pathogen. Because we have established a critical role of TIRAP in E. coli-induced lung infection (22), these data (22) together with our current findings demonstrate that both MyD88-dependent and MyD88-independent cascades of TLR4 are required for the induction of host defense against pulmonary E. coli infection.

In the current investigation, we revealed that TRIF in the MyD88-independent cascade does contribute to lung inflammation after E. coli challenge. Given the fact that TRIF<sup>−/−</sup> neutrophils have an unimpaired actin assembly (Fig. 2) and TRIF<sup>−/−</sup> neutrophils and AMs have an intact bactericidal capability (Fig. 7), the defective influx of neutrophils into the airways is most likely the result of attenuated LIX, TNF-α, and IL-6 production in the lungs of TRIF<sup>−/−</sup> mice. However, our findings rule out the role of TRIF in E. coli LPS-induced neutrophil accumulation. In this context, E. coli LPS is known to activate TLR4 signaling cascade to induce lung inflammation. Using C3H/HeJ mice, which have mutated/nonfunctional TLR4, we have established that E. coli LPS lung inflammation requires TLR4 (27). We have also illustrated that the TIRAP adaptor molecule in the MyD88-dependent cascade is critical to induce lung inflammation after viable E. coli and E. coli LPS challenge (22). The differential requirement of TLR4-TRIF signaling axis in the induction of lung inflammation between E. coli and E. coli LPS is unexpected, and it brings up several possibilities, as follows: 1) the affinity and/or avidity of E. coli binding to TLR4 is different than E. coli LPS; 2) the presence of another receptor in addition to TLR4 is responsible for E. coli-induced effects; and 3) E. coli, but not LPS, binding to TLR4 or other cell surface receptor recruits one or more intracellular signaling molecules that signal via TRIF. A proposed scheme for this is presented in Fig. 8. Future studies are required to examine these possibilities.

NF-κB is one of the well-studied transcription factors that mediate the activation of numerous inflammatory cytokines and chemokines (33–36, 38, 39). Elegant studies have demonstrated that NF-κB activation is a pivotal event in the pathogenesis of bacterial pneumonia (33–35). It has been established that adaptors transduce cell surface TLR signals to NF-κB, which results in NF-κB-dependent transcription (6–8). The MyD88-dependent cascade induces early NF-κB translocation, whereas the MyD88-independent cascade causes late NF-κB activation (6–8). Our findings suggest that TRIF-dependent NF-κB activation is a critical mediator to induce LIX, TNF-α, and IL-6 expression in the lungs in response to E. coli (Figs. 4 and 5). Although our investigation demonstrates a role for TRIF in E. coli-induced lung inflammation, a recent study using H. influenzae indicates that TRIF does not play a role in cytokine and chemokine expression in the lungs (19). The discrepancy between our findings and their observations (19) could be explained by the nature of the pathogens and time points used to measure chemokines/cytokines in the lungs.

Our results demonstrate that TRIF is an important molecule for MAPK activation in BMMs (Fig. 6). A remarkable feature of MAPK activation is that only the late MAPK activation was attenuated or abolished in BMMs obtained from TRIF<sup>−/−</sup> mice after E. coli and E. coli LPS challenge. These results are consistent with previous reports stating that activation of TLR4 leads to different phases of MAPK activation (11–15). Whereas activation of the TLR4-mediated MyD88-dependent signaling leads to an immediate-phase activation of MAPKs, TLR4-mediated MyD88-independent signaling causes a late-phase activation of MAPKs (11–15). Our findings also suggest that MAPK signaling is important for TNF-α and IL-6 production in BMMs in response to E. coli and E. coli LPS. Although IL-6 production in BMMs obtained from TRIF<sup>−/−</sup> mice in response to LPS was abolished, we did not observe a difference of IL-6 levels in BALF obtained from TRIF<sup>−/−</sup> and TRIF<sup>−/−</sup> mice. Although several reasons could have contributed to this discrepancy of IL-6 expression in BMMs vs BALF, we speculate that IL-6 production caused by LPS in nonmyeloid (resident) cells is not TRIF dependent. This explanation is supported by the fact that LIX production by viable E. coli is TRIF dependent, whereas LIX induction by E. coli LPS is TRIF independent (Fig. 5, E and J). In this context, we have demonstrated that LIX is predominantly produced by resident cells, i.e., alveolar type II cells after LPS challenge (25, 26).

The sentinel event in lung defense is the clearance of bacterial pathogens from the lungs and limitation of bacterial dissemination in the bloodstream. In our model of murine pneumonia, we have highlighted the importance of TRIF for antibacterial defense in the lung against E. coli. Although a previous study investigated the role of TRIF in H. influenzae infection, TRIF does not contribute to antibacterial defense in the lung in their model (19). To our knowledge, we have demonstrated for the first time that TRIF is capable of limiting bacterial growth in the lungs. The main reason underlying discrepancy between Wieland et al. (19) and ours are the time points used to measure bacterial CFUs in the lungs. The higher bacterial growth in the lungs of TRIF<sup>−/−</sup> mice and its dissemination may be due to attenuated neutrophil accumulation in the lungs (Fig. 2A) due to reduced LIX production (Fig. 5). Another potential mechanism for higher bacterial growth in the lungs and more bacterial dissemination in TRIF<sup>−/−</sup> mice may be decreased activation of recruited neutrophils by TNF-α and IL-6. Thus, the role of TRIF in pulmonary host defense against E. coli infection is most likely dependent on the interplay among E. coli-induced inflammatory mediators in the lung.

We conclude that the TLR4-mediated TRIF-dependent pathway is important to initiate a prompt and effective host response against E. coli, which is essential for bacterial clearance in the lung and bloodstream. Specific targeting of TRIF may prove to be a feasible option to attenuate excessive neutrophil recruitment in the lung during Gram-negative bacterial pneumonia.
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Disclosures

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