Both TRIF- and MyD88-Dependent Signaling Contribute to Host Defense against Pulmonary *Klebsiella* Infection

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Both TRIF- and MyD88-Dependent Signaling Contribute to Host Defense against Pulmonary Klebsiella Infection

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*Klebsiella pneumoniae causes extensive lung damage. TLR signaling involves adaptors TRIF and MyD88. However, the relative contribution of TRIF and MyD88 signaling in host defense against pulmonary K. pneumoniae infection has not been elucidated. Therefore, we investigated the role of TRIF and MyD88 in K. pneumoniae pneumonia. TRIF−/− mice infected with K. pneumoniae showed impaired survival and reduced bacterial clearance, neutrophil influx, histopathologic evidence of inflammation, and TNF-α, IL-6, KC, MIP-2, but not LIIX, expression in the lungs. In addition, K. pneumoniae-induced late NF-κB activation and phosphorylation of MAPKs was attenuated in the lungs of TRIF−/− mice. However, MyD88−/− mice infected with K. pneumoniae showed a much more remarkable phenotype, including impaired survival and reduced bacterial clearance, histopathology, and TNF-α, IL-6, KC, MIP-2, and LIX expression with almost no neutrophil influx in the lungs. In MyD88−/− mice, K. pneumoniae-induced early NF-κB and MAPK activation in the lungs was also reduced. Furthermore, the role of MyD88 is dominant over TRIF because TRIF/MyD88 double knockout mice displayed a more pronounced phenotype than TRIF−/− mice. Moreover, human alveolar macrophages pretreated with MyD88 blocking peptide showed attenuated TNF-α, IL-6, and IL-8 expression. Also, C57BL/6 mice pretreated with MyD88 blocking peptide exhibited attenuation in K. pneumoniae-induced neutrophil influx and enhanced bacterial burden in the lungs and dissemination. Overall, this investigation provides new insights into the TRIF and MyD88 signaling triggered by pulmonary K. pneumoniae infection in the lungs and demonstrate the therapeutic potential of MyD88 in reducing excessive neutrophil influx in human disease during Gram-negative bacterial pneumonia. The Journal of Immunology, 2009, 183: 0000–0000.

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Bacterial pneumonia is a serious illness with substantial morbidity and mortality (1–3). Klebsiella pneumoniae is a frequent cause of severe pneumonia with extensive lung destruction. Neutrophil recruitment to the lung, the pathological hallmark of bacterial pneumonia (4, 5), is required to augment host defense (1, 4). However, excessive neutrophil accumulation can result in acute lung injury or acute respiratory distress syndrome (6). Therefore, therapeutic strategies to modulate uncontrolled neutrophil influx in bacterial pneumonia and acute lung disease are sought to minimize lung damage.

Pathogens can be detected by receptors that recognize common pathogen-associated molecular patterns (7, 8). TLRs are vital sensors of these molecular patterns and are transmembrane proteins found on the cell surface or within endocytic vesicles (9, 10). For example, TLR2, TLR4, and TLR5 recognize bacterial peptidoglycan, endotoxin (LPS), and flagellin, respectively (8–10). Upon ligand binding to TLRs, MyD88, and Toll/IL-1R (TIR)3 domain-containing adaptor protein (TIRAP) are recruited to the TLR signaling complex, which results in the activation of MAPKs and NF-κB leading to production of cytokines/chemokines. This cascade is called the MyD88-dependent pathway (11, 12). Activation of TLRs also recruits other adaptor proteins including TIR domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). This pathway activates NF-κB and a type I IFNs and is called the TRIF-dependent (MyD88-independent) pathway (11, 12).

The MyD88-dependent cascade of TLRs involving MyD88 and TIRAP has been the primary focus of previous studies on bacteria-induced lung inflammation. In this context, MyD88 has been shown to be important for pulmonary host defense against Pseudomonas aeruginosa (13–15), nontypeable Haemophilus influenzae (16), Escherichia coli (17), Burkholderia pseudomallei (18), and Legionella pneumophila (19–21), whereas TIRAP plays a critical role in host defense in the lungs against E. coli (17) and K. pneumoniae (22). Although we have shown previously that MyD88−/− mice had attenuated neutrophil influx in response to K. pneumoniae infection, the host defense mechanisms associated with MyD88 have not been elucidated against K. pneumoniae (22). Regarding the TRIF-dependent signaling, TRIF has been shown to be important for host defense against some bacterial pathogens, such as E. coli (23) and P. aeruginosa (24), although it is not essential to host defense against a nontypeable H. influenzae (16) and B. pseudomallei (18). The role of the TRIF-dependent signaling cascade against K. pneumoniae has not been established.

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TRIF-related adaptor molecule; LIIX, LPS-induced CXC chemokine; KC, keratinocyte cell-derived chemokine; AM, Alveolar macrophage; BP, blocking peptide; CP, control peptide; BALF, bronchoalveolar lavage fluid; WT, wild type.
In the current study, we characterized the role of TRIF and MyD88 in pulmonary host defense against *K. pneumoniae*. Although we observed that activation of both TRIF and MyD88 signaling cascades is required for neutrophil-mediated host defense in the lungs against *K. pneumoniae*, the MyD88-dependent cascade seems more important. Our results demonstrate that the MyD88-dependent signaling is dominant over the TRIF pathway because TRIF/MyD88−/− mice showed a phenotype identical with MyD88−/− mice. Our findings reveal that MyD88 has a therapeutic potential in humans because 1) MyD88 blocking peptide (BP) attenuates chemokine/cytokine expression in human alveolar macrophages (AMs), and 2) C57BL/6 mice pretreated with MyD88 BP showed a reduction in neutrophil recruitment and a higher bacterial burden in the lungs and dissemination. Taken together, our findings support a model in which these two cascades play essential and independent roles in host defense in the lungs against *K. pneumoniae*, with the MyD88 signaling being dominant over the TRIF cascade. These findings also support the therapeutic potential of MyD88 in attenuating excessive lung inflammation in human disease.

Materials and Methods

**Mice**

TRIF−/−, MyD88−/− and TRIF/MyD88−/− mice (12, 25) were on a C57BL/6 background. Therefore, C57BL/6 mice were used as controls. All animal studies were approved by the Louisiana State University Animal Care and Use Committee. The mice were 8- to 10-wk-old females, ranging in weight from 19 to 25 g.

**Infection model**

*K. pneumoniae* intratracheal inoculation was performed as we have previously described (22, 26). *K. pneumoniae* serotype 2 (strain 43816; American Type Culture Collection) was grown for 16 h at 37°C in tryptic soy broth. Bacteria were harvested by centrifugation, washed twice in sterile isotonic saline, and resuspended in saline at a concentration of 20 × 10^5 CFU/ml. Mice were anesthetized with i.p. ketamine/xylazine and the trachea was exposed through a midventral incision followed by intratracheal inoculation of 50 μl of bacteria (10^6 CFU/50 μl/mouse). The neck incision was closed with sterile staples. Control mice were inoculated intratracheally 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 plates. Bacterial colonies were counted after incubation at 37°C for 18 h. Culture medium was collected and used for immunoblotting. To ensure equal amounts of protein onto the gel, a Bradford protein assay was performed (Bio-Rad). Equal amounts of protein from lung homogenates were loaded and separated by SDS-PAGE according to the method of Lammeli and electroblotted on to nitrocellulose membrane (Hybond ECL; Amersham Biosciences). Membranes were blocked for 1 h in TBS (containing 0.1% Tween 20) with 5% nonfat dry milk at room temperature for 1 h, followed by overnight incubation with primary Ab. The primary Abs to VCAM-1, ICAM-1, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), and phospho-SAPK/JNK (Thr183/Tyr185) (data not shown).

**Bronchoalveolar lavage fluid (BALF) collection**

BALF was obtained from the whole lung to collect cells in the airspace and to determine cytokine and chemokine levels as previously described (27–30). Approximately 3.0 ml of BALF was retrieved from each mouse, and 0.1 ml of BALF was sedimented by centrifugation and stained with Diff-Quik staining (Fisher) to determine leukocyte subtypes. A total of 500 cells were counted in this respect. Leukocytes in BALF were determined using a hemocytometer. For determination of cytokines/chemokines, the remainders (2 ml) of the undiluted cell-free BALF was passed via a 0.22-μm filter and used immediately or stored at −20°C.

**Myeloperoxidase assay**

Myeloperoxidase, a marker of neutrophil accumulation in the lungs, was measured as previously described (27–30). Excised whole lungs were weighed, kept frozen at −70°C, and then homogenized. The resulting homogenates were centrifuged and the pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0) (supplemented with 0.5% hexadecyl trimethyl ammonium bromide) to determine the myeloperoxidase level. Lungs were homogenized, incubated at 60°C for 2 h, and assayed for activity in a hydrogen peroxide/O-dianisidine buffer at 460 nm at 0 and 90 s. The myeloperoxidase activity was calculated between 0 and 90 s (time (min) × 1.13 × 10^−2). Samples were processed within 2 wk after collection.

**NF-κB activation**

NF-κB/p65 binding assays (TransAM ELISA kit) were performed according to the manufacturer’s protocol. A total of 7.5 μg of nuclear extract obtained from each lung was collected at 24 and 48 h postadministration of *K. pneumoniae* or saline, mixed with binding buffer, added to the precoated plate (with the DNA binding motif of NF-κB) and incubated for 1 h at room temperature. Wells were then washed, and plates were incubated with NF-κB/p65 Ab for 1 h. Plates were washed three times with wash buffer, and HRP-conjugated anti-rabbit IgG was added to each well and incubated for 1 h. Plates were read at 450 nm after adding the developing reagent (27–30).

**Cytokine and chemokine measurement**

Cytokine and chemokine concentrations were measured in BALF or lung homogenates using a cytokine- or chemokine-specific sandwich ELISA as described in our earlier publications (27–30). The minimum detection limit is 2 pg/ml cytokine or chemokine protein.

**Lung pathology**

The lungs were perfused from the right ventricle of heart with 10 ml of isotonic saline. Lungs were then removed and fixed in 4% phosphate-buffered formalin for 24 h. Fixed tissues were embedded in paraffin, and 5-μm sections were prepared and stained with H&E. These H&E sections were evaluated by a veterinary pathologist in a blinded fashion according to the following scoring system for inflammation: 0, No inflammatory cells (macrophages or neutrophils) present in section; 1, <5% of section is infiltrated by inflammatory cells; 2, 5–10% of section is infiltrated by inflammatory cells, and 3, >10% of section is infiltrated by inflammatory cells. These lung sections were also evaluated for bacterial burden with the following scoring: 0, no bacteria; 1, <5 bacteria per 10 high power fields (<×40 objective); 2, 5–20 bacteria per 10 high power fields; and 3, >20 bacteria per 10 high power fields.

**Immunoblotting**

At the designated time points, the lungs were homogenized for 45 s in 1 ml of buffer containing 0.1% Triton X-100 in PBS, complete protease inhibitor cocktail (Thermo Scientific), complete phosphate inhibitor cocktail (Thermo Scientific), and 1 mM DTT, followed by centrifugation at maximum speed in a microcentrifuge at 4°C. The resulting supernatants were used for immunoblotting. To ensure equal amounts of protein onto the gel, a Bradford protein assay was performed (Bio-Rad). Equal amounts of protein from lung homogenates were loaded and separated by SDS-PAGE according to the method of Lammeli and electroblotted on to nitrocellulose membrane (Hybond ECL; Amersham Biosciences). Membranes were blocked for 1 h in TBS (containing 0.1% Tween 20) with 5% nonfat dry milk at room temperature for 1 h, followed by overnight incubation with primary Ab. The primary Abs to TNF-α, IL-6, and IL-8 protein measurement by ELISA. Medium was centrifuged at 500 × g for 10 min to discard remaining cell debris, and supernatants were stored at −80°C until use. We found that MyD88 BP or CP did not alter the viability of cells or bacterial growth after pretreatment (data not shown).

**Human AM isolation and stimulation with K. pneumoniae**

AMs were isolated from lungs of humans who had no history of lung diseases, as described in our previous publication (17). Thereafter, the human AMs in each well (2 × 10^6 cells/well in 6-well plate in 2 ml of medium) were pretreated with either 200 μg of MyD88 BP (100 μg/ml) or control peptide (CP), or left untreated for 2 h, followed by stimulation with 1 × 10^5 CFU/ml *K. pneumoniae* for 18 h. Culture medium was collected for TNF-α, IL-6, and IL-8 protein measurement by ELISA. Medium was centrifuged at 500 × g for 10 min to discard remaining cell debris, and supernatants were stored at −80°C until use. We found that MyD88 BP or CP did not alter the viability of cells or bacterial growth after pretreatment (data not shown).
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Statistical analysis

All data are expressed as mean ± SE. Data were analyzed with Student's t test (between two groups) or with the one-way ANOVA (>2 groups). Survival curves were compared by Wilcoxon rank sign test. Differences in data values were defined significant at a value for \( p < 0.05 \) using Kaleidagraph (Synergy Software).

Results

TRIF is required for pulmonary host defense against K. pneumoniae

To determine the importance of TRIF in mucosal host immunity in the lung, we used an experimental model of pulmonary K. pneumoniae infection. We first examined the importance of TRIF in survival from K. pneumoniae infection. Mice deficient in TRIF (TRIF\(^{-/-}\)) and their littermate controls (TRIF\(^{+/+}\)) were challenged with intratracheal K. pneumoniae (10\(^3\) CFU/mouse), and survival was monitored up to 14 days. As demonstrated in Fig. 1A, TRIF\(^{-/-}\) mice showed accelerated mortality as compared with their wild-type (WT) counterparts (Fig. 1A).

Having established that TRIF is important for host defense, we sought to investigate the mechanisms associated with enhanced mortality in TRIF\(^{-/-}\) mice followed by K. pneumoniae infection. Mice were infected with K. pneumoniae (10\(^3\) CFU) intratracheally and sacrificed at 24 and 48 h postinfection. The lungs and spleens were isolated to determine the bacterial CFU. TRIF\(^{-/-}\) mice had a greater number of CFU in the lungs and spleens at 48 h postinfection (Fig. 1, B and C).

We then investigated whether TRIF mediates K. pneumoniae-induced neutrophil influx in the lungs to augment host defense. In TRIF\(^{-/-}\) mice, neutrophil influx into the airspaces (BALF) and lung parenchyma (myeloperoxidase activity) was reduced in response to 10\(^3\) CFU/mouse K. pneumoniae at 24 and 48 h postinfection (Fig. 1, D–F), demonstrating that TRIF is important for neutrophil-mediated lung defense against K. pneumoniae. TRIF\(^{-/-}\) mice similarly showed moderate suppurative broncho-pneumonia (score of 2.0) with intralesional bacteria (score of 1.0) (Fig. 1G), whereas TRIF\(^{+/+}\) mice displayed mild suppurative pneumonia (score of 1.0) with high intralesional bacteria (score of 2.0). No pathological changes were, however, observed in saline challenged (control) lungs obtained from both TRIF\(^{-/-}\) and TRIF\(^{+/+}\) animals (Fig. 1G).

Cytokine and chemokine production in response to K. pneumoniae requires TRIF

It has been demonstrated that cytokines and ELR\(^+\) (glutamic acid-leucine-arginine) CXC chemokines contribute to neutrophil influx into the lungs (31–33). In this regard, BALF studies were performed following challenge with K. pneumoniae to determine cytokine and chemokine levels. Although K. pneumoniae-induced TNF-\(\alpha\), IL-6, keratinocyte cell-derived chemokine (KC), MIP-2 production in BALF was reduced in TRIF\(^{-/-}\) mice at 48 h (Fig. 2, A–D), LPS-induced CXC chemokine (LIX)
expression was not differed between TRIF$^{-/-}$ and TRIF$^{-/-}$ mice at this time point (Fig. 2 E).

Because IL-23 and IL-17 can regulate ELR$^+$ CXC chemokines, such as KC and MIP-2, in response to K. pneumoniae infection (34, 35), we have determined the levels of IL-23 and IL-17 in our model. Our data show less IL-17 in TRIF$^{-/-}$/H11002/H11002 mice, although IL-23 levels were not different between TRIF$^{-/-}$/H11002/H11002 and TRIF$^{-/-}$/H11001/H11001 mice (Fig. 2, F and G). We measured these cytokines in lung homogenates and BALF, however, their levels were not detectable in BALF (data not shown).

**FIGURE 2.** Impaired cytokine and chemokine responses in the airspaces of TRIF$^{-/-}$ mice in response to infection with K. pneumoniae (Kp). A–E, Cytokine and chemokine levels in BALF were measured by sandwich ELISA after infection with K. pneumoniae. Protein levels expressed as mean $\pm$ SE with $n = 4–6$ animals used at each time point. *, $p < 0.05$ for significant difference between TRIF$^{-/-}$/H11001/H11001 and TRIF$^{-/-}$/H11002/H11002 mice. F and G, Levels of IL-23 and IL-17 in lung homogenates from TRIF$^{-/-}$/H11001/H11001 and TRIF$^{-/-}$/H11002/H11002 mice. Data are expressed as mean $\pm$ SE with $n = 4–6$ animals used at each group. *, $p < 0.05$.

**FIGURE 3.** Activation of NF-$\kappa$B, up-regulation of ICAM-1 and VCAM-1, and activation of MAPK against infection with K. pneumoniae (Kp). A, Reduced NF-$\kappa$B activation in TRIF$^{-/-}$ mice following K. pneumoniae infection. Nuclear translocation of the p65 subunit of NF-$\kappa$B as detected by p65 ELISA of nuclear extracts of mouse lungs at 24 and 48 h following K. pneumoniae infection for $n = 3–5$ mice per group at a time point. Values that are significantly different between TRIF$^{-/-}$/H11001/H11001 and TRIF$^{-/-}$/H11002/H11002 are indicated by asterisks ($p < 0.05$). B, Attenuated up-regulation of ICAM-1 and activation of MAPK in TRIF$^{-/-}$ mice. Total protein in the lungs was prepared from TRIF$^{-/-}$/H11001/H11001 and TRIF$^{-/-}$/H11002/H11002 mice at 24 and 48 h following infection with K. pneumoniae, run on SDS-PAGE gel and the membrane was blotted with the appropriate Ab as described in Materials and Methods. Results are representative of three separate experiments with identical results. C, Densitometric analysis of Western blots from three performed experiments to quantify the protein levels of adhesion molecules (ICAM-1 and VCAM-1) and phospho-MAPKs following K. pneumoniae infection. The results obtained were normalized against GAPDH and expressed as mean $\pm$ SE.
TRIF deficiency impairs NF-κB activation, ICAM-1 and VCAM-1 up-regulation and MAPK activation in the lung against K. pneumoniae

To investigate further mechanisms underlying attenuated neutrophil recruitment to the lungs in TRIF<sup>−/−</sup> mice, we investigated NF-κB activation, ICAM-1 and VCAM-1 expression, and MAPK activation in the lungs following K. pneumoniae infection. Although substantial NF-κB activation was observed in the lungs of TRIF<sup>+/+</sup> mice, a modest reduction in NF-κB activation was observed in the lungs of TRIF<sup>−/−</sup> mice against K. pneumoniae (Fig. 4A). In addition, ICAM-1, but not VCAM-1, expression was consistently reduced in TRIF<sup>−/−</sup> mice at 24 and 48 h following K. pneumoniae challenge (Fig. 3, B and C). Furthermore, TRIF<sup>−/−</sup> mice infected with K. pneumoniae showed reduced activation of JNK and p38 kinases at 24 h, whereas ERK kinase was substantially attenuated only at 48 h (Fig. 3, B and C).

MyD88-dependent cascade regulates host defense against K. pneumoniae

We next examined the importance of MyD88-dependent signaling cascade in host defense against K. pneumoniae infection because 1) TRIF-independent (MyD88-dependent) -dependent cascades use different signaling mechanisms to boost antibacterial defense against K. pneumoniae, and 2) to test whether these two cascades use the same mechanism to augment host defense against K. pneumoniae. As revealed in Fig. 4A, MyD88<sup>−/−</sup> mice showed early mortality (85% animals died on day 2 postinfection) compared with control mice (no death till day 2) and therefore, we performed experiments only at 24 h postinfection in MyD88<sup>−/−</sup> mice. In addition, MyD88<sup>−/−</sup> mice showed higher CFUs in the lungs and spleens compared with controls (Fig. 4B). Furthermore, MyD88<sup>−/−</sup> mice had minimal neutrophil accumulation in airspaces and showed reduced neutrophil recruitment to lung parenchyma (Fig. 4, C–E). Moreover, MyD88<sup>−/+</sup> mice showed moderate suppurative bronchopneumonia (score of 2.0) with intralesional bacteria (score of 1.0) (Fig. 4F), whereas MyD88<sup>−/+</sup> mice showed no detectable histopathological changes (score of 0) with high intraleusal bacteria (score of 3.0). Importantly, no significant histopathological changes were observed in the lungs of either MyD88<sup>−/−</sup> or MyD88<sup>+/+</sup> mice in response to saline challenge (data not shown).

Cytokine and chemokine expression in response to K. pneumoniae requires MyD88

BALF studies were then conducted in mice following challenge with K. pneumoniae. In MyD88<sup>−/−</sup> mice, TNF-α, IL-6, KC, MIP-2, and LIX levels in the BALF in response to intratracheal K. pneumoniae infection were decreased compared with those of WT mice (Fig. 5, A–E). We also observed that both IL-23 and IL-17 proteins were reduced in MyD88<sup>−/−</sup> mice in response to infection with K. pneumoniae (Fig. 5, F and G).

MyD88 regulates NF-κB activation, ICAM-1 and VCAM-1 up-regulation and MAPK activation in the lung against K. pneumoniae

We further delineated the mechanisms associated with less neutrophil influx in MyD88<sup>−/−</sup> mice following K. pneumoniae infection. In this context, we determined the role of NF-κB and cell adhesion molecules. NF-κB was activated in the lungs of MyD88<sup>−/−</sup> mice against K. pneumoniae infection whereas a substantial reduction in NF-κB activation was observed in the lungs of MyD88<sup>−/−</sup> mice at 24 h (Fig. 6A). Furthermore, both ICAM-1 and VCAM-1 expression was reduced in MyD88<sup>−/−</sup> mice at 24 h postinfection with K. pneumoniae (Fig. 6, B and C). When MyD88<sup>−/−</sup> mice were infected with K. pneumoniae, activation of JNK, ERK, and p38 kinases was abrogated (Fig. 6, B and C).
Neutrophil accumulation in the lungs in response to K. pneumoniae requires both TRIF and MyD88, but MyD88 has a predominant role.

From our results, it appears that TRIF-dependent cascade induces a late phase activation of NF-κB and expression of cytokines/chemokines, but not LIX, and VCAM-1, whereas MyD88-dependent pathway induces an early phase activation of NF-κB and expression of cytokines/chemokines, including LIX and VCAM-1 in response to K. pneumoniae. Based on these findings, we hypothesized that the MyD88-dependent cascades are dominant over TRIF cascade. To test the hypothesis, we generated mice lacking both TRIF and MyD88 (double knockout mice; TRIF/MyD88−/−).
MyD88^{--}\). In TRIF/MyD88^{--}\) mice, \textit{K. pneumoniae}-induced neutrophil influx was almost abolished, whereas neutrophil accumulation was attenuated in TRIF^{--}\) mice (Fig. 7, A and B). Furthermore, cytokine/chemokine expression, including LIX was reduced in TRIF/MyD88^{--}\) mice at 24 h (Fig. 7, C–G). These results show a more pronounced phenotype in TRIF/MyD88^{--}\) mice than in TRIF^{--}\) mice.

**Effect of MyD88 blocking in human AMs in response to \textit{K. pneumoniae}**

Because AMs play critical roles in the induction of host response against bacteria, we examined the importance of MyD88-dependent signaling cascades in cytokine/chemokine responses using primary human AMs (2 × 10^6/well) in response to 2 × 10^4 \textit{K. pneumoniae}. Human AMs were stimulated with \textit{K. pneumoniae}, in the presence of MyD88 BP or CP, and cytokine/chemokine expression was measured in culture medium. Live \textit{K. pneumoniae} stimulation of AMs resulted in expression of TNF-\(\alpha\), IL-6, and IL-8 (Fig. 8), and these responses were attenuated by the BP (Fig. 8). In contrast, CP had no influence on chemokine and cytokine gene expression in response to MyD88^{--}\) stimulation (data not shown). In addition, BP or CP alone did not induce cytokine/chemokine expression in AMs (data not shown). These observations demonstrate that MyD88 is a central regulator in the expression of cytokines and neutrophil chemoattractant in response to \textit{K. pneumoniae} challenge.

**Effect of MyD88 blocking in the lung in response to \textit{K. pneumoniae}**

To exhibit the importance of MyD88 in pathological settings, control (C57BL/6) mice were pretreated with 500 \mu g of MyD88 BP or CP 2 h before \textit{K. pneumoniae} administration. When these mice were pretreated with MyD88 blocking peptide before \textit{K. pneumoniae} infection, neutrophil influx was reduced in the lungs of these mice compared with mice treated with CP at 48 h (Fig. 9, A and B). We have also observed enhanced bacterial burden in the lungs and bacterial dissemination in the spleens (Fig. 9, C and D).

**Discussion**

\textit{K. pneumoniae} can cause life-threatening pneumonia with extensive lung damage. TLRs are well-characterized family of pattern recognition receptors that provide host defense against pathogens. Ligand binding to TLRs initiates a series of downstream signaling cascades via the interaction of TLRs with the TIR domains of adaptors, which ultimately results in the synthesis and secretion of cytokines and chemokines. Although TLR4 (36) and TLR9 (37) have been shown to play roles in \textit{K. pneumoniae}-induced pneumonia, the roles of adaptor molecules in TLR signaling cascades remain to be elucidated.
Infections (10^3 CFU/mouse). Peptide-treated and subsequently infected and spleens of BP-treated mice following intratracheal K. pneumoniae than resident cells in the initial antibacterial host defense in the lungs (4, 5, 38). Although TRIF is important for neutrophil recruitment against E. coli (23) and P. aeruginosa (24), it does not seem to be important for neutrophil migration to the lungs against nontypeable H. influenzae (16) and B. pseudomallei (18). These findings could demonstrate the pathogenic specific role of TRIF in neutrophil-mediated pulmonary host defense. Furthermore, we revealed that MyD88 is also important for antibacterial host defense against a pulmonary pathogen (K. pneumoniae) and these data are in line with other investigations showing the crucial role of MyD88 in bacterial clearance during infection with both Gram-positive and Gram-negative bacteria (13–16, 18, 22). It is important to note that the TRIF signaling cascade activated through TLR4 is MyD88-independent and that TRAM is critical for the TLR4-TRIF cascade. TRAM-TRIF signaling occurs from an endosomal compartment after internalization of TLR4-TRAM complex and results in IFN-γ production (39). The role of endocytosis in TRIF signaling in the lungs against K. pneumoniae infection should be a subject of future investigations.

Neutrophil sequestration within capillaries and migration into lung parenchyma during lung infection is a multistep process that involves neutrophil stiffening, retention in capillaries, adhesion to endothelium, and extravasation to the alveolus (40, 41). Neutrophils bind to various adhesion molecules, such as ICAM-1, E-selectin, and VCAM-1 expressed on endothelial cells. Most importantly, VCAM-1 and ICAM-1 are up-regulated by TNF-α during infection/inflammation (42, 43). The data presented in this study constitute a strong argument that K. pneumoniae-induced TRIF signaling leads to the expression of TNF-α and subsequent up-regulation of ICAM-1 on endothelial cells. Most importantly, VCAM-1 and ICAM-1 are up-regulated by TNF-α during infection/inflammation (42, 43). The data presented in this study constitute a strong argument that K. pneumoniae-induced TRIF signaling leads to the expression of TNF-α and subsequent up-regulation of ICAM-1 on endothelial cells. Most importantly, VCAM-1 and ICAM-1 are up-regulated by TNF-α during infection/inflammation (42, 43).

Leukocyte migration into tissues and subsequent activation is regulated by NF-κB activation and the production of cytokines and chemokines. In particular, critical roles for ELR⁺ CXC chemokines have been demonstrated in murine models of bacterial pneumonia (44–46). It has been established that TLR signaling can activate NF-κB (47, 48). Our results suggest that TRIF-dependent late NF-κB activation is a critical mediator of TNF-α and IL-6 expression in the lungs in response to K. pneumoniae. Although similar findings have been reported in investigations using E. coli (23) and P. aeruginosa (24), investigations using H. influenzae (16) and B. pseudomallei (18) have revealed that TRIF is not required for cytokine/chemokine expression in the lungs. The discrepancy between these findings may be explained by the nature of the pathogens and time points used to measure chemokines/ cytokines in the lungs. We also provide evidence that MyD88 is an important mediator of K. pneumoniae-induced early NF-κB activation and cytokine/chemokine production in the lungs. Unlike TRIF, MyD88 is important for the production LXI and VCAM-1 probably via IL-23 response to K. pneumoniae. These results demonstrate that MyD88, as compared with TRIF, has additional and essential mechanisms to induce neutrophil influx to the lungs in K. pneumoniae infection. Because we observed more dramatic attenuation of early NF-κB in MyD88−/− mice as compared with TRIF−/− mice against K. pneumoniae infection, it appears that early phase of NF-κB activation is required for the induction of LIX and VCAM-1.

The current study also shows that TRIF and MyD88 are important for K. pneumoniae-induced MAPK activation in the lungs. It is important to mention that we have performed our studies in lung
Other TLR(s) → MyD88 → TLR2/4 → Plasma membrane

Early IFN-β activation
TNF-α, IL-6, KC, MIP-2 and LIX

Late IFN-β activation
TNF-α, IL-6, KC, and MIP-2

ICAM-1 and VCAM-1 upregulation

Neutrophil influx

Bacterial clearance

K. pneumoniae

Proposed scheme for TRIF and MyD88 signaling cascades leading to neutrophil influx in the lungs (in vivo) against K. pneumoniae. K. pneumoniae activates single or multiple TLRs to induce downstream signaling. TRIF signaling induces late NF-κB activation and subsequent TNF-α, IL-6, KC, and MIP-2 production and ICAM-1 up-regulation, whereas MyD88 signaling induces early NF-κB activation and subsequent TNF-α, IL-6, KC, MIP-2, and LIX production and ICAM-1 and VCAM-1 up-regulation.

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Disclosures
The authors have no financial conflict of interest.

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