

Toll/IL-1R Domain-Containing Adaptor Protein (TIRAP) Is a Critical Mediator of Antibacterial Defense in the Lung against *Klebsiella pneumoniae* but Not *Pseudomonas aeruginosa*¹

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Bacterial pneumonia is a leading cause of mortality and is associated with extensive neutrophil accumulation. Major pathogens associated with this disease include nonflagellated *Klebsiella pneumoniae* (Kp) and flagellated *Pseudomonas aeruginosa* (Pa). TLRs are essential for innate immune defense. TIRAP (Toll/IL-1R domain-containing adaptor protein) is an adaptor in TLR1, TLR2, TLR4, and TLR6 signaling, whereas MyD88 is an adaptor for all TLRs. However, the importance of TIRAP in pulmonary defense against Kp or Pa has not been examined. To demonstrate the role of TIRAP, TIRAP-deficient and wild-type littermates were intratracheally inoculated with Kp or Pa. We found that TIRAP^{-/-} mice had substantial mortality, higher bacterial burden in the lungs, and enhanced dissemination following Kp challenge. Furthermore, Kp-induced neutrophil sequestration, histopathology, and MIP-2, TNF- α , IL-6, and LIX (lipopolysaccharide-induced CX₂C chemokine) production were attenuated in the lungs of TIRAP^{-/-} mice. In contrast, TIRAP is not required for Pa-induced mortality, pulmonary bacterial burden, bacterial dissemination, neutrophil accumulation, or histopathology, yet it is necessary for MIP-2, TNF- α , and IL-6 production, but not LIX production. However, both Kp- and Pa-induced neutrophil influxes are MyD88 dependent. To determine the mechanisms associated with Pa-induced neutrophil accumulation, we inoculated mice with a flagellin C mutant of Pa (Pa Δ fliC) or purified flagellin, a TLR5 agonist. Pa Δ fliC-induced neutrophil sequestration and LIX expression are dependent on TIRAP, whereas flagellin-induced neutrophil influx and LIX expression are independent of TIRAP. These novel findings illustrate a pathogen-specific role for TIRAP in pulmonary defense and suggest that TLR5 plays an essential role for Pa-induced neutrophil influx via LIX production. *The Journal of Immunology*, 2006, 177: 538–547.

Pneumonia is a leading infectious cause of mortality in immunocompetent individuals in the United States (1–3). Pneumonia is also a common disease among patients infected with HIV (3). Gram-negative bacteria, including *Klebsiella pneumoniae* (Kp)³ and *Pseudomonas aeruginosa* (Pa), are well-described causes of pneumonia. Bacterial pneumonia is associated with an inflammatory response in the lung, including neutrophil sequestration. However, excessive neutrophil accumulation leads to severe lung pathology during bacterial pneumonia. Even though proper antibacterial treatment may be provided, impaired innate host defense leads to defective clearance of microbial pathogens

from the lungs, indicating that pulmonary innate defense is a critical element in controlling microbial infections in the lung (4).

The first line of defense in the lung is provided by the innate immune system, which is required to mount an efficient immune response against microbial pathogens (5, 6). The innate immune system uses a set of germline-encoded cell membrane receptors that are referred to as Toll-like receptors or TLRs, which contribute to the induction of immune responses via recognition of conserved patterns in microbes (7, 8). To date, a total of 11 TLRs have been characterized, and several TLRs recognize bacterial components (7, 8). For example, TLR2 recognizes peptidoglycan derived from Gram-positive bacteria, whereas TLR4 recognizes LPS derived from Gram-negative bacteria (5–8). In addition, TLR5 recognizes flagellins of Gram-negative and Gram-positive bacteria (5–8).

Cascades downstream of TLRs are very similar to that of IL-1R signaling (9). Upon interaction with their ligands, the cytoplasmic adaptor proteins TIRAP (Toll/IL-1R domain-containing adaptor protein) and MyD88 are recruited to the TLR complex, which results in the production of proinflammatory cytokines, and this pathway is termed the MyD88-dependent pathway (10–12). TLR stimulation also recruits the adaptor proteins TRIF (Toll/IL-1R domain-containing adaptor inducing IFN- β) and TRAM (TRIF-related adaptor molecule), and this cascade is termed the MyD88-independent pathway (13, 14). Whereas TIRAP is an adaptor molecule for TLR1-, TLR2-, TLR4-, and TLR6-mediated signaling cascades, MyD88 is an adaptor molecule for all characterized TLRs (10–14).

Despite the importance of TLRs in the induction of pulmonary innate immune responses against microbial assault, relatively few

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³ Abbreviations used in this paper: Kp, *Klebsiella pneumoniae*; BALF, bronchoalveolar lavage fluid; i.t., intratracheal(ly); LIX, lipopolysaccharide-induced CX₂C chemokine; MPO, myeloperoxidase; Pa, *Pseudomonas aeruginosa*; recFLA-ST, recombinant *Salmonella typhimurium* flagellin; TIRAP, Toll/IL-1R domain-containing adaptor protein; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN- β ; TSA, tryptic soy agar.

investigations have revealed the role of TLRs in the lung. Using TLR2-deficient mice, our group and others have demonstrated that TLR2 is critical to lung immune responses against *Mycoplasma pneumoniae* (15) and *Streptococcus pneumoniae* (16, 17). We and others have used TLR4 mutant (C3HeJ) mice to show the importance of TLR4 to induce lung defense against *Haemophilus influenzae* (18), *S. pneumoniae* (19), and Kp (20, 21). However, the role of TLR2 and TLR4 in Pa lung infection is still debatable. For example, Power et al. (22) have shown that both TLR2 and TLR4 are involved in pulmonary defense against a mucoid strain of Pa. In contrast, another study by Ramphal et al. (23) concluded that TLR4 and TLR2 do not contribute to host defense during lung infections against the PAK strain of Pa.

Knowledge of the role of adaptor molecules in the TLR cascade in pulmonary host defense against bacterial pathogens is even more limited, despite their importance in cell signaling. Unlike cell surface TLRs, adaptor molecules offer a therapeutic advantage because each adaptor is involved in several TLR-associated signaling cascades. This is a particularly important point, because each bacterium possesses a plethora of virulence factors that may activate multiple TLRs simultaneously. MyD88 is shown to be important for host defense in the lung against Pa (23, 24), although the role of MyD88 in Kp-induced lung infections is not clear. In addition, TRIF gene mutant mice were used to demonstrate that TRIF does not play a role in pulmonary defense against nontypeable *H. influenzae* (25). Although we demonstrated the critical role of TIRAP in host defense in the lung against *Escherichia coli* in a previous report (26), it is not known whether TIRAP is important for successful host defense in pulmonary infection with other highly important Gram-negative respiratory pathogens, including Kp and Pa. This is an important issue considering the fact that each bacterial species expresses a unique set of virulence factors during *in vivo* infection. Furthermore, it is not known whether TIRAP plays a differential role among Gram-negative pathogens. A comprehensive understanding of the role of these adaptors is essential for designing better treatment strategies aimed at modulating pulmonary defense.

In the present investigation we have examined the involvement of TIRAP in pneumonia induced by two clinically important etiologic agents, namely Kp, a nonflagellated bacterium, and Pa, a flagellated bacterium. To determine the function of TIRAP in lung innate immune responses against these Gram-negative pathogens, genetically engineered TIRAP gene-deficient mice (TIRAP^{-/-}) and their wild-type littermates were challenged with intratracheal (i.t.) inoculation of Kp or Pa. Our findings demonstrate that TIRAP signaling is essential for an effective pulmonary defense against Kp but not Pa infection, despite the fact that MyD88 signaling is critical for both. Our work demonstrated that flagellin is among the bacterial factors that modulate the TIRAP-independent but MyD88-dependent host response in the lung against Pa via TLR5. Murine models of infection form the groundwork to examine the importance of TIRAP in the pathogenesis of pulmonary diseases in humans caused or complicated by Gram-negative bacteria.

Materials and Methods

Mouse strains

TIRAP gene-disrupted mice (TIRAP^{-/-}) and their wild-type controls (TIRAP^{+/+}) (10) were obtained by breeding TIRAP^{+/-} after backcrossing six times with C57BL/6 to ensure they had similar genetic backgrounds. MyD88 gene-deficient mice were used after backcrossing 12 times with C57BL/6 (11, 12) and, therefore, C57BL/6 mice were used as littermate controls. The animal experiments were approved by the Animal Care and Use Committee of the National Jewish Medical and Research Center. Pathogen-free, 8- to 10-wk-old female mice ranging from 20 to 24 g in

weight were used in all of our experiments. Mice were kept on a 12-h light/12-h dark cycle with normal mouse chow and water *ad libitum*.

Mouse experiments

Kp and Pa were used for mouse inoculation. Kp serotype 2 (American Type Culture Collection strain 43816) was grown for 16 h at 37°C in tryptic soy broth as described in previous publications (20, 21, 27, 28). A well-characterized Pa strain (PA01) or the *flhC* mutant of PA01 (Pa Δ *flhC*); kindly provided by D. Hassett at the University of Cincinnati College of Medicine, Cincinnati, OH) was cultured in the absence (for wild-type Pa) or the presence of 20 μ g/ml gentamicin (for Pa Δ *flhC* mutant) for 16 h at 37°C in Luria-Bertani broth as previously described (29). Bacteria were harvested by centrifugation at 1200 \times g for 2 min and washed twice in sterile isotonic saline. Bacteria were resuspended at various concentrations in saline for animal inoculation. The initial inocula were confirmed by plating serial 10-fold dilutions on MacConkey and tryptic soy agar (TSA) plates. Mice were anesthetized i.p. with tribromoethanol (Avertin; 250 mg/kg), followed by i.t. administration of 50 μ l of the bacterial suspension (containing 10³ or 0.25 \times 10³ CFU of Kp or 10⁶ or 0.25 \times 10⁶ CFU of Pa). These bacterial concentrations were based on previously published studies using Kp (20, 21, 27, 28) or Pa (29). Control mice were i.t. inoculated with 50 μ l of saline. In a similar fashion, 1 μ g of endotoxin-free recombinant flagellin from *Salmonella typhimurium* (recFLA-ST; InvivoGen) was used in mice. According to the manufacturer, this purified flagellin activates TLR5 but not TLR4 or TLR2.

Enumeration of leukocytes in bronchoalveolar lavage fluid (BALF)

At the designated time points, the animals were euthanized with 100 mg/kg pentobarbital and exsanguinated by cardiac puncture. A midventral incision was used to open the thorax, and the trachea was isolated and cannulated with a 20-gauge catheter that was immobilized with 2-0 silk suture materials. BALF was collected from the whole lung to obtain cells in the airspace and procure proteins for cytokine and chemokine determination as described in our previous publications (30–32). 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 cytospin slides that were then stained by the Diff-Quick method (Fisher) to determine leukocyte subtypes based on their cellular and nuclear morphologies. Total leukocytes in BALF were determined using a hemocytometer. Two milliliters of the undiluted cell-free BALF was centrifuged, passed via a 0.22- μ m filter, and used immediately or kept at -20°C for the determination of cytokines and chemokines by ELISA.

Measurement of cytokine and chemokine levels in BALF

Cytokine and chemokine levels were measured in BALF using a specific sandwich ELISA as previously described (30–32). The minimum detection limit is 2 pg/ml protein.

Lung isolation

At 2, 8, and 24 h postchallenge, the whole (nonlabeled) lungs were excised from mice and immediately snap frozen followed by storage at -70°C. The lungs were used for making lung homogenates for myeloperoxidase (MPO) assay, Western blotting, or bacterial counts.

MPO assay

Determination of lung MPO activity was performed as previously described (33). MPO is an enzyme found in myeloid cells and has been used largely as a marker of neutrophil accumulation in the lung. 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% hexadecyltrimethylammonium bromide to determine the MPO activity. Lung samples were then sonicated, 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 MPO activity was calculated between these time points. The lung samples were used for MPO activity within 2 wk after they were harvested.

Western blotting of lung homogenates

Lung tissue was homogenized in 1 ml of buffer containing 0.1% Triton X-100 in PBS supplemented with complete protease inhibitor mixture (Roche) for 30 s and centrifuged at maximum speed in a microcentrifuge at 4°C. The resulting supernatant fluids were filtered and used for Western blotting. To ensure equal amounts of protein onto the gel, a Bradford protein assay was performed before loading (Bio-Rad). The lung homogenates (20 μ g) were analyzed on 8–15% Tris-glycine gels, and the gels were

transferred onto a polyvinylidene difluoride membrane. The mAb to mouse VCAM-1, ICAM-1 or β -actin was added at a 1/500, 1/200, or 1/1000 dilution, respectively. After washes, the membranes were incubated with appropriate secondary Ab at a dilution of 1/10,000 and developed with ECL plus Western blot detection (Amersham Biosciences).

Bacterial culture

Whole lungs were homogenized in 10 ml of sterile saline for 30 s, and the resulting homogenates were plated as serial 10-fold dilutions on MacConkey and TSA plates. Bacterial colonies were counted after incubation at 37°C for 24 h. Spleen homogenates were made in a similar manner for bacterial culture, except that they were homogenized for 15 s in 2 ml of sterile PBS.

Lung histology

At 24 h postbacterial inoculation, the whole mouse lungs were inflated and fixed with Streck tissue fixative (Streck Laboratories) for 12 h at room temperature as described (26). The specimens were then embedded in paraffin, and 5- μ m serial sections were made. These sections were then stained with H&E for histological examination. Nonquantitative histological analysis was performed by a pathologist who was blinded for groups.

In vitro uptake/killing assay

To evaluate the ability of neutrophils to uptake and/or kill Kp, an uptake/bactericidal assay was performed as described previously (26, 32). The Kp obtained after two washings with sterile 0.9% saline was used for the assay. Viable Kp at a concentration of 10^6 CFU/ml was mixed with murine bone marrow-derived neutrophils at a concentration of 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS in a 1.5-ml microfuge tube and rotated at 50 rpm at 37°C for 2 or 6 h. At the end of incubation, the bacterial colonies were plated after serially diluting the medium at several 10-fold dilutions and culturing 20 μ l of culture on MacConkey and TSA plates.

Actin polymerization measurement

To measure actin assembly in response to inflammatory mediators, neutrophils were exposed to MIP-2 and TNF- α as described in our previous publications (34, 35). Briefly, mouse neutrophils were incubated with 5 ng/ml MIP-2 for 15 min, 1 μ g/ml TNF- α for 1 h, or left unstimulated for 1 h at 37°C. After incubation the cells were fixed, and actin cytoskeleton changes were recorded using a fluorescent microscope.

Neutrophil depletion

The protocol used to deplete neutrophils (GR1⁺) in mice has been described previously (36) with minor modifications. A total of 50 μ g of anti-GR1⁺ mAb (sodium azide-free and low LPS content; RB6-8C5 from BD Pharmingen) per mouse in 50 μ l was administered i.p. at 12 and 2 h before bacterial challenge. As a control, 50 μ g of isotype control mAb in equal volumes was administered at the same time points before bacterial infection. To validate the efficiency of neutrophil depletion, blood neutrophils were counted every 12 h up to 3 days, and 2% neutrophils were found up to 3 days after depletion.

Statistical analysis

All data are expressed as means \pm SE. For comparison between two groups, Student's *t* test was used. Statistical significance was defined as *p* < 0.05 using Kaleidagraph version 6.0 (Synergy Software).

Results

TIRAP^{-/-} mice have decreased survival following i.t. Kp but not Pa inoculation

To assess the protective role of TIRAP in Gram-negative bacterial pneumonia, mice in the absence (TIRAP^{-/-}) or presence (TIRAP^{+/+}) of a functional TIRAP protein were administered two doses of Kp or Pa through the i.t. route, and survival was monitored for up to 14 days. TIRAP^{-/-} mice had decreased survival, and all mice died within 4 days postinfection of a dose of 10^3 CFU of Kp per mouse (Fig. 1A). In contrast, 80% of Kp-infected TIRAP^{+/+} mice survived. A similar survival pattern was observed in TIRAP^{-/-} and TIRAP^{+/+} mice with 250 CFU of Kp per mouse (Fig. 1B). By contrast, there were no differences in survival rates between TIRAP^{-/-} and TIRAP^{+/+} animals after the administra-

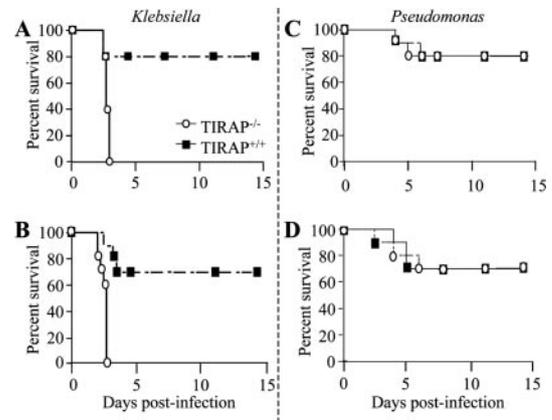


FIGURE 1. Increased mortality following i.t. Kp but not Pa infection in TIRAP^{-/-} mice. TIRAP^{-/-} and TIRAP^{+/+} mice were i.t. inoculated with either 10^3 CFU of Kp (A) or 0.25×10^3 CFU of Kp (B) per mouse or 10^6 CFU of Pa (C) or 0.25×10^6 CFU of Pa (D) per mouse, and survival was monitored for up to 14 days. Data are from two independent experiments performed on each group (*n* = 10 mice). *, *p* < 0.05; determined by Wilcoxon rank sign test between groups.

tion of 10^6 CFU (Fig. 1C) or 0.25×10^6 CFU of Pa per mouse (Fig. 1D). None of the saline-challenged animals died within 24 days (data not shown). These observations illustrate that TIRAP is a critical molecule contributing to antimicrobial defense in the lung during acute bacterial pneumonia induced by Kp but not Pa.

TIRAP^{-/-} mice have impaired clearance of Kp but not Pa

To obtain more insights into the mechanisms by which TIRAP deficiency resulted in increased mortality during Gram-negative pneumonia, TIRAP^{-/-} and TIRAP^{+/+} mice were sacrificed 24 and 48 h after Kp (10^6 CFU/mouse) or 6 and 24 h after Pa (10^3 CFU/mouse) inoculation. Earlier time points were selected for Pa, because preliminary studies indicated that organisms were cleared from the lungs by 48 h after inoculation (data not shown). As compared with TIRAP^{+/+} mice, TIRAP^{-/-} animals had a higher bacterial burden in the lungs at 48 h and more bacterial dissemination as measured by CFU in the spleen at 24 and 48 h after Kp challenge (Fig. 2, A and B). In sharp contrast, there were no significant differences in bacterial growth in the lungs or bacterial dissemination observed between TIRAP^{-/-} and TIRAP^{+/+} mice after Pa inoculation (Fig. 2, C and D). These findings support the conclusion that TIRAP contributes to Kp containment in the lungs and its dissemination in the bloodstream during pneumonia. TIRAP does not appear to contribute to control of Pa.

TIRAP deficiency impairs neutrophil recruitment in the lung against Kp but not Pa

We next investigated whether the absence of functional TIRAP influenced early leukocyte (particularly neutrophil) recruitment in the lung against extracellular Gram-negative bacterial pathogens. We conducted BALF studies and lung MPO measurements as an index of neutrophil accumulation in lung parenchyma following challenge with 10^3 CFU of Kp or 10^6 CFU of Pa. Total leukocyte and neutrophil influx in BALF and lung parenchyma was examined in TIRAP^{-/-} and TIRAP^{+/+} mice after pathogen challenge. In TIRAP^{-/-} mice, total white blood cell and neutrophil accumulation was attenuated at 24 and 48 h as compared with wild-type counterparts after Kp challenge (Fig. 3, A–C). In sharp contrast to the observations with Kp, Pa-induced total white blood cell and neutrophil sequestration in the lungs was not influenced by TIRAP deficiency at 6 or 24 h (Fig. 3, D–F). As expected, no significant

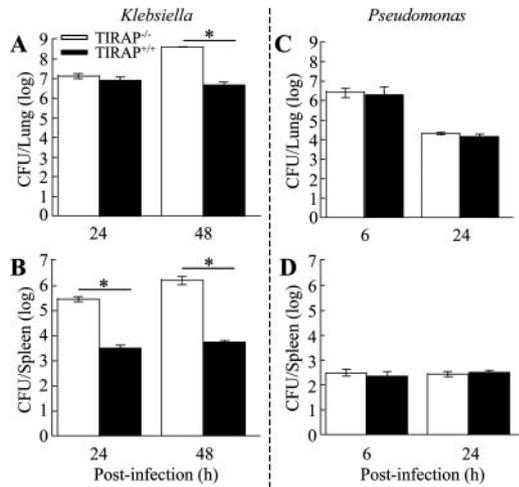


FIGURE 2. Impaired bacterial clearance in the lungs and blood stream in *TIRAP*^{-/-} mice after i.t. Kp but not Pa challenge. Infected *TIRAP*^{-/-} and *TIRAP*^{+/+} mice were determined for bacterial burden in lung and spleen homogenates after Kp (10^3 CFU/mouse) (A and B) or Pa (10^6 CFU/mouse) (C and D) inoculation. CFU data are expressed as mean \pm SE of six mice from three separate experiments at each time point. *, $p < 0.05$; significant differences between *TIRAP*^{-/-} and *TIRAP*^{+/+} mice.

cellular influx in the lungs was detected after saline exposure (Fig. 3, G–I).

Unlike TIRAP, MyD88 is an adaptor molecule for all known TLRs (7, 8). Therefore, we used *MyD88*^{-/-} mice to demonstrate whether Kp- and Pa-induced neutrophil accumulation is dependent on other TLRs where TIRAP is not an adaptor molecule. Fig. 4 indicates that both Kp-dependent (10^3 CFU/mouse) and Pa-dependent (10^6 CFU/mouse) neutrophil sequestration is entirely dependent on MyD88. Thus, our findings support the conclusion that TIRAP is necessary for Kp- but not Pa-induced neutrophil influx into the lungs. Our observations support the involvement of other TLRs for Pa where MyD88, but not TIRAP, is an adaptor molecule.

As an alternative mechanism, higher CFU counts observed in the lungs of *TIRAP*^{-/-} mice after Kp inoculation may also be due to impairment of the uptake/bactericidal capacity of migrated neutrophils into the lungs. To address this issue, we conducted an in vitro uptake/killing assay of Kp by neutrophils. Kp was cultured in the presence of neutrophils from *TIRAP*^{-/-} and *TIRAP*^{+/+} mice, and the CFUs of bacteria after 2 and 6 h were enumerated. As shown in Fig. 5, no differences between CFUs from *TIRAP*^{-/-} and *TIRAP*^{+/+} mice were observed at 2 (A) and 6 h (B), suggesting that *TIRAP*^{-/-} neutrophils have an intact ability to uptake/kill Kp.

TIRAP^{-/-} mice have attenuated up-regulation of VCAM-1 after Kp infection, but VCAM-1 up-regulated in *TIRAP*^{-/-} mice after Pa challenge

In previous studies, it has been demonstrated that activation of the pulmonary endothelium in lung vasculature by LPS derived from *E. coli* and viable *E. coli*-induced proinflammatory mediators results in up-regulation of VCAM-1 and ICAM-1 and that this process is important for neutrophil sequestration (26, 37, 38). Therefore, in the current investigation we determined the up-regulation of these adhesion molecules in *TIRAP*^{-/-} and *TIRAP*^{+/+} mice after Gram-negative bacterial infections. Although significant up-regulation of VCAM-1 was observed in *TIRAP*^{+/+} mice in response to Kp, this up-regulation was attenuated in *TIRAP*^{-/-} mice at 24 h postinfection (Fig. 5, C and D). By contrast, significant

up-regulation of VCAM-1 was noted in the lungs of both *TIRAP*^{-/-} and *TIRAP*^{+/+} mice after Pa challenge at 24 h (Fig. 5, C and D). As expected, no VCAM-1 was up-regulated in saline-challenged mice (Fig. 5, C and D). Our observations support the hypothesis that TIRAP is an important mediator for VCAM-1 up-regulation, which is likely important for neutrophil influx in the lung in response to Kp but not Pa. However, in this study we would not be able to demonstrate up-regulation of ICAM-1, because a high baseline was observed with saline-challenged lungs (data not shown).

The decreased number of neutrophils migrating to the lungs of *TIRAP*^{-/-} mice during Kp-induced infection could also be due to the impairment of actin assembly on these cells in response to inflammatory mediators. To address this mechanism, we used isolated neutrophils from *TIRAP*^{-/-} and *TIRAP*^{+/+} mice and stimulated them with MIP-2 and TNF- α . As depicted in Fig. 5E, these mediators caused a similar degree of actin assembly, suggesting that defective actin polymerization is not a mechanism for attenuated neutrophil accumulation in the lungs of *TIRAP*^{-/-} mice in response to Kp.

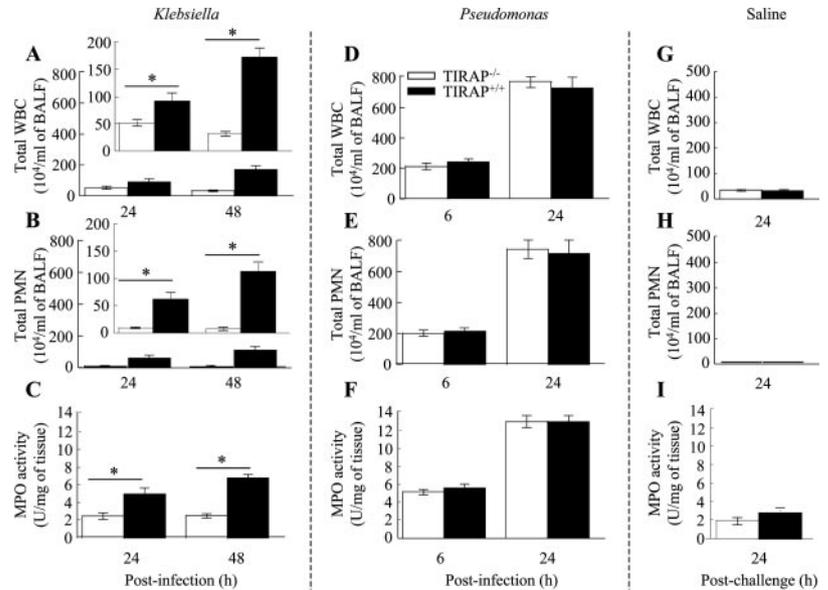
Neutrophils are critical in controlling Gram-negative bacterial burden in the lungs and bacterial dissemination in *TIRAP*^{-/-} and *TIRAP*^{+/+} mice

Because neutrophil (GR1⁺ cell) recruitment in the lungs was impaired in *TIRAP*^{-/-} mice exposed to Kp, we determined whether this cell type may influence Kp burden in the lungs and blood dissemination in *TIRAP*^{+/+} mice. Therefore, we depleted the mice of GR1⁺ cells and infected mice with Kp. Although all of the neutrophil-depleted mice were found dead within 4 days after Kp inoculation, 80% of isotype-matched control mAb-treated animals were alive up to 14 days after the inoculation (Fig. 6A). GR1⁺ mAb-treated mice also showed more bacterial burden in the lungs and bacterial dissemination after Kp inoculation (Fig. 6, B and C). Because similar degrees of neutrophil accumulation were observed in the lungs of *TIRAP*^{+/+} and *TIRAP*^{-/-} mice in response to Pa, we also conducted experiments in GR1⁺ cell-depleted *TIRAP*^{+/+} mice to demonstrate the role of neutrophils in bacterial burden in the lungs and dissemination following Pa challenge. Our observations indicate that neutrophil-depleted *TIRAP*^{+/+} mice were found dead within 1 day, whereas none of the isotype-control mAb treated *TIRAP*^{+/+} mice were found dead up to 14 days after Pa challenge (Fig. 6D). Furthermore, GR1⁺ mAb-treated *TIRAP*^{+/+} mice show more bacterial burden in the lungs and higher bacterial dissemination after Pa challenge in *TIRAP*^{+/+} mice (Fig. 6, E and F). Similar results were obtained using neutrophil-depleted *TIRAP*^{-/-} mice after Pa challenge (data not shown). Thus, GR1⁺ cells (neutrophils) clear Kp and Pa from the lungs and blood circulation.

TIRAP^{-/-} mice have reduced histopathology in the lungs in response to Kp but not Pa

Experiments were conducted to determine the importance of TIRAP in the induction of histopathological changes. No appreciable histopathological changes were observed in *TIRAP*^{-/-} mice after Kp inoculation (Fig. 7A). Whereas diffuse neutrophilic inflammation with focal areas of consolidation was observed in *TIRAP*^{+/+} mice at low power (Fig. 7D), neutrophil infiltration in the interstitium and alveolus was evident at high power 24 h after Kp challenge (Fig. 7D). By contrast, a similar degree of neutrophilic inflammation was observed in *TIRAP*^{+/+} and *TIRAP*^{-/-} mice after Pa challenge (Fig. 7, B and E). In addition, saline-administered *TIRAP*^{-/-} and *TIRAP*^{+/+} mice had no significant histopathology at 24 h (Fig. 7, C and F). These findings demonstrate that

FIGURE 3. Attenuated pulmonary leukocyte and neutrophil accumulation in TIRAP^{-/-} mice after Kp but not Pa inoculation. The TIRAP^{-/-} and TIRAP^{+/+} animals underwent BALF collection after bacterial (A–F) or saline (G–I) challenge. PMN, polymorphonuclear leukocyte; WBC, white blood cell. Data are expressed as mean ± SE of five to seven animals from three separate experiments in each group at each time point. *, *p* < 0.05.



TIRAP is also an important molecule in the initiation of lung pathology after Kp but not Pa infection.

TIRAP deficiency in vivo results in attenuated cytokine and chemokine response in the lung after Kp and Pa infections

Because the presence of cytokines and chemokines at high concentrations in the lung contributes to neutrophil accumulation, we used BALF to assess the production of MIP-2, LIX (lipopolysaccharide-induced CX₂C chemokine), TNF- α , and IL-6 levels by ELISA after pathogen challenge. Production of MIP-2, TNF- α , and IL-6 was impaired in the lungs of TIRAP^{-/-} mice after Kp challenge (Fig. 8, A, C, and D) and Pa inoculation (Fig. 8, E, G, and H). More interestingly, LIX, a potent neutrophil chemoattractant, levels after Pa infection were not significantly different between TIRAP^{-/-} and TIRAP^{+/+} mice after Pa inoculation (Fig. 8F).

Another mechanism for attenuated neutrophil accumulation in the lungs of TIRAP^{-/-} mice is the chemotactic defect of neutrophils to chemokines. In this regard, we have previously shown that TIRAP^{-/-} neutrophils are equally chemotactic to chemokines as the TIRAP^{+/+} neutrophils (26), ruling this out as a mechanism for attenuated neutrophil influx in the lungs of TIRAP^{-/-} mice.

TIRAP independent neutrophil accumulation in the lungs in response to Pa involves flagellin

Unlike Kp, Pa has flagellin (FliC). Therefore, we used a *fliC* mutant of Pa (Pa Δ *fliC*) and recFLA-ST, a TLR5 agonist, in TIRAP^{-/-} and TIRAP^{+/+} mice to determine the mechanisms associated with Pa-induced, TIRAP-independent pulmonary defense. Because the influx of neutrophils into the lung is a sentinel event during bacterial pneumonia (1–3), we used neutrophil accumulation as an end point. We used MyD88^{-/-} mice because MyD88 is an adaptor molecule of all TLRs (7–8). The absence of neutrophil accumulation in the lungs of MyD88^{-/-} mice in response to wild-type Pa (Fig. 4) or Pa Δ *fliC* (data not shown) was observed, indicating the involvement of TIRAP-independent TLR.

Fig. 9 demonstrates that the flagellin knocked-out strain (Pa Δ *fliC*) causes attenuated total cellular and neutrophil accumulation in the lung at both 6 and 24 h postinfection in TIRAP^{-/-} mice (Fig. 9, A and B) and suggests that this neutrophil accumulation occurs via LIX production (Fig. 9C). The finding that there was no difference in survival between TIRAP^{-/-} and TIRAP^{+/+}

mice in response to Pa Δ *fliC* (Fig. 9D) indicates that a TIRAP-dependent response is not required for pulmonary antibacterial defense against this strain of Pa. In addition, purified flagellin-induced total cellular (Fig. 9E) and neutrophil accumulation (Fig. 9F) as well as LIX production (Fig. 9G) in the lungs in TIRAP^{-/-} and TIRAP^{+/+} mice was not different, but they were entirely dependent on MyD88 (Fig. 9, H–J). We used recFLA-ST in our experiments instead of Pa flagellin for the following reasons: 1) commercial flagellin (recFLA-ST) has no contaminating endotoxin and lipoproteins; 2) Pa flagellin preparations are generally contaminated with lipoproteins, a TLR2 agonist, and LPS, a TLR4 agonist, and are extremely difficult to remove from flagellin preparations; and 3) purified Pa flagellin is not commercially available. More interestingly, Pa Δ *fliC*-induced LIX expression is TIRAP dependent (Fig. 9C), whereas wild-type Pa-induced LIX expression is TIRAP independent (Fig. 8F). Together, these observations suggest that TLR5 contributes to the TIRAP-independent neutrophil accumulation in the lungs against Pa via production of LIX.

Discussion

Pneumonia is a major threat in the United States (1–3, 39). The important causative Gram-negative pathogens of pneumonia are Kp and Pa (3). Although Kp is an important pathogen of hospital- and community-acquired pneumonia, Pa is the predominant pathogen in cystic fibrosis patients and nosocomial pneumonia. Pa is particularly virulent in patients with structural or functional defects in immune molecules. The high incidence of pneumonia along with the emergence of antibiotic-resistant bacterial pathogens warrants designing novel treatment strategies to control this illness. In this context, modulation of host immunity is an attractive target for improving prognosis, particularly among patients with immune system deficiencies and who are infected with antibiotic-resistant bacterial strains. However, gaining more insights into pulmonary innate immunity against pathogens is a necessity for designing improved strategies to modulate host defense.

Mounting evidence highlights the importance of the innate immune system as an efficient host defense against bacterial infections, including pneumonia (5–8). TLRs have recently emerged as important immune molecules for immediate immune responses in the lung against microbial pathogens via the recognition of pathogen-associated molecular patterns (5–8). TLRs are type I transmembrane proteins that contain two important domains, namely

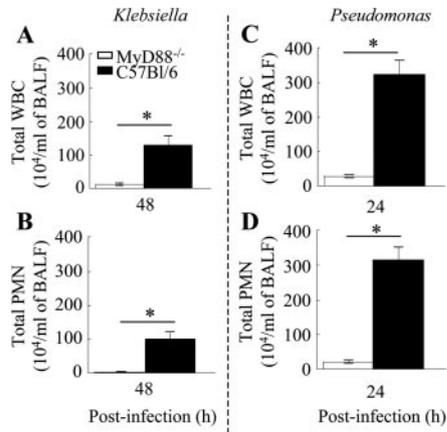


FIGURE 4. Defective leukocyte and neutrophil sequestration in the lungs of MyD88^{-/-} mice after Kp (10³ CFU/mouse) and Pa (10⁶ CFU/mouse) infection. BALF was collected in MyD88^{-/-} and MyD88^{+/+} (C57BL/6) mice 24 and 48 h after infection. PMN, polymorphonuclear leukocyte; WBC, white blood cell. Data expressed as mean \pm SE of six animals in each group at each time point from three independent experiments. *, $p < 0.05$; significant differences between TIRAP^{-/-} and TIRAP^{+/+} mice.

extracellular leucine-rich repeats for ligand binding and an intracellular region for signaling (5–8). Whereas TLR4, TLR2, and TLR5 are expressed on the cell surface, TLR7, TLR8, and TLR9 are expressed on the endosomes (5–8). TLR-mediated signaling involves adaptor molecules, including TIRAP and MyD88 (5–8). While TIRAP is an adaptor for TLR1, TLR2, TLR4, and TLR6, MyD88 is an adaptor for all TLRs (5–8). Although each adaptor molecule mediates signaling to several TLRs, little is known about the role of TLR adaptors during pulmonary infections, including pneumonia. The goal of this study was to evaluate the role of TIRAP in lung defense against Kp and Pa using genetically deficient TIRAP mice. Several new findings are reported in the present investigation. In our murine model of pneumonia, we have demonstrated the importance of TIRAP to host defense in the lung against Kp. We found that genetic deletion of TIRAP did not affect lung host defense against wild-type Pa despite the fact that MyD88 is required. Our observations suggest that TLR5 plays an essential role for neutrophil accumulation in the lung against Pa via production of LIX.

In clinical settings, early neutrophil influx is a common feature during pneumonia induced by bacterial pathogens (40, 41). It is known that neutropenia is an essential predisposing factor for bacterial pneumonia (42). In this context, our findings demonstrate that neutrophil depletion was accompanied by enhanced mortality and increased bacterial burden in the lung during Kp- and Pa-induced pneumonia in a murine model (Fig. 6). These data clearly demonstrate that neutrophils are crucial for controlling these pulmonary infections and augmenting host survival.

Neutrophil migration to the lung during inflammation is dependent on modulation of the pulmonary endothelium by inflammatory mediators and assembly of the actin cytoskeleton on neutrophils by these mediators (34, 43). Recent studies have shown that TNFR1, TNFR2, and IL-1R1 are essential to induce significant neutrophil sequestration in the lungs during pneumonia in response to both Gram-negative and Gram-positive pathogens, including *E. coli* (44, 45) and *S. pneumoniae* (46). Thus, our current observations suggest that attenuated neutrophil accumulation in TIRAP^{-/-} mice after Kp infection may be

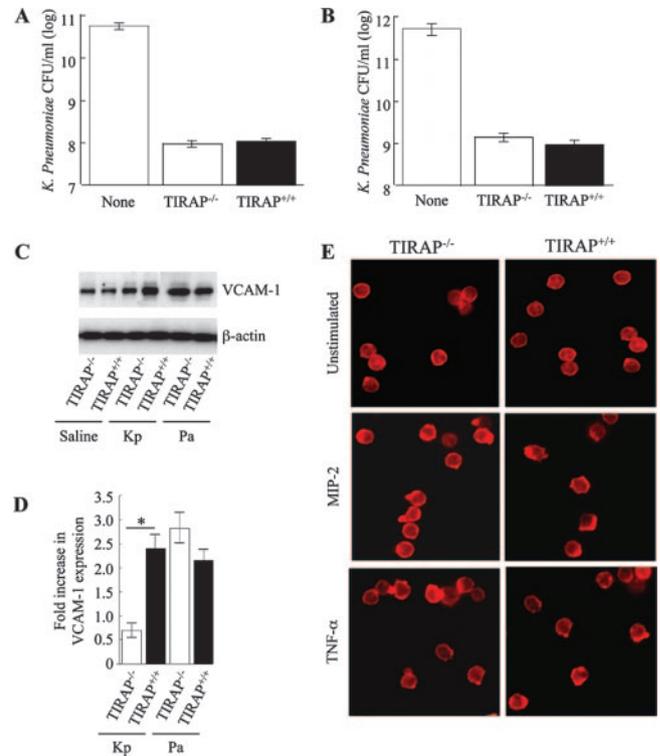
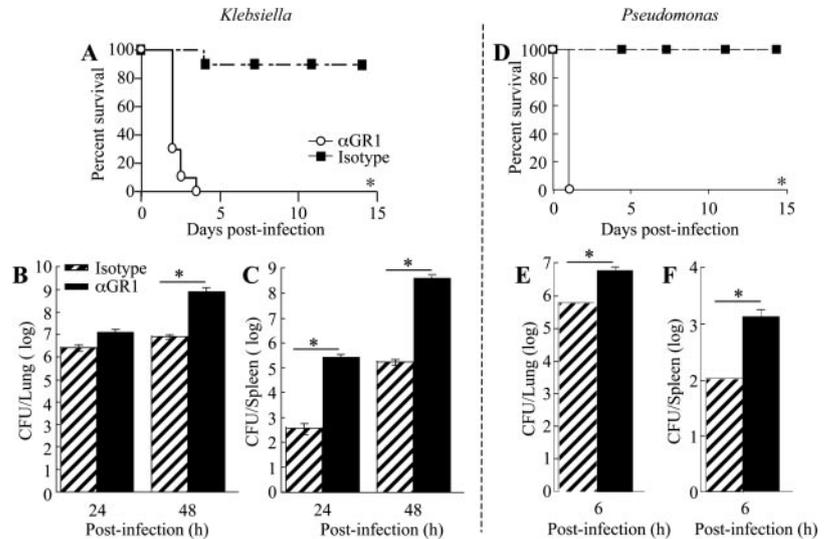


FIGURE 5. A and B, Unimpaired capacity of TIRAP^{-/-} and TIRAP^{+/+} neutrophils to uptake/kill Kp. A total of 10⁶ CFU of Kp was cultured alone or in the presence of 10⁶ CFU of bone marrow-derived polymorphonuclear neutrophils from TIRAP^{-/-} or TIRAP^{+/+} mice in 1 ml of medium. Viable bacteria were then enumerated at 2 and 6 h after culture. The CFU values shown represent mean \pm SE of a single experiment performed in triplicate from three mice per group. C and D, Impaired up-regulation of VCAM-1 in the lung after Kp but not Pa inoculation. The lungs were homogenized, total protein in the lungs was quantitated, and proteins were resolved on an 8–15% gel. The autoradiograph of VCAM-1 is a representative blot of six mice from three separate experiments (C). The graph shows VCAM-1 (D) for each sample expressed relative to the densitometric value of saline-treated sample at 24 h. *, $p < 0.05$; significant differences between TIRAP^{-/-} and TIRAP^{+/+} mice. E, Unaltered actin assembly in TIRAP^{-/-} and TIRAP^{+/+} neutrophils after chemokine and cytokine stimulation. Bone marrow-derived neutrophils were isolated and stimulated with MIP-2 and TNF- α . Cells were labeled with nitrobenzoxadiazole-phalloidin, and actin cytoskeleton changes were assessed by an immunofluorescence microscope. Photographs are representative of the pictures of cells from two separate experiments (five mice in each group) with identical results.

due to the involvement of TNFR1, TNFR2, and IL-1R1 as downstream signaling mediators.

Pa possesses several virulence factors, including LPS, lipoproteins, flagellin, phospholipase C, and molecules that are translocated through the type III secretion system (47, 48). The interaction between the bacterium and lung cell receptors is the first and critical event in the multistep sequence leading to the pathophysiology of pneumonia. Our current work demonstrates a critical role of MyD88, but not TIRAP, in initial neutrophil sequestration in an intrapulmonary challenge with wild-type Pa (Fig. 3–4). Furthermore, our investigation suggests that TIRAP-independent neutrophil sequestration in the lungs against Pa is due to the activation of TLR5 by flagellin (Fig. 9). In this context, studies have demonstrated that flagellin encoded by the *fljC* gene is a major virulence factor. It has also been shown that purified flagellin activates cells

FIGURE 6. Diminished survival, enhanced bacterial burden in the lungs, and more bacterial dissemination in neutrophil-depleted TIRAP^{+/+} mice following Kp (A–C) and Pa (D–F) infection. Mice were pretreated with anti-GR1 or isotype control mAb at 12 and 2 h before inoculation with 10³ CFU of Kp per mouse or 10⁶ CFU of Pa per mouse, and their survival (10 mice from two separate experiments), bacterial burden in the lungs, and dissemination were assessed (six animals from three independent experiments at each time point). *, $p < 0.05$; determined by Wilcoxon rank sign test between groups for survival. For bacterial CFU Student's t test was used, and significant differences between TIRAP^{-/-} and TIRAP^{+/+} mice are indicated by asterisks; *, $p < 0.05$.



via TLR5 (49). Furthermore, a polymorphism in human TLR5 predisposes individuals to Legionnaires' disease because of attenuated proinflammatory cytokine production (50). Considering the current information on TLR signaling in response to Pa, studies have predominantly used transformed epithelial cell lines in vitro. However, findings that confirm these in vitro observations are still limited. Therefore, our experiments using wild-type and PaΔ*flhC* strains of Pa should add new information that is more relevant to the pneumonia caused by Pa. Our findings raise the hypothesis that blocking TLR5 during cystic fibrosis may represent a strategy to minimize excessive neutrophil influx and subsequent lung damage during this disease. Although Pa-induced neutrophil accumulation was abolished in MyD88^{-/-} mice, the observation that the *flhC*-dependent neutrophil response in TIRAP^{-/-} mice was attenuated but not completely lacking in the lungs against Pa demonstrates the activation of TLR5 by other flagellin(s) of Pa and/or the involvement of other unidentified TLR(s) in which Pa components can bind and signal.

Bacteria-induced neutrophil sequestration in the lung is dependent on the efficient production of cytokines and chemokines by both resident and myeloid lung cells in response to the interaction between the bacteria and host cell receptors (51). Production of these proinflammatory mediators by bacteria is due to direct stimulation of chemokine-producing cells or the indirect stimulation by chemokines such as TNF-α. ELR⁺ CXC chemokines, which include keratinocyte cell-derived chemokine (52, 53), MIP-2 (54), lungkine (55), and LIX (30, 56) in the murine lung, are important for chemotaxis to neutrophils and bind to the receptor CXCR2 (57,

58). We found that the production of MIP-2, TNF-α, LIX, and IL-6 are dependent on TIRAP (Fig. 7). Because STAT4 is also shown to be important for cytokine expression in the lung against Kp infection (28), our observations may suggest that STAT4 is a likely downstream target in the TIRAP-mediated signaling cascade in response to Kp.

Our results demonstrate that Pa-induced MIP-2, TNF-α, and IL-6 production after Pa infection is also TIRAP^{-/-} dependent. This is likely due to LPS and/or lipoproteins derived from Pa, because they can activate the TLR2 cascade, which is TIRAP dependent (5–8). The finding that wild-type Pa, but not PaΔ*flhC*, and flagellin (recFLA-ST) alone produced high levels of LIX in a TIRAP-independent fashion supports the hypothesis that TLR5 activation by bacterial flagellin is required for the induction of LIX. In this context, LIX is expressed during lung inflammation after Pa challenge (57). We have recently reported that LIX is expressed in the lungs by resident alveolar type II cells (30, 56). Furthermore, using recombinant LIX alone and blocking LIX using an Ab, we demonstrated that LIX by itself could induce neutrophil accumulation in the lungs during Gram-negative bacterial product (LPS)-induced inflammation (30, 56). Because persistent neutrophil accumulation in the airways is observed during cystic fibrosis lung disease in humans (59), our observations suggest the possibility that excessive expression of the human LIX homologues ENA-78 (epithelial neutrophil-activating peptide 78) and GCP-2 (granulocyte chemotactic protein 2) by flagellin binding to TLR5 is an important event during this disease pathogenesis

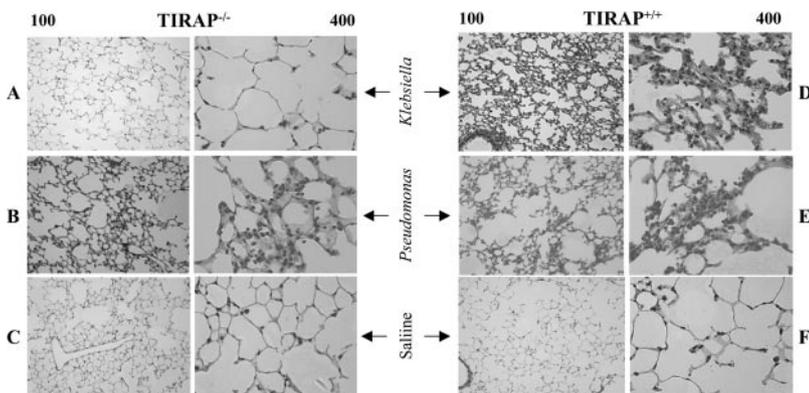


FIGURE 7. Reduced lung histopathology in TIRAP^{-/-} mice following Kp but not Pa inoculation. Lung sections were made 24 h after bacterial (A, B, D, and E) or saline challenge (C and F) and stained with H&E. These photographs are representative sections of six mice from three independent experiments in each condition with identical results.

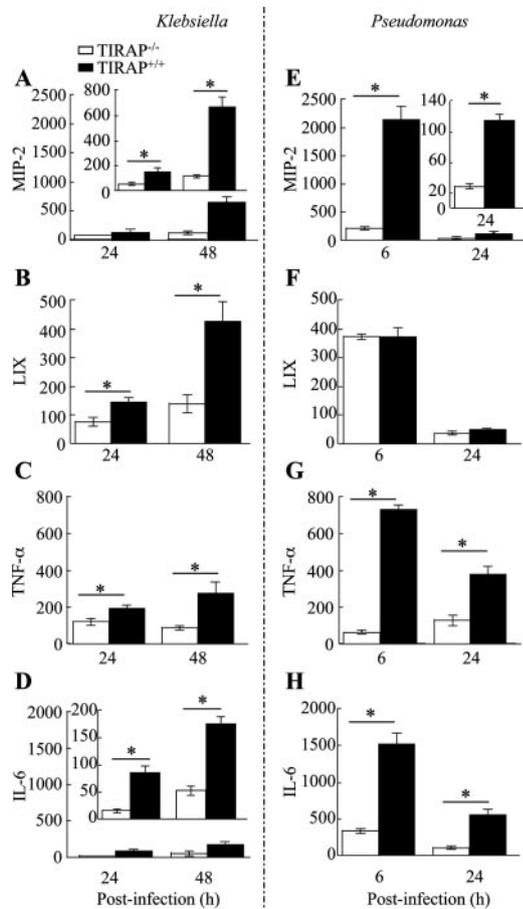


FIGURE 8. Impaired cytokine and chemokine responses in airspaces of TIRAP^{-/-} mice after Kp and Pa infection. Cytokine and chemokine levels in BALF were measured by ELISA after Kp or Pa infection. Protein levels (pg/ml) are expressed as mean ± SE, and six animals were used from three independent experiments at each time point. Significant differences between knockout and wild-type mice are indicated by asterisks; *, $p < 0.05$.

Neutrophil accumulation in the lung involves up-regulation of cell adhesion molecules on vascular endothelium (26, 37, 38). In particular, VCAM-1 and ICAM-1 are not constitutively expressed on the endothelium (26, 37, 38), and VCAM-1 and ICAM-1 up-regulation occurs during inflammation in the lung via proinflammatory mediators such as MIP-2 and TNF-α (37, 38). Our findings suggest that Kp-induced, TIRAP-mediated signaling leads to the expression of chemokines and cytokines and up-regulation of VCAM-1 on the endothelium, which contributes to neutrophil influx in the lungs. Our report illustrates that defective actin assembly of neutrophils from TIRAP^{-/-} mice is not a mechanism for attenuated neutrophil sequestration in the lung during Kp infection. In our previous studies, we demonstrated that TIRAP^{-/-} neutrophils have no chemotactic defect, ruling this out as a mechanism for defective neutrophil accumulation in TIRAP^{-/-} mice after Kp challenge (26).

Observations from our current investigation have broad implications in searching for new therapeutic targets to modulate host defense during bacterial pneumonia. In this context, targeting earlier steps at the level of TLRs could modulate the up-regulation of adhesion molecules on the endothelium that influences neutrophil sequestration. Because each bacterium possesses a plethora of virulence factors to interact with multiple TLRs, and each TLR signals via multiple adaptor proteins, targeting adaptor molecules to

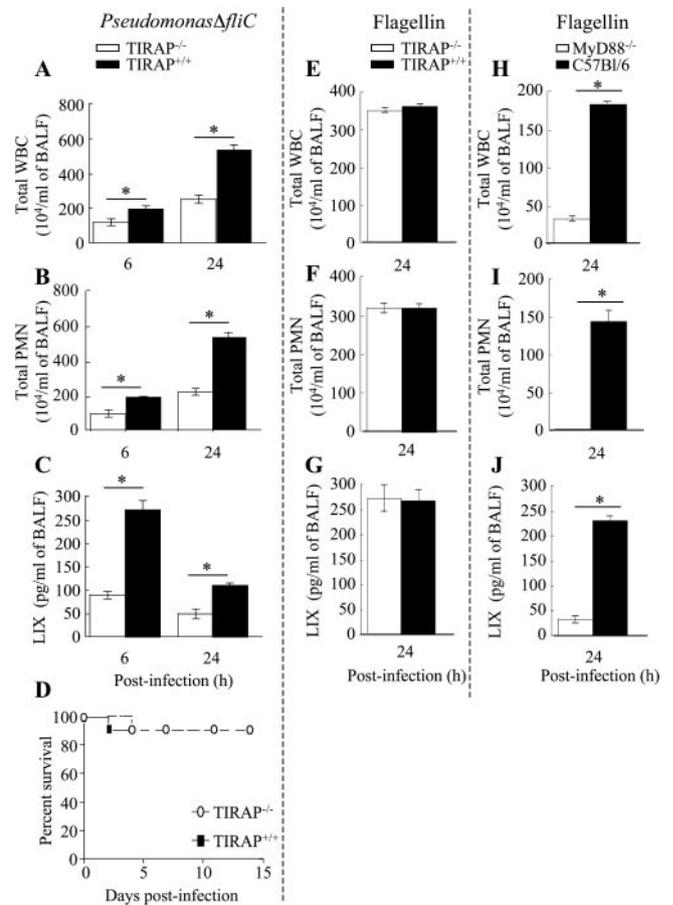


FIGURE 9. A and B, Attenuated pulmonary cellular infiltration in the lungs of TIRAP^{-/-} and TIRAP^{+/+} mice after PaΔfliC administration. The TIRAP^{-/-} and TIRAP^{+/+} animals underwent BALF collection at the designated time points after bacterial (A–C) challenge. Data are expressed as mean ± SE of six animals in each group at each time point from three independent experiments. *, $p < 0.05$; significant differences between TIRAP^{-/-} and TIRAP^{+/+} mice. C, TIRAP-dependent LIX expression in the lung after PaΔfliC inoculation. Data are expressed as mean ± SE of six animals in each group at each time point from three independent experiments; *, $p < 0.05$. D, Unaltered survival between TIRAP^{-/-} and TIRAP^{+/+} mice after PaΔfliC infection (10⁶ CFU/mouse). A total of 10 mice from two separate experiments were used in each group. *, $p < 0.05$; determined by Wilcoxon Rank Sign Test between groups. E–G, Unchanged flagellin-induced cellular and neutrophil influx in the lungs and LIX expression of TIRAP^{-/-} and TIRAP^{+/+} after being i.t. instilled with 1 μg of flagellin. BALF was obtained at 24 h after inoculation, and total leukocyte and neutrophil counts and LIX levels were calculated. Cell numbers and LIX levels are expressed as means ± SE of five animals in each group at each time point from three separate experiments. H–J, MyD88-dependent cellular and neutrophil influx and LIX expression after 1 μg of flagellin inoculation. BALF was obtained at 24 h after inoculation, and total cell and neutrophil counts and LIX levels were calculated. Cell numbers and LIX levels are expressed as means ± SE of five animals in each group at each time point from three separate experiments; *, $p < 0.05$. PMN, polymorphonuclear leukocyte; WBC, white blood cell.

modulate host defense in the TLR cascade could be a viable strategy.

In conclusion, our data reveal the inherent complex nature of the innate immune system in which indispensable and dispensable roles for a particular component are dependent on the specific extracellular pathogen within the group of Gram-negative bacteria. More interestingly, the results in this study suggest that TIRAP

polymorphism may have functional defects in innate immune responses in humans against Gram-negative, bacteria-induced pulmonary infections.

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Disclosures

The authors have no financial conflict of interest.

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