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Toll-IL-1 Receptor Domain-Containing Adaptor Protein Is Critical for Early Lung Immune Responses against *Escherichia coli* Lipopolysaccharide and Viable *Escherichia coli*[1]

Samithamby Jeyaseelan,[2]*‡ Rizwan Manzer,† Scott K. Young,* Masahiro Yamamoto,§ Shizuo Akira,§ Robert J. Mason,†‡ and G. Scott Worthen*‡

Pulmonary bacterial diseases are a leading cause of mortality in the U.S. Innate immune response is vital for bacterial clearance from the lung, and TLRs play a critical role in this process. Toll-IL-1R domain-containing adaptor protein (TIRAP) is a key molecule in the TLR4 and 2 signaling. Despite its potential importance, the role of TIRAP-mediated signaling in lung responses has not been examined. Our goals were to determine the role of TIRAP-dependent signaling in the induction of lung innate immune responses against *Escherichia coli* LPS and viable *E. coli*, and in lung defense against *E. coli* in mice. LPS-induced neutrophil sequestration; NF-κB translocation; keratinocyte cell-derived chemokine, MIP-2, TNF-α, and IL-6 expression; histopathology; and VCAM-1 and ICAM-1 expression were abolished in the lungs of TIRAP$^{-/-}$ mice. A cell-permeable TIRAP blocking peptide attenuated LPS-induced lung responses. Furthermore, immune responses in the lungs of TIRAP$^{-/-}$ mice were attenuated against *E. coli* compared with TIRAP$^{+/+}$ mice. TIRAP$^{-/-}$ mice also had early mortality, higher bacterial burden in the lungs, and more bacterial dissemination following *E. coli* inoculation. Moreover, we used human alveolar macrophages to examine the role of TIRAP signaling in the human system. The TIRAP blocking peptide abolished LPS-induced TNF-α, IL-6, and IL-8 expression in alveolar macrophages, whereas it attenuated *E. coli*-induced expression of these cytokines and chemokines. Taken together, this is the first study illustrating the crucial role of TIRAP in the generation of an effective early immune response against *E. coli* LPS and viable *E. coli*, and in lung defense against a bacterial pathogen. ***The Journal of Immunology,*** **2005, 175: 7484–7495.**

Uncontrolled immune responses leading to inflammatory lung diseases caused by Gram-negative bacteria and their products are an important cause of human death (1, 2). The pathophysiology of lung responses is characterized by enhanced capillary permeability, up-regulation of cell adhesion molecules, expression of proinflammatory mediators, and accumulation of neutrophils in the alveolar spaces with diffuse damage of alveolar epithelium and endothelium (3–6). Local or systemic LPS released from the outer cell wall of replicating and dying Gram-negative bacterial pathogens is a characteristic feature of several diseases, including acute lung injury (ALI)[3] and acute respiratory distress syndrome (ARDS). Although LPS is not itself a neutrophil chemoattractant, it can trigger an inflammatory cascade in the lung via the production of proinflammatory mediators by both myeloid and nonmyeloid cells. In turn, the production of TNF-α and chemokines, such as IL-8, can elicit an early neutrophil sequestration in the human lung to augment local defense in response to LPS. Clearly, a comprehensive understanding of the interaction between LPS and lung cells is required to design better treatment strategies to minimize excessive immune responses in the lung.

The first line of defense in the lung against microbial pathogens is provided by the innate immune system (7, 8). Cell surface TLRs play a vital role in the induction of innate immune responses in the local environment of the lung against pathogen-associated molecular patterns (7, 8). The potent Gram-negative bacterial stimulus, LPS, is primarily recognized by a complex of proteins, including TLR4, LPS-binding protein, MD-2, and CD14 (9–12). Because CD14, LPS-binding protein, and MD-2 have no intrinsic signaling capabilities (9–12), TLR4 is required to induce signaling cascades (13). The pathways underlying TLR4-induced signaling are identical with that of IL-1R activation (14). LPS binding to TLR4 activates both MyD88-dependent and MyD88-independent signaling cascades to cause, respectively, expression of cytokine and chemokine genes, and expression of IFN-inducible genes, ultimately modulating the innate immune response of the lung (15–20). Recent studies have illustrated that the novel adaptor protein, Toll-IL-1R domain-containing adaptor protein (TIRAP), is a critical component of the TLR4 signaling cascade to LPS in isolated cells (21). However, little is known about the role of these adaptor molecules in MyD88-dependent and MyD88-independent cascades in the induction of lung immune responses against LPS in vivo. A thorough understanding of the role of these receptors and adaptors is important for early and better therapeutic strategies to modulate excessive host responses.

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[3] Abbreviations used in this paper: ALI, acute lung injury; AM, alveolar macrophage; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BP, blocking peptide; CP, control peptide; i.t., intratracheal; KC, keratinocyte cell-derived chemokine; KO, knockout; MPO, myeloperoxidase; PMN, polymorphonuclear cell; TIRAP, Toll-IL-1R domain-containing adaptor protein; TSA, tryptic soy agar; WT, wild type.

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The role of pattern recognition receptors and their adaptors in the induction of pulmonary host responses against bacterial pathogens is under intense investigation. For example, CD14 is reported to be critical to induction of the local innate immune response in the lung to *Escherichia coli* (22). In addition, we and others have demonstrated that TLR2 is necessary to the pulmonary innate immune response against *Mycoplasma pneumoniae* (23) and *Streptococcus pneumoniae* (24, 25). Furthermore, TLR4 contributes to lung defense against *S. pneumoniae* (26). Regarding the adaptor molecules of TLR2 and TLR4, an elegant study by Skerrett et al. (27) illustrated that MyD88-dependent signaling is crucial for antibacterial defense in the lung against *Pseudomonas aeruginosa*. Although previous investigations have shown that pattern recognition receptors and the intracellular adaptor molecule MyD88 play important roles in host defense, less is known about the role of other adaptor molecules, including TIRAP, in the induction of lung innate immune responses against bacterial pathogens.

Given the central role of the MyD88-dependent cascade as a regulator of proinflammatory mediators in several isolated cell types, and the specificity of TIRAP for the TLR4 and TLR2 signaling cascades, we hypothesized that TIRAP activation is a proximal and critical event for innate immune responses in the lung against bacterial pathogens. To study the role of TIRAP-dependent signaling in pulmonary innate immune responses, mice deficient of TIRAP by homologous recombination ($TIRAP^{-/-}$) were challenged with *E. coli* LPS or the intact extracellular pathogen, *E. coli*. Our reports demonstrate that TIRAP signaling is critical for LPS-induced pathological immune responses in the lung. These observations were validated using a cell-permeable TIRAP blocking peptide (BP) in vivo. In association with diminished lung immune response to LPS in $TIRAP^{-/-}$ mice, there was attenuated immune response to *E. coli*, and early mortality and higher bacterial burden in the lungs and spleen following intratracheal (i.t.) *E. coli* inoculation. To demonstrate the role of TIRAP signaling in the human system, we used primary human alveolar macrophages (AMs) and found that TIRAP signaling is critical to induce immune mediators by those cells in response to *E. coli* LPS and viable *E. coli*. These observations detail a heretofore unrecognized vital role for TIRAP-mediated signaling in innate immune responses in the lung against *E. coli* LPS and viable *E. coli*, and antibacterial defense against a clinically important pathogen. We speculate that these novel observations may have important implications for modification of host-injurious responses in the lung to Gram-negative bacteria and/or their products.

Materials and Methods

Animals

TIRAP gene-deficient mice ($TIRAP^{-/-}$) and their wild-type (WT) control mice ($TIRAP^{+/+}$) (21) were backcrossed six times with C57BL/6 and were used afterward. All animal experiments were conducted using a protocol, which was approved by the National Jewish Medical and Research Center Committee on animal studies. Eight- to 10-wk-old female mice, ranging from 21 to 24 g in weight, were used in our experiments. Mice were maintained under specific pathogen-free conditions and kept on a 12:12-h light:dark cycle with free access to food and water.

Reagents

Ultrapure *E. coli* LPS (O111:B4) was purchased from Sigma-Aldrich and was dissolved in preservative- and pyrogen-free isotonic (0.9%) saline to yield a concentration of 300 μg/ml for the induction of lung innate immune responses in mice. The TIRAP BP (NH_2-RQIKIWFQNRRMKWKKL QLRDAAPGGAIVS-COOH) included the *Drosophila antennapedia* protein leader sequence positioned at the NH_2 terminus (28), and the negative control for TIRAP BP consisted of the *antennapedia* sequence positioned at the NH_2 terminus of the reversed TIRAP peptide sequence. Both were purchased from Calbiochem, and are cell permeable when added exogenously (28). Peptides were reconstituted in the culture medium (for human macrophage in vitro experiments) or PBS (for mouse in vivo experiments). LPS-free glass and plastic ware were used in experiments. rMIP-2 was purchased from R&D Systems, and immune response modifier, 1-(4-amino-2-methyl-1*H*-imidazo[4, 5-*c*]quinolin-1-yl)-2-methylpropan-2-ol hydrochloride (a TLR7 agonist, S-27609) was obtained from 3M Pharmaceuticals.

LPS challenge

The mouse model to induce lung immune responses against LPS-derived from *E. coli* has been described in our previous publications (29–32). Briefly, mice were exposed to 300 μg/ml LPS in 0.9% saline by aerosolization for 20 min in a plexiglass chamber using a Bang nebulizer (CH Technologies). Control groups of mice were treated according to the same procedure, except they received isotonic saline instead of LPS. In another set of experiments, the animals were anesthetized with i.p. avertin (250 mg/kg), followed by i.t. administration of 500 μg of blocking, control peptide (CP) in 50 μl of 0.9% saline or 50 μl of saline alone (control) 2 h before LPS aerosolization. At 2, 8, and 24 h postinhalation, the whole lungs were collected, because LPS-induced inflammatory features in the airspace and lung parenchyma were distinct at 2-, 8-, and 24-h time points, as previously described (29–32).

Instillation of MIP-2 and S-27609

Animals were anesthetized with 250 mg/kg avertin, and a 1-cm midventral skin incision was performed. After isolation of muscles surrounding the trachea, the trachea was isolated and 50 μl of MIP-2 or S-27609 in 0.9% saline was inoculated. The skin incision was closed with sterile staples. The bronchoalveolar lavage (BAL) fluid was collected at 2 and/or 4 days postinoculation for cellular influx, and cytokine and chemokine analysis.

BAL fluid collection

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 thoracic cavity, and the trachea was isolated and cannulated with a 20-gauge catheter, which was immobilized with 2-0 silk suture material. BAL fluid was collected from the whole lung to obtain cells in the airspace and to obtain proteins for cytokine examination, as previously described (29–32). A total of 3.0 ml of BAL fluid was obtained from each mouse, and 0.5 ml of BAL fluid was centrifuged and placed on glass cytospin slides, which were then stained by Diff-Quick reagents (Fisher Scientific) to enumerate leukocyte subtypes based on their cellular and nuclear morphological features. A total of 2 ml of the undiluted BAL fluid was centrifuged, passed via a 0.22-μm filter, and used immediately or kept at −20°C for the determination of cytokines and chemokines by ELISA.

Cytokine determination

Cytokine and chemokine levels were measured in BAL fluid using a specific sandwich ELISA, as previously described (29–32). The minimum detection limit is 2 pg/ml cytokine protein (29–32).

Lung harvesting

At 2, 8, and 24 h postchallenge, the whole (nonlavaged) lungs were excised from mice and were immediately snap frozen, followed by storage at −70°C. The lungs were used immediately for the detection of NF-κB translocation, myeloperoxidase (MPO) assay, total RNA isolation, and lung histopathology.

NF-κB translocation

An ELISA-based NF-κB assay was performed, according to the manufacturer's protocol (Active Motif), to detect the translocation of the p65 subunit of NF-κB into the nucleus of lung cells, as described previously (23, 30). Briefly, a total of 20 μg of nuclear extract in each lung sample at 2 h after LPS or after saline was added to the NF-κB-specific oligonucleotide-coated 96-well plate and incubated for 1 h at room temperature. After washing the plate three times, a primary Ab specific for p65 was added and incubated for 1 h at room temperature. After three washes to remove excess primary Ab, an anti-HRP conjugate was added to the plate and the color development was monitored. Color development was measured at the OD of 450 nM.

MPO assay

Determination of lung MPO activity was performed, as previously described (29, 30). MPO is an enzyme found in myeloid cells and has been used largely as a marker of neutrophil accumulation into 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.

Lung RNA

Fresh mouse lungs subjected to LPS or saline aerosolization were harvested at designated time points, and crude total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). Crude RNA was subsequently purified using the RNAeasy kit (Qiagen) with DNase I treatment, according to the manufacturer's recommendations (Ambion). These RNA were used for semiquantitative RT-PCR experiments, as described below.

Semiquantitative RT-PCR analysis of lungs

Reverse transcription was performed using 1 μg of total RNA, as previously described (33). Semiquantitative PCR was performed using the following specific primers: 5′-TGA-GCT-GCG-CTG-TCA-GTG-CCT-3′ and 5′-AGA-AGC-CAG-CGT-TCA-CCA-GGA-3′ for keratinocyte cell-derived chemokine (KC) (product size: 256 bp); 5′-TGC-CTG-AAG-ACC-CTG-CCA-AGG-3′ and 5′-GTT-AGC-CTT-GCC-TTT-GTT-CAG-3′ for MIP-2 (355 bp); 5′-CCA-GGC-GGT-GCC-TAT-GTC-TC-3′ and 5′-AGC-AAA-TCG-GCT-GAG-GGT-GT-3′ for TNF-α (407 bp); and 5′-ATG-GAT-GAC-GAT-ATC-GCT-C-3′ and 5′-GAT-TCC-ATA-CCC-AGG-AAG-G-3′ for β-actin (550 bp). A total of 10 μl of product was electrophoresed on a 1% agarose gel containing ethidium bromide, and bands were visualized and photographed using an UV transilluminator.

Lung histology

Twenty-four hours after LPS or saline inhalation, mouse lungs were inflated and fixed with Streck tissue fixative (Streck Laboratories) 12 h at room temperature. The specimens were then embedded in paraffin, and 5-μm serial sections were made. These sections were then stained with H&E for histological examination using a light microscope. Nonquantitative histological evaluation was performed by a pulmonologist/pathologist in a blinded fashion.

Immunoblot analysis of lung homogenates

At the designated times, the harvested lungs were homogenized in 1 ml of buffer containing 0.1% Triton X-100 in PBS and complete protease inhibitor mixture (Roche) for 30 s and centrifuged at maximum speed in a microcentrifuge at 4°C. The resulting supernatant fluids were used for immunoblotting. To ensure equal amounts of protein onto the gel, a Bradford protein assay was performed (Bio-Rad). The lung homogenates were analyzed on two 8–15% Tris-glycine gels. Each gel was transferred on polyvinylidene difluoride membrane using standard protocols. The mAb to mouse VCAM-1, ICAM-1, or β-actin was added at a 1/500, 1/200, or 1/1,000 dilution, respectively. Immunostaining was performed using appropriate secondary Ab at a dilution of 1/10,000 and developed with ECL plus Western blot detection system (Amersham Biosciences).

Ex vivo neutrophil actin cytoskeleton determination

To determine whether neutrophils from TIRAP$^{-/-}$ mice display equal actin assembly as TIRAP$^{+/+}$ to sequester neutrophils into the lungs, we measured actin polymerization in response to KC, MIP-2, TNF-α, or LPS because this process is involved in several mechanisms used by neutrophils for lung recruitment. Bone marrow-derived polymorphonuclear cells (PMNs) were isolated, as described in our previous publications (32, 34). Isolated PMNs (10^6/ml) were assayed for actin polymerization in response to *E. coli* LPS (1 μg/ml; 1 h at 37°C), TNF-α (1 μg/ml; 1 h at 37°C), KC (5 ng/ml; 15 min, 37°C), or MIP-2 (5 ng/ml; 15 min, 37°C), was quantitated as relative fluorescence intensity, as previously described in our studies (32, 34).

E. coli *challenge*

To assess the role of TIRAP in lung host defense against a live bacterium, we used an *E. coli* strain (ATCC 25922), which was previously used to examine lung innate immune responses in mice (35). A frozen 500 μl (10^7/ml) of aliquot of the bacteria was grown for 6 h to midlogarithmic phase at 37°C in 50 ml of tryptic soy broth (BD Biosciences) while shaking at 200 rpm, harvested by centrifugation at 1200 $\times$ *g* for 12 min, and washed twice in sterile 0.9% saline. The bacteria were then resuspended in sterile 0.9% saline at a concentration of 10^6 or 10^7 CFU/50 μl/mouse. The TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice were anesthetized with avertin (250 mg/kg), and a midventral incision was performed. After isolation of muscles, the trachea was exposed, and each mouse was inoculated with 10^6 or 10^7 CFU in 50 μl of *E. coli* suspension in 0.9% saline (pH 7.4). A 50-μl aliquot of serially diluted suspension of initial inoculum was plated onto a tryptic soy agar (TSA) plate and a MacConkey plate for validation. The animals were humanely sacrificed at 6 and 24 h postinfection, and their whole lungs were excised and homogenized in 10 ml of 0.9% saline. Several 10-fold dilutions of the homogenates were plated on TSA and MacConkey plates, and colonies were enumerated after overnight incubation at 37°C. In a separate set of experiments, BAL fluid was harvested from the mice after *E. coli* infection for total WBC and neutrophil counts, and cytokine and chemokine measurements, and the lungs were harvested for NF-κB translocation and chemokine and cytokine expression. At 24 h after *E. coli* LPS or *E. coli* administration, 5-μm lung sections were prepared and stained with H&E for histopathology, as previously described (36). Descriptive histopathology was performed by a pulmonologist/pathologist in a blinded fashion.

In another set of experiments, TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice were inoculated i.t. with a lethal dose of (10^9 CFU in 50 μl of 0.9% saline) *E. coli* and survival was monitored. In addition, in a separate set of experiments, the bacterial burden in the lungs and bacterial dissemination (as measured as spleen CFUs) were measured at 3 h post-*E. coli* infection to the lethal dose.

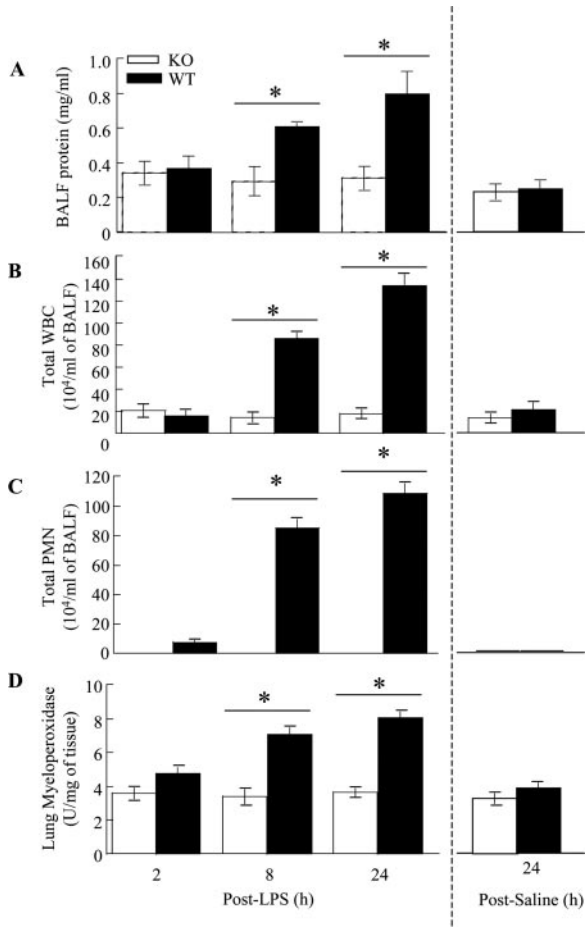

FIGURE 1. Early innate immune responses in the lung after LPS challenge. Animals were aerosolized with 300 μg of LPS in saline or saline alone (control) for 20 min, and BAL fluid was collected at 2, 8, and 24 h postchallenge. BAL fluid total protein (*A*), white blood cell (*B*), and neutrophil counts (*C*) and on MPO activity in lung parenchyma (*D*) were measured in TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice. Bars depict means $\pm$ SD of eight animals from three separate experiments in each group at 2, 8, and 24 h, and asterisk (*) denotes significant differences between TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice ($p < 0.05$).

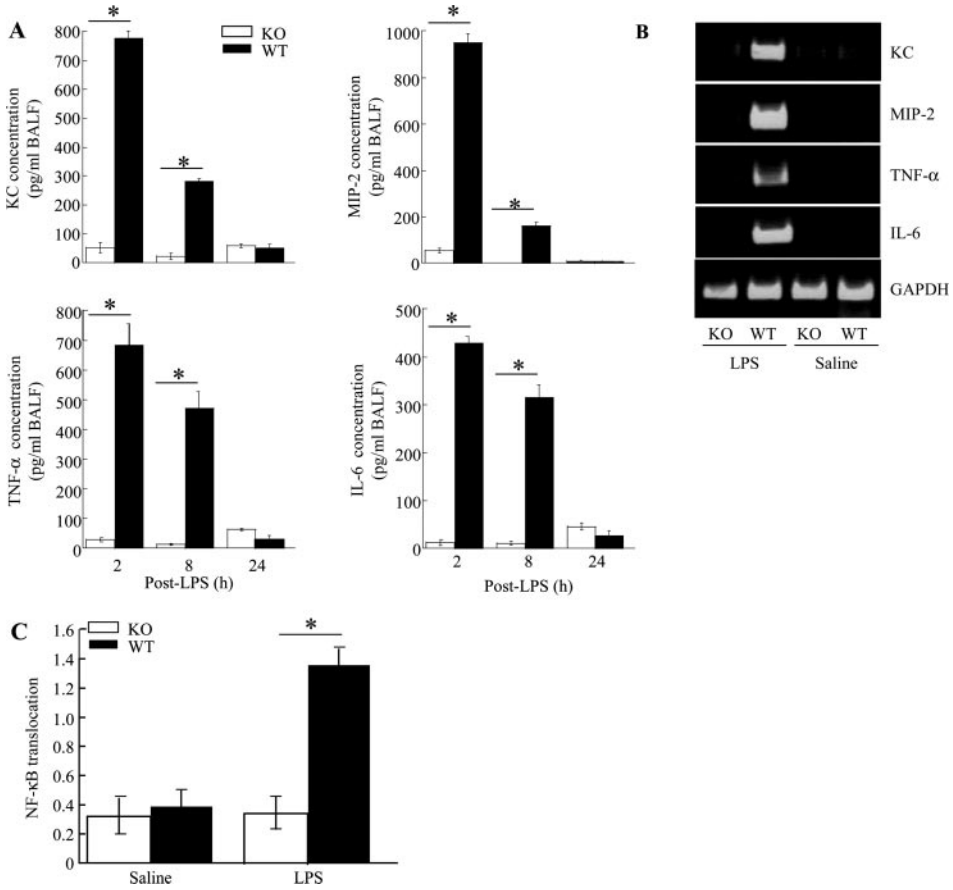

FIGURE 2. Effect of aerosolized LPS on cytokine and chemokine expression in BAL fluid and NF-κB translocation in the whole lungs of TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice at 2, 8, and 24 h post-LPS or postsaline, as measured in BAL fluid by ELISA (*A*). $n = 8$ mice from three independent experiments in each group at 2, 8, and 24 h. *B*, Cytokine and chemokine expression in the whole lungs of TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice at 2 h after LPS or after saline, as determined by semiquantitative RT-PCR. This is a representative gel picture of eight mice from three separate experiments in each group of LPS and saline challenge at 2 h. *C*, NF-κB translocation in the whole lungs of TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice 2 h after LPS or saline aerosolization, as measured in nuclear extracts by an ELISA-based NF-κB p65 assay. OD value at 450 nm was used to represent the p65 subunit of NF-κB translocation levels. $n = 6$ mice from three separate experiments in each group of LPS and saline challenge. ∗, Significant differences between TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice ($p < 0.05$).

E. coli *killing assay*

To evaluate the ability of neutrophils to kill *E. coli*, a killing assay was performed, as described previously (31). The *E. coli* obtained after two washings with sterile 0.9% saline was used for the assay. Viable *E. coli* at a concentration of 10^4 or 10^5 CFU/ml was mixed with murine PMN at a concentration of 10^4 or 10^5 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 h. At the end of incubation, the CFUs were determined by serially diluting the culture at several 10-fold dilutions on a MacConkey and TSA plate.

Human AM isolation and LPS stimulation

AMs were isolated from normal lungs of humans who had no history of lung diseases, as described in our previous publication (37). The lungs were obtained from organ donors whose lungs were not suitable for transplantation. The donors are identified, and the use of their tissue was approved by the Institutional Review Board at National Jewish. Briefly, lungs were lavaged with sterile 150 mM sodium chloride supplemented with 5 mM HEPES and 5 mM EDTA. The lavage was collected in 50-ml sterile tubes. The lavaged lungs were finely chopped in the presence of the instillation, and the pieces were vortexed to dislodge the cells. The cells were then passed through different layers of gauze, followed by a 20-μm filter to remove the lung debris. The cell suspension from the lavage and the lungs was pooled and centrifuged at 250 × g for 10 min, and the resulting pellet was resuspended in D-10 medium (DMEM containing 10% FBS, 2 mM glutamine, 2.5 μg/ml amphotericin B, 100 μg/ml streptomycin, 100 μg/ml penicillin G, and 10 μg/ml gentamicin). The percentage of AMs was determined by cell morphology, and cell viability was assessed by trypan blue exclusion (Cambrex), which was varied between 86 and 94% and 94–96%, respectively. Two million viable cells were plated in each well at a density of 10^6 cells/ml on six-well tissue culture plastic plate. After 4-h incubation at 37°C, the nonadherent cells were removed by two vigorous washings with 1 ml of D-10 medium (without antibiotics and antifungals) resulting in >98% of macrophages, as assessed by Wright-Giemsa stain (Diff-Quik; Dade Diagnostics), and then 2 ml of medium was added in each well and incubated for 40 h at 37°C to make them quiescent. Thereafter, the human AMs in each well were pretreated with 200 μg of TIRAP BP (100 μg/ml) or CP, or left untreated for 2 h, followed by stimulation with 200 ng of LPS (100 ng/ml) or 2 × 10^4 *E. coli* (1 × 10^4/ml), for 18 h. Cells were then harvested for total RNA isolation, and cell supernatants were collected for cytokine and chemokine protein measurement by ELISA. Supernatants were centrifuged at 500 × g for 10 min to discard remaining cell debris, and supernatants were harvested and stored at −20°C until use. The viability of AMs was assessed using trypan blue before and after treatment with TIRAP BP or CP. We found that TIRAP BP or CP did not alter the viability of cells or bacterial growth after pretreatment (data not shown). Two hours after LPS stimulation, total RNA from human AMs was extracted using TRIzol, followed by purification using Rneasy kit, according to the manufacturer's protocol. Reverse transcription was performed using 1 μg of total RNA, as previously described (33). Semiquantitative PCR was performed using the following specific primers: TNF-α, 5′-AGC CCATGTTGTAGCAAACC-3′ and 5′-TTTGGGAAGGTTGGATGTTC-3′ (463 bp); IL-6, 5′-AAAGAGGCACTGGCAGAAAA-3′ and 5′-AAAGCT GCGCAGAATGAGAT-3′ (350 bp); IL-8, 5′-CCACCGGAAGGAACCA TCTC-3′ and 5′-CCAGTTTTCCTTGGGGTCCA-3′ (285 bp); and GAPDH, 5′-ACAGTCAGCCGCATCTTCTT-3′ and 5′-TGTGGTCATGAGTCCTT CCA-3′ (600 bp).

Statistical analysis

Data were expressed as means ± SD. For comparison between two groups, Student's *t* test was used. A *p* value of <0.05 was considered significant using Kaleidagraph version 6.0 (Synergy Software).

Table I. *TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice show unaltered leukocyte and neutrophil influx in the airspaces in response to MIP-2*[a]

Mouse Strain	Total WBC (×10^4)	Total PMN (×10^4)
KO	33.0 ± 4.2	3.1 ± 0.6
WT	35.7 ± 0.4	2.4 ± 0.5

[a] Total WBCs and PMNs enumerated in BAL fluid at 4 h after i.t. inoculation with 10 ng of MIP-2/mouse. Data are presented as mean ± SD. A total of five mice were used in each group from three separate studies.

Table II. *TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice demonstrate unaltered leukocyte and neutrophil sequestration, and cytokine and chemokine production in the airspaces in response to a TLR7 agonist S-27609*[a]

Mouse Strain	Total WBC ($\times 10^4$)	Total PMN ($\times 10^3$)	MIP-2 (pg/ml)	TNF-α (pg/ml)	IL-6 (pg/ml)
2 h posttreatment					
KO	9.9 ± 1.8	1.9 ± 1.0	270.7 ± 32.0	108.5 ± 16.6	48.8 ± 5.5
WT	9.0 ± 3.1	2.2 ± 0.6	307.4 ± 16.6	140.6 ± 16.3	55.5 ± 5.6
4 h posttreatment					
KO	14.7 ± 2.7	10.5 ± 2.1	46.8 ± 6.0	18.7 ± 4.5	24.8 ± 3.7
WT	16.3 ± 1.8	9.5 ± 6.6	45.2 ± 4.7	14.8 ± 1.9	26.3 ± 5.0

[a] Total WBC and neutrophil numbers, and cytokines and chemokines measured in BAL fluid at 2 and 4 h after i.t. inoculation with 40 μg of the TLR7 agonist/mouse. Data are expressed as mean ± SD. A total of six mice were used in each group from three independent experiments.

Results

The absence of functional TIRAP results in reduced leukocyte accumulation in the lung in response to E. coli *LPS*

To determine the role of TIRAP in early immune responses in the lung, we used LPS, the most potent cell stimulus of Gram-negative bacteria. We conducted BAL fluid and lung homogenate studies at 2, 8, and 24 h following LPS challenge in response to 300 μg/ml LPS in saline or saline alone (control) for 20 min. Total white blood cells and neutrophils in BAL fluid were quantitated in TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice exposed to LPS or saline. In TIRAP$^{-/-}$ mice, total protein, total white blood cells, and neutrophil numbers were not increased above the level of saline-treated mice up to 24 h (Fig. 1). By contrast, total white blood cells and neutrophils were increased significantly in TIRAP$^{+/+}$ mice as compared with saline-challenged TIRAP$^{+/+}$ mice (Fig. 1). In parallel, lung MPO activity, an index of total lung neutrophil sequestration in parenchyma and microvasculature, was not increased in TIRAP$^{-/-}$ mice by LPS compared with saline-treated TIRAP$^{-/-}$ mice (Fig. 1). These data support the conclusion that TIRAP is necessary for LPS-induced microvascular capillary leakage and neutrophil recruitment into the airspace and lung parenchyma.

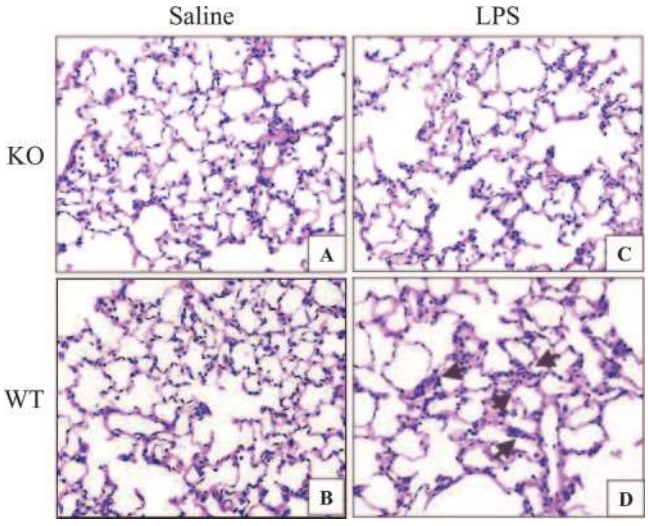

FIGURE 3. Effect of TIRAP deficiency on LPS-induced lung histology. H&E-stained paraffin-embedded lung sections from TIRAP$^{+/+}$ (WT) (*A* and *B*) and TIRAP$^{-/-}$ (knockout (KO)) mice (*C* and *D*). *A* and *B* were saline-exposed lungs, whereas *C* and *D* were LPS-exposed lungs at 24 h postchallenge. These are representative sections of five mice from three separate experiments in each condition with identical results. The arrow indicates infiltrating inflammatory cells and edema. Original magnification: ×400.

TIRAP deficiency in vivo results in decreased KC, MIP-2, TNF-α, and IL-6 expression in the lung in response to LPS

Because the presence of cytokines and chemokines at high concentrations in the lung contributes to neutrophil accumulation, we used BAL fluid and whole lung homogenates to quantify expression of KC, MIP-2, TNF-α, and IL-6 levels at, 2, 8, and 24 h after LPS challenge. These cytokines and chemokines were minimally detected at the protein level in TIRAP$^{-/-}$ mice as compared with TIRAP$^{+/+}$ mice (Fig. 2*A*). Expression of mRNA for these cytokines was also impaired in the lungs of TIRAP$^{-/-}$ mice (Fig. 2*B*). Furthermore, no significant cytokine or chemokine production was observed in BAL fluid obtained from saline-challenged mice (data not shown).

We used several controls to ascertain that the impaired cytokine and chemokine responses in response to LPS in the airspace and lung parenchyma are specific for TIRAP signaling: 1) to determine whether chemotactic activity of neutrophils from TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice was similar, we introduced exogenous MIP-2 i.t. to TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice and observed significant neutrophil influx in both strains of mice (Table I); and 2) the TLR7 agonist (S-27609) was instilled to activate a TLR pathway that does not induce TIRAP (21). We observed comparable levels of neutrophil influx, and MIP-2, TNF-α, and IL-6 in both TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice (Table II). These observations indicate that TIRAP signaling mediates the expression of KC, MIP-2, TNF-α, and IL-6 in the lung by LPS, suggesting a mechanism for the absence of neutrophil sequestration in the lungs of TIRAP$^{-/-}$ mice.

Deficiency of TIRAP leads to attenuation of LPS-induced NF-κB activation in the lung

Decreased cytokine and chemokine expression in the lung in response to LPS in TIRAP$^{-/-}$ mice could reflect attenuated activation of NF-κB, one of the most important transcription factors activated by LPS. We therefore examined NF-κB activation in the lungs of TIRAP$^{-/-}$ mice. In TIRAP$^{-/-}$ mice, no difference of NF-κB translocation was observed in the nuclei of lung cells between LPS-challenged and saline-treated mice (Fig. 2*C*). By contrast, significant NF-κB translocation was detected in the lung nuclear proteins of TIRAP$^{+/+}$ mice (Fig. 2*C*). These data suggest that the absolute dependence upon TIRAP for LPS-induced cytokine and chemokine expression may be due, in part, to the effects of TIRAP on NF-κB translocation.

TIRAP deficiency results in diminished histologic changes in the lung to LPS

The effect of TIRAP-dependent signaling on the histopathological changes in response to LPS was evaluated in the absence (TIRAP$^{-/-}$) or presence (TIRAP$^{+/+}$) of functional TIRAP protein

at 24 h. We selected this time point because we demonstrated in our previous studies that histopathologic changes in the lungs to LPS (300 μg/ml LPS for 20 min) are maximal at 24 h (29). In TIRAP$^{-/-}$ mice, minimal histological changes were observed in response to LPS (Fig. 3). By contrast, in TIRAP$^{+/+}$ mice, LPS induced interstitial and intra-alveolar sequestration of neutrophils and edema that were significant when compared with saline-challenged mice, as assessed in a qualitative manner (Fig. 3). These observations support that TIRAP is an important element in inflammatory responses in the lungs.

Impaired lung expression of VCAM-1 and ICAM-1 in TIRAP$^{-/-}$ mice after LPS challenge

Neutrophil migration to lung is dependent, in part, on modulation of lung vasculature by secondary mediators, and upon neutrophil responsiveness to these mediators (38). LPS-induced VCAM-1 and ICAM-1 up-regulation has been observed during endothelial activation in lung vasculature (39, 40), and therefore is used in this study as a marker of endothelial activation. We determined the endothelial activation in TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice in response to LPS. Up-regulation of VCAM-1 (Fig. 4, *A* and *B*) and ICAM-1 expression (Fig. 4, *C* and *D*) in TIRAP$^{-/-}$ mice was significantly attenuated in response to LPS, suggesting another potential mechanism for impaired neutrophil accumulation in TIRAP$^{-/-}$ mice. No up-regulation of VCAM-1 or ICAM-1 was detected in the lungs of saline-challenged TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice (data not shown).

Alternatively, neutrophil sequestration within pulmonary capillaries in lung parenchyma and subsequently to the airspace from the blood vessels is dependent on assembly of actin cytoskeleton in neutrophils in response to cytokines and chemokines (34, 41). To demonstrate whether the absence of neutrophil accumulation seen in the lungs of TIRAP$^{-/-}$ mice is due to impairment of neutrophil cytoskeleton remodeling in response to KC, MIP-2, and TNF-α, we used isolated neutrophils from TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice. KC, MIP-2, and TNF-α cause similar degrees of actin polymerization in neutrophils of both TIRAP$^{+/+}$ and TIRAP$^{-/-}$ mice (Fig. 4*C*), suggesting that impairment of neutrophil actin assembly is not a mechanism for the absence of neutrophil sequestration in the lungs of TIRAP$^{-/-}$ mice.

Inhibition of TIRAP signaling in the lung attenuated LPS-induced immune responses

To further demonstrate the importance of TIRAP in lung innate immune responses, normal C57BL/6 mice were pretreated with 500 μg of TIRAP BP or CP before LPS or saline challenge. Parameters of lung responses, including total WBC and neutrophil counts, and cytokine and chemokine expression in the BAL fluid, NF-κB activation, and VCAM-1 expression in the whole lung,

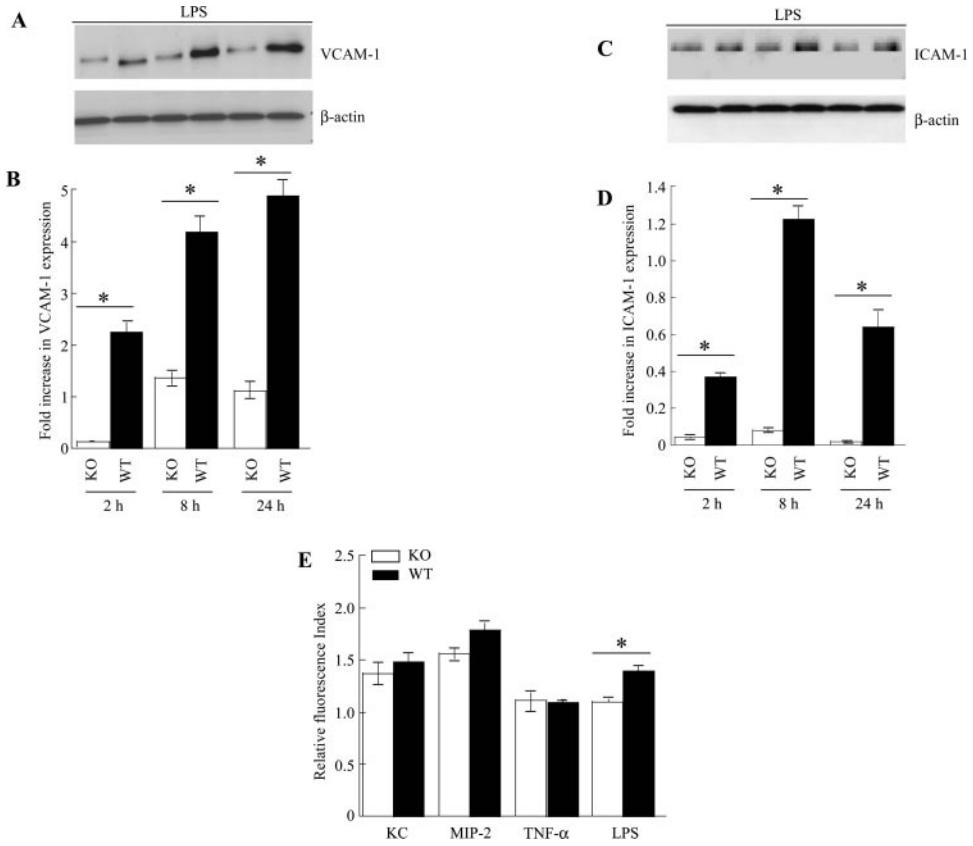

FIGURE 4. *A*, Effect of TIRAP signaling on LPS-induced VCAM-1 and ICAM-1 expression in the whole lungs. Total protein in the lungs was prepared from TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice after 2, 8, and 24 h post-LPS or postsaline treatment. The autoradiograph demonstrates representative blot of VCAM-1 (*A*) and ICAM-1 (*C*) protein of five mice from three independent studies in each group at 2, 8, and 24 h post-LPS or postsaline with identical results. The graph shows VCAM-1 (*B*) and ICAM-1 (*D*) for each sample expressed relative to the densitometric value of saline-treated sample at each time point. These values are mean ± SD of five mice in each group from three independent experiments at 2, 8, and 24 h. *E*, Effect of TIRAP deficiency in actin assembly. Bone marrow-derived band 3 cells were isolated and stimulated with KC, MIP-2, TNF-α, and LPS. Cells were labeled with nitrobenzoxadiazole-phallacidin, and actin assembly was assessed by flow cytometry. Five animals from three separate experiments were used in each group, and the data were normalized to unstimulated controls, giving a relative fluorescence index of 1. Significant differences between KO and WT mice are indicated by asterisks ($p < 0.05$).

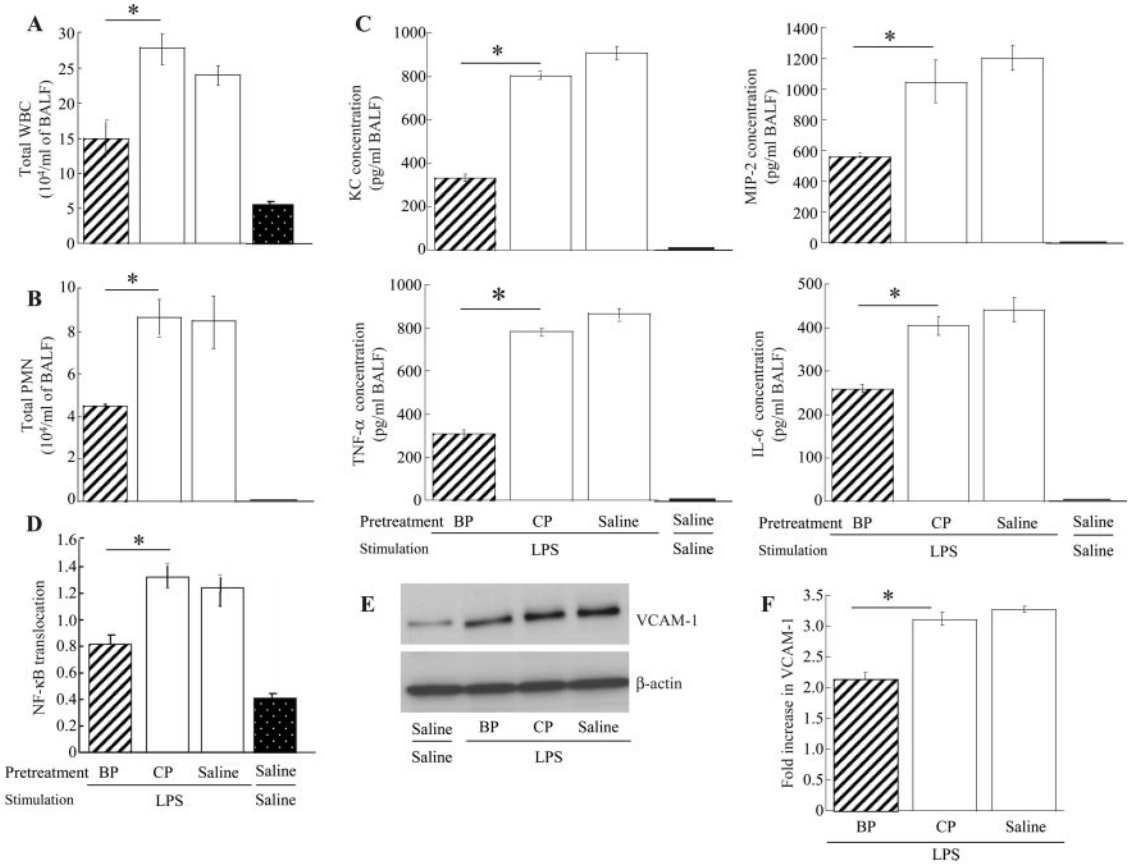

FIGURE 5. A TIRAP BP attenuates LPS-induced total WBC (*A*) and neutrophil counts (*B*), cytokine and chemokine expression in (*C*), NF-κB translocation (*D*), and VCAM-1 expression in the lung (*E* and *F*). C57BL/6 mice were pretreated with 500 μg of BP in 50 μl of PBS (BP), CP in 50 μl of PBS, or 50 μl of PBS alone 2 h before LPS challenge. The BAL fluid was harvested 2 h post-LPS or postsaline challenge to determine total WBC and neutrophil counts and cytokine and chemokine measurements ($n = 7$ from three separate experiments in each group), and the lungs were excised to measure NF-κB translocation ($n = 6$ from three independent experiments in each group) and VCAM-1 protein up-regulation ($n = 8$ per from three separate studies in each group). *E*, The autoradiograph demonstrates representative expression of VCAM-1 protein of eight mice from three independent experiments with identical results. *F*, The graph shows mean ± SD of eight mice from three separate experiments. VCAM-1 expression for each sample is expressed relative to the densitometric value of saline-treated sample. ∗, Significant differences between BP-treated and CP-treated mice ($p < 0.05$).

were measured in both TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice. Significant reduction in all of the above measures was observed in TIRAP$^{-/-}$ mice as compared with TIRAP$^{+/+}$ mice as early as 2 h (Fig. 5). BP or CP treatment alone did not influence any of these measures in the lungs of TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice (data not shown). These observations further confirm the importance of TIRAP-dependent signaling in the induction of innate lung immune response against LPS and suggest that TIRAP may be a pharmacological target to modify the excessive host response to Gram-negative pathogens.

Effect of TIRAP signaling on E. coli*-induced immune responses in the lung*

We next investigated whether the absence of functional TIRAP influenced lung innate immune responses to an extracellular bacterial pathogen, *E. coli*. We conducted BAL fluid studies at 6 and 24 h following challenge with 10^7 *E. coli*. Total protein and cellular responses in BAL fluid were examined in TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice after *E. coli* challenge. In TIRAP$^{-/-}$ mice, total protein, total white blood cells, and neutrophil accumulation were attenuated at 6 and 24 h as compared with WT mice (Fig. 6, *A–C*). Similarly, KC, MIP-2, TNF-α, and IL-6 were also attenuated in TIRAP$^{-/-}$ mice at 6 and 24 h compared with WT mice (Fig. 6*D*). No significant concentrations of cytokines and chemokines were detected in BAL fluid of saline-challenged mice (data not shown). Furthermore, more leukocytes and edema were observed in the lungs of TIRAP$^{+/+}$ mice, as evidenced by histopathology in a nonquantitative manner (Fig. 6*E*). These findings conclude that TIRAP is a key mediator for viable *E. coli*-induced innate immune responses.

Effect of TIRAP signaling on host defense against E. coli

The importance of TIRAP signaling during Gram-negative bacterial clearance in the lung was determined using TIRAP$^{-/-}$ and WT counterparts. TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice were sacrificed at 6 and 24 h following 10^6 or 10^7 i.t. *E. coli* administration, and their lungs were used for CFU determination. TIRAP$^{-/-}$ mice had more bacterial burden in the lung at the 24-h, but not at the 6-h time point (Fig. 7, *A* and *B*).

Although our findings reflect that neutrophil recruitment to the murine lungs is important for bacterial clearance, as observed with i.t. inoculation of *E. coli*, they may also be explained by impairment of neutrophil bactericidal ability. To address the latter possibility, we performed an in vitro killing assay of *E. coli* by neutrophils. *E. coli* was quantitatively cultured in the presence of neutrophils in vitro, and the CFUs of viable bacteria at 2 h after *E. coli* inoculation were enumerated. As depicted in Fig. 7, *C* and *D*, no significant differences in CFU between neutrophils from TIRAP$^{-/-}$ and TIRAP$^{+/+}$ were observed. These findings demonstrate that TIRAP$^{-/-}$ and TIRAP$^{+/+}$ neutrophils have comparable capacity to kill *E. coli*.

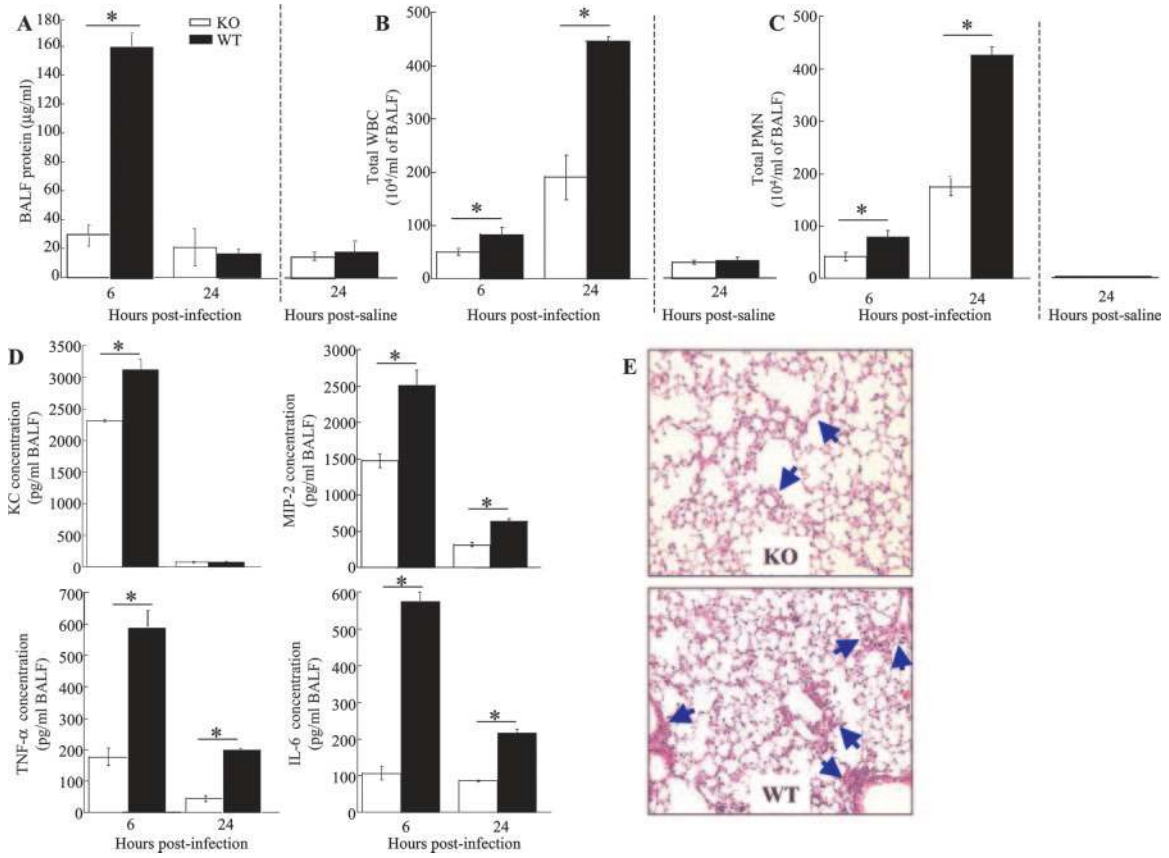

FIGURE 6. Effect of viable *E. coli* (10^7/mouse) on total protein, WBC and neutrophil counts, and cytokine and chemokine expression in TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice at 6 and 24 h postinfection. *A*, Total protein in the airspaces of lungs. *B*, Total WBC counts in the airspaces. *C*, Total neutrophil counts. *D*, Cytokine and chemokine expression. *E*, Histology of lungs at 24 h postinfection, showing neutrophil accumulation in the alveolar spaces, as indicated by the arrow. Original magnification: ×400. *A–D*, $n = 9$ mice from three separate studies in each group at 6 h, and seven mice from three separate experiments in each group at 24 h. Significant differences between KO and WT mice are indicated by asterisks ($p < 0.05$).

To further demonstrate the importance of TIRAP signaling in host defense, mice were administered a lethal dose of *E. coli* (10^9/mouse) i.t. for survival studies because of the following limitations. First, it has been shown (35) that *E. coli* does not actively multiply in murine lungs after i.t. inoculation. Second, *E. coli* at sublethal doses (10^6 and 10^7/mouse) is completely cleared in the

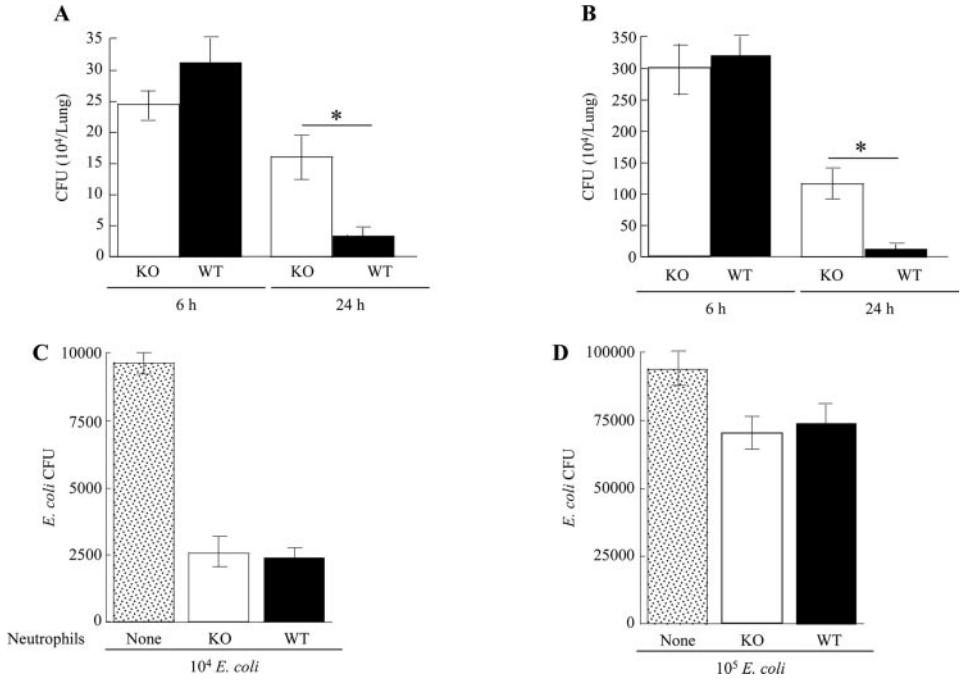

FIGURE 7. Bacterial clearance of *E. coli* after i.t. inoculation. The TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice were i.t. administered with *E. coli* on day 0 at a concentration of 10^6 (*A*) or 10^7/mouse (*B*), and bacterial burden in the lungs was determined at 6 (*A*) and 24 h (*B*) postinfection mice ($n = 6$ mice from three separate studies in each group at 6 h, and seven mice from three separate experiments in each group at 24 h). Neutrophil-killing assay. A total of 10^4 (*C*) or 10^5 (*D*) of *E. coli* was cultured alone (*B*) or cocultured with 10^6 bone marrow-derived PMNs from TIRAP$^{-/-}$ or TIRAP$^{+/+}$ mice. Bacteria were then quantified at 2 h after infection. The data shown represent mean CFU ± SD of six mice from three consecutive experiments. *, Significant differences between TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice ($p < 0.05$).

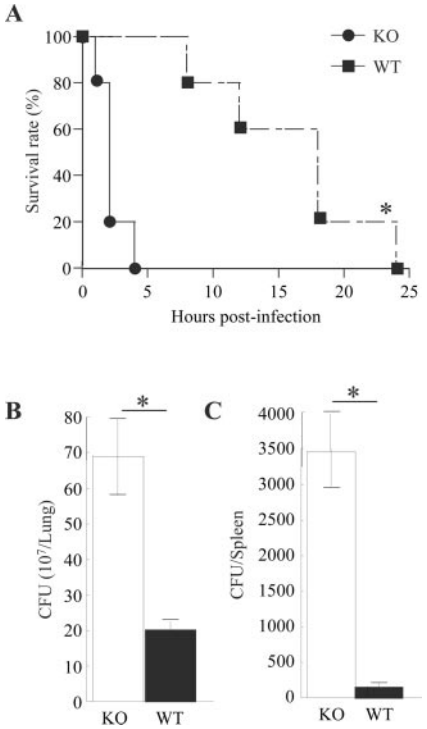

FIGURE 8. *A*, Survival in TIRAP$^{-/-}$ and TIRAP$^{+/+}$ animals following i.t. *E. coli* infection. Animals were i.t. inoculated with a lethal dose of *E. coli* (10^9 CFU/mouse) (*A*) on day 0, and assessed for survival. $n = 14$ mice from two separate experiments in each group. $*$, $p < 0.05$ determined by Wilcoxon Rank Sign test between groups. *B* and *C*, Lung (*B*) and spleen CFU at 3 h postinfection of 10^9 *E. coli*/mouse. A total of seven animals from three independent experiments was used to obtain the lungs and spleens. Significant differences between KO and WT mice were calculated using Student's *t* test and are indicated by asterisks ($p < 0.05$).

murine lungs in 5 days after i.t. inoculation (S. Jeyaseelan and G. Worthen, unpublished data). With the lethal dose, all TIRAP$^{-/-}$ animals died by 4 h, whereas WT mice started dying at 6 h and all died by 24 h (Fig. 8*A*). No mortality was observed with saline-challenged TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice up to 24 h (data not shown). These findings suggest that TIRAP is a critical mediator in the host defense against an extracellular pathogen, *E. coli*. To evaluate the mechanisms by which the absence of functional TIRAP resulted in early mortality in TIRAP$^{-/-}$ mice to a lethal dose of *E. coli*, we examined the bacterial burden in the lungs and *E. coli* dissemination in the spleens of TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice. TIRAP$^{-/-}$ mice had more bacterial burden in the lungs (Fig. 8*B*) and *E. coli* dissemination (Fig. 8*C*). These observations support the conclusion that TIRAP signaling is important for bacterial clearance from the lung and minimizing bacterial dissemination, a mechanism most likely associated with survival of mice.

Effect of TIRAP blocking in human AMs in response to LPS and viable E. coli

Because AMs play a central role in the early host response against bacterial pathogens and/or their products in the human lung (1), we determined the role of TIRAP-dependent signaling in the induction of immune responses using primary human AMs (2×10^6/well) in response to 200 ng of LPS or 2×10^4 *E. coli*. Human AMs were stimulated with LPS or live *E. coli*, in the presence of TIRAP BP or CP, and cytokine and chemokine expressions were measured. LPS-induced cytokine and chemokine responses were abolished by pretreatment with 200 μg of TIRAP BP per well (Fig. 9*A*). Live *E. coli* stimulation of AMs also resulted in expression of IL-8, IL-6, and TNF-α (Fig. 9, *B* and *C*), and these responses were attenuated by the BP (Fig. 9*B*). In contrast, the CP had minimal influence on chemokine and cytokine gene expression in response to *E. coli* LPS or viable *E. coli* (Fig. 9). BP or CP alone did not induce cytokine or chemokine expression in AMs (data not shown). These observations demonstrate that TIRAP is a critical regulator in the expression of immune mediators, including the dominant neutrophil chemoattractant IL-8 in human AMs, in response to *E. coli* LPS and viable *E. coli*.

Discussion

Pulmonary diseases caused by Gram-negative bacterial pathogens are the leading cause of mortality from infectious diseases in the U.S. (42). The difficulties associated with effective treatment of these diseases include increasing numbers of immunocompromised patients, a growing proportion of elderly individuals, and the emergence of antibiotic-resistant bacterial pathogens. Modulation of the host immune response itself is an attractive target for improving clinical outcomes, particularly among patients with dysregulation of the immune system or infected with antibiotic-resistant bacteria. However, understanding the role of innate immune responses in the lung against Gram-negative pathogens and their prototypical products is a prerequisite to designing early therapeutic interventions to minimize the mortality associated with severe lung damage.

TLRs are one of the most important family of cell surface proteins that provide early innate immune responses in the lung to invading pathogens (7, 8). TLRs recognize pathogen-associated molecular patterns such as microbial components, including LPS (7, 8). Although few recent studies have focused on the role of TIRAP in isolated individual cells in vitro (21, 43), none have examined its role in the complex environment of the lung in vivo. The aim of the present investigation was to define the role of TIRAP in pulmonary inflammation and host defense, and thereby clarify its candidacy as a molecular target in modulating the immune response. In Gram-negative bacterial infection, LPS induces a plethora of immune responses (7, 8). Our data indicate a critical role for TIRAP in early lung immune responses to LPS.

Of the mechanisms that support the initial host defense system in the lung against pathogens, the most important one is the successful recruitment of leukocytes from the blood (44). Neutrophil sequestration within capillaries and migration into lung parenchyma and subsequently to the alveolar spaces during early immune responses in the lung are a multistep process that involves neutrophil stiffening, retention in capillaries, firm adhesion, and migration into the alveolus (38). Expression of cell adhesion molecules on the vascular endothelium is the first step in the sequence of events (38). Leukocytes bind to ICAM-1, E-selectin, and VCAM-1 expressed on endothelial cells. In particular, VCAM-1 and ICAM-1 are not constitutively expressed on endothelium (38, 44). Furthermore, VCAM-1 and ICAM-1 up-regulation during the inflammatory process results from induction by LPS and other inflammatory mediators, such as TNF-α (39, 40). Although there are several potential mechanisms explaining impaired influx of neutrophils into the TIRAP$^{-/-}$ lung, recent reports indicate that lack of up-regulation of adhesion molecules on endothelium (39, 40) and impaired neutrophil cytoskeleton assembly (34, 41) are sufficient mechanisms. Our current observations suggest that LPS-induced TIRAP signaling leads to the expression of chemokines and cytokines and subsequent up-regulation of VCAM-1 and ICAM-1 on endothelium, which contributes to neutrophil influx in the lungs in response to LPS. In this context, previous studies have also demonstrated that VCAM-1 (40) and ICAM-1 (45) were up-regulated in the lungs in response to LPS. Quite interestingly,

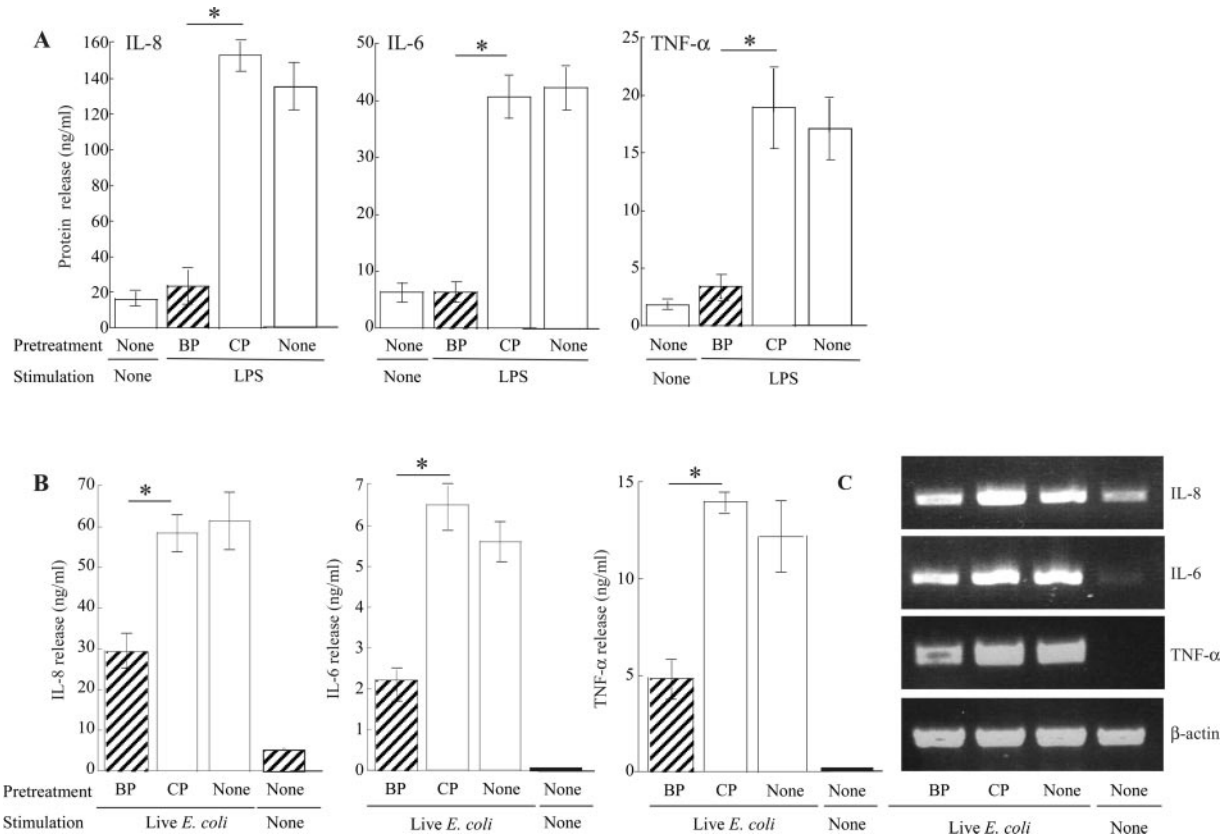

FIGURE 9. TIRAP BP attenuates LPS derived from *E. coli* and live *E. coli*-induced expression of IL-8, IL-6, and TNF-α in human AMs. AMs in each well were pretreated with 200 μg of a cell-permeable TIRAP BP or CP for 2 h before stimulating with 200 ng of LPS (*A*) or live *E. coli* (2×10^4) (*B*) in the presence of peptide. Supernatants were collected 18 h after stimulation, and IL-8, IL-6, and TNF-α release were measured by ELISA. Both peptides were reconstituted with the macrophage medium. Data are mean $\pm$ SD of nine wells of three independent experiments. Significant differences between BP and CP are indicated by asterisks ($p < 0.05$). *C*, Peptide-treated and untreated macrophages were lysed at 2 h poststimulation, total RNA was extracted, and RT-PCR was performed using specific primers, as described in *Materials and Methods*. This is a representative gel of nine wells of three separate experiments with identical results.

TIRAP$^{-/-}$ neutrophils were not defective in actin polymerization in response to chemokines and cytokines, which are required for cell stiffening and retention in capillaries for eventual neutrophil extravasation.

Early immune responses are frequently illustrated as a double-edged sword in which they are important for recruitment of immune cells to deal with the microbial assault; however, an excessive response leads to host injury. A number of pulmonary diseases, notably ALI and ARDS, are caused by excessive immune responses to Gram-negative bacterial products, such as LPS. Therefore, modulation of the immune response in the lung is potentially of great utility. Because our results demonstrate LPS-induced innate immune responses in the lung that appear totally dependent on TIRAP signaling, we examined whether blocking TIRAP is a feasible strategy to minimize immune responses in vivo. Blocking TIRAP via a specific cell-permeable peptide attenuated the lung inflammatory response in healthy C57BL/6 mice (Fig. 5). These observations may present new therapeutic options for TIRAP inhibitors in the treatment of ALI and ARDS. However, the potential for harmful effects, such as bacterial burden when targeting such fundamental cascades of the host defense, needs to be carefully evaluated.

From the therapeutic point of view, due to the identified and unidentified complex adhesion mechanisms associated with neutrophil accumulation in the lung by LPS, blocking an individual adhesion molecule may not be a viable approach to minimize neutrophil sequestration during LPS-mediated inflammation. However, blocking the initial signaling steps possibly at the level of pattern recognition receptor or adaptor molecules could plausibly attenuate the activation of adhesion molecules on endothelium. In this context, our results, using TIRAP$^{-/-}$ mice in response to LPS and TIRAP BP before LPS challenge, demonstrate that targeting upstream signaling, such as TIRAP-mediated cascades, could minimize neutrophil influx into the lungs.

Innate immune responses to intact pathogens are more complex than their individual products, such as LPS, and continue to be a point of attention. Although the present study demonstrates the critical role of TIRAP in the induction of innate immune responses in the lung against *E. coli*, previous studies have demonstrated the importance of cytokine receptors, such as TNFR1, TNFR2, and IL-1R1, in early immune responses in the lung against *E. coli* (46, 47). Our results therefore suggest that attenuated downstream cytokine responses in TIRAP$^{-/-}$ mice may contribute to the reduction in neutrophil accumulation in the lungs in response to *E. coli*.

The observation that lung innate immune responses were diminished, albeit not a complete lack, against viable *E. coli* is intriguing, and suggests the presence of a TIRAP-independent signaling cascade to the intact bacterium. The molecular mechanisms underlying TIRAP-independent signaling remain to be elucidated, but may include TIRAP-independent, but TLR4-dependent signaling cascades via molecules, such as Toll/IL-1R domain-containing adaptor inducing IFN-β and TRIF-related adaptor molecule, as well as TIRAP-independent but TLR4- or 2-independent signaling pathways via another virulence factor of the bacterium. Because *E. coli* is a flagellated bacterium, the most likely TLR to be involved in TIRAP-independent signaling is TLR5, which is known to bind

to bacterial flagellin (48, 49). Future studies are required to explore these possibilities.

In this investigation, TIRAP$^{-/-}$ mice had early mortality as compared with WT mice, demonstrating the important role of TIRAP in induction of the antibacterial defense in the lung against *E. coli*. Because we noticed that TIRAP$^{-/-}$ mice had impaired *E. coli* clearance in the lung to *E. coli* and, most importantly, more bacterial dissemination (Fig. 8), this may be the mechanism for early mortality in TIRAP$^{-/-}$ mice observed following pulmonary *E. coli* infection. Clearance of bacterial pathogens in the lung during infection is mediated primarily via an efficient innate immune response. Although several cell subsets are most likely involved, neutrophils are the most important phagocytic cells for early bacterial clearance in the lung (50). In this context, the present study demonstrates that the recruitment of neutrophils most likely contributes to improved *E. coli* clearance in TIRAP$^{+/+}$ mice (Fig. 8). This conclusion is supported by the fact that neutrophils from TIRAP$^{-/-}$ and TIRAP$^{+/+}$ mice have comparable bactericidal capacity (Fig. 8).

As sentinel cells in the airspace, AMs are the first cells to encounter pathogens and respond to pathogenic assault via producing a spectrum of innate immune mediators. However, there is no previous evidence that TIRAP plays a central role in the expression of immune mediators by human AMs. Therefore, in this investigation, we examined the importance of TIRAP signaling in the induction of early immune responses in human AMs. Our data illustrate that inhibition of TIRAP by a specific BP abolishes LPS-induced cytokine and chemokine responses in human primary AMs in vitro, suggesting a prominent role of macrophage TIRAP in local immune responses in the human lung. In this context, the sole previous study on TIRAP in human primary cultures demonstrated that TIRAP is important for cytokine and chemokine production in nonmyeloid synovial fibroblasts, but not in human peripheral blood-derived macrophages in response to LPS (51). In their investigation (51), the authors used dominant negatives and overexpressors to demonstrate the role of TIRAP. The discrepancy between our findings and the reported observations (51) might be explained by differences in cell types, and usage of different methods to attenuate TIRAP-mediated signaling cascades in cells. Furthermore, we are the first to demonstrate the importance of TIRAP in the expression of important chemokines and cytokines to a pathogen, *E. coli*, in primary human AMs.

In conclusion, our study has produced several key findings: 1) TIRAP is an integral component required for the induction of innate immune responses against *E. coli* LPS and viable *E. coli*; 2) TIRAP is important for host defense against *E. coli* in the lung; and 3) TIRAP-mediated signaling is crucial to induce immune mediators in human pulmonary myeloid cells against *E. coli* LPS and *E. coli*. These findings form the groundwork to investigate the role of TIRAP in the pathogenesis of lung diseases in humans caused by Gram-negative bacteria and their products. Most importantly, these findings suggest that polymorphism in TIRAP may have important functional consequences in the induction of innate immune responses in the human population against bacterial pathogens.

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Disclosures

The authors have no financial conflict of interest.

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