Roles of Tumor Necrosis Factor Receptor Signaling during Murine *Escherichia coli* Pneumonia

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We hypothesized that tumor necrosis factor (TNF)-α signaling is essential to inflammation and host defense during *Escherichia coli* pneumonia. We tested this hypothesis by instilling *E. coli* into the lungs of wild-type (WT) mice and gene-targeted mice that lack both p55 and p75 receptors for TNF-α. The emigration of neutrophils 6 h after instillation of *E. coli* was not decreased, but rather was significantly increased (167% of WT), in TNF receptor (TNFR)–deficient mice. This increased neutrophil emigration did not result from peripheral blood neutrophilia or enhanced neutrophil sequestration, inasmuch as the numbers of neutrophils in the circulating blood and in the pulmonary capillaries did not differ between TNFR-deficient and WT mice. The accumulation of pulmonary edema fluid was not inhibited in TNFR-deficient compared with WT mice. Nuclear factor-κB (NF-κB) translocation in the lungs was not prevented in TNFR-deficient mice. Thus, signaling pathways independent of TNFRs can mediate the acute inflammatory response during *E. coli* pneumonia. However, despite this inflammatory response, bacterial clearance was impaired in TNFR-deficient mice (109 ± 8% versus 51 ± 14% of the original inoculum viable after 6 h in TNFR-deficient and WT mice, respectively). Increased neutrophil emigration during *E. coli* pneumonia in TNFR-deficient mice may thus result from an increased bacterial burden in the lungs. During acute *E. coli* pneumonia, the absence of TNFR signaling compromised bacterial killing, but did not prevent inflammation, as measured by the accumulation of edema fluid and neutrophils. **Mizgerd, J. P., J. J. Peschon, and C. M. Doerschuk. 2000. Roles of tumor necrosis factor receptor signaling during murine *Escherichia coli* pneumonia. Am. J. Respir. Cell Mol. Biol. 22:85–91.**

Tumor necrosis factor (TNF)-α is a pleiotropic early response cytokine (reviewed in Reference 1). In response to inflammatory stimuli such as gram-negative bacteria or bacterial lipopolysaccharide (LPS), TNF-α is rapidly produced. TNF-α binds to two different membrane receptors, TNF receptor (TNFR)1 (also known as p55, p60, or CD120a) and TNFR2 (also known as p75, p80, or CD120b), and induces the transcription of downstream genes that regulate acute inflammation, including the early response cytokine interleukin (IL)-1β, C-X-C chemokines such as KC and macrophage inflammatory protein (MIP)–2, and adhesion molecules such as intercellular adhesion molecule (ICAM)–1, vascular cell adhesion molecule–1, and E-selectin. The transcription of these downstream genes is regulated by nuclear factor (NF)-κB (2–12), which can be activated by either of the TNFRs (13–16).

The pulmonary deposition of gram-negative bacteria or LPS induces the nuclear translocation of NF-κB and the expression of TNF-α (17–20). Several genes that are both induced by TNF-α and regulated by NF-κB are essential to the acute inflammatory response elicited by bacteria or LPS in the lungs, including KC (21), MIP-2 (22, 23), and ICAM-1 (24, 25).

We hypothesized that signaling through the receptors for TNF-α was essential to host defense during acute *Escherichia coli* pneumonia. To test this hypothesis, we compared neutrophil emigration, edema accumulation, NF-κB translocation, and bacterial killing in the lungs of wild-type (WT) mice and gene-targeted mutant mice deficient in both TNFR1 and TNFR2 (TNFR-deficient mice).

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Abbreviations: colony-forming units, CFU; interleukin, IL; lipopolysaccharide, LPS; nuclear factor, NF; red blood cell, RBC; tumor necrosis factor, TNF; TNF receptor, TNFR; wild-type, WT.

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Materials and Methods

Protocol 1: Acute Inflammation during *E. coli* Pneumonia

The inflammatory response to *E. coli* in the alveolar air spaces was compared in TNFR-deficient mice (26) and WT mice of similar random hybrid genetic background (C57BL/6 x 129). Mice were maintained in a full barrier facility until experimental use. Neutrophil emigration was

studied 6 h and 24 h after bacterial instillation. At the 6-h time-point, emigration was detectable but not yet maximal, facilitating the study of early responses and the initiation of pulmonary inflammation. Previous studies have examined the roles of inflammatory mediators relevant to the pulmonary emigration of neutrophils at this time point (27–33). After mice were anesthetized by intramuscular injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg), ^{125}I-labeled human albumin (Mallinckrodt Medical; Hazelwood, MO) was injected intravenously as a marker for plasma content. The trachea was surgically exposed, and 2.3 ml/kg of *E. coli* suspended to approximately 10^8 colony-forming units (CFU)/ml in 0.9% saline with 5% colloidal carbon (to mark the site of deposition) were delivered by intratracheal instillation 15 min after the injection of ^{125}I-albumin. After 5 h and 58 min, mice received intravenous injections of ^{51}Cr-labeled murine red blood cells (RBCs) as a marker for blood content. Mice were killed 6 h after bacterial instillation by inhalation of a lethal overdose of halothane. The hearts were tied off to maintain pulmonary blood, and peripheral blood samples were collected from the inferior vena cava. Lungs were removed and fixed by intratacheal instillation of 6% glutaraldehyde at a pressure of 23 cm H_2O.

Circulating neutrophils were quantified in peripheral blood samples. After RBC lysis, leukocytes were enumerated using a hemocytometer, and differential counts were obtained from blood smears stained with LeukoStat (Fisher Scientific, Pittsburgh, PA).

Emigrated and sequestered neutrophils were quantified by morphometry in histologic sections (34). Colloidal carbon–containing lung regions were embedded in paraffin, and 5-μm-thick sections were cut and stained with hematoxylin and eosin. A counting grid (10×10, covering 70,000 μm^2 of the magnified field) was reflected onto the field of view using a drawing tube, and pneumonic regions of peripheral lung that were largely free of noncapillary blood vessels and bronchioles or larger airways were examined. Three grids (300 points) were counted for each lung, and each point was classified as landing on (*1*) air space or tissue and (*2*) on a neutrophil or non-neutrophil. The quantities of neutrophils in alveolar air space or septal tissue were expressed as volume percentages of the respective compartments.

Pulmonary edema, as measured by the vascular leakage of radiolabeled albumin, was quantified before dissection of the lungs for morphometry, as previously described (34). In short, the specific activities of [^{125}I]albumin and ^{51}Cr-RBCs were measured for blood and plasma samples and for excised, fixed lungs from each mouse. The hematocrit was calculated from the [^{125}I]albumin activities in the blood and plasma samples. Pulmonary blood volume was calculated from the ^{51}Cr-RBC activity in the blood sample and the lungs. The total volume of plasma equivalents in the lungs was calculated from the [^{125}I]albumin activities in the plasma sample and the lungs. The volume of intravascular plasma in the lungs was derived from the hematocrit and the pulmonary blood volume. The volume of extravascular plasma equivalents in the lungs was calculated as the difference between the total volume of plasma equivalents and the volume of intravascular plasma. Pulmonary edema was expressed as microliters of extravascular plasma equivalents per lung.

Protocol 2: NF-κB Translocation during *E. coli* Pneumonia

WT and TNFR-deficient mice were anesthetized as described earlier, and 2.3 ml/kg of *E. coli* suspended to approximately 10^8 CFU/ml with 5% colloidal carbon were delivered by intratracheal instillation. After 3 h, mice were killed by halothane overdose. Their lungs were excised, snap-frozen in liquid nitrogen, and stored at −80°C until nuclear protein extraction. Nuclear proteins were collected from colloidal carbon–containing lung lobes from mice instilled with *E. coli*, as well as from lungs of mice that did not receive instillation. Protein concentrations were measured using a bicinchonic acid assay with bovine serum albumin as the standard. Equal concentrations (0.5 mg/ml) of nuclear protein were incubated with 3.5 nM γ[^{32}P]adenosine triphosphate–labeled NF-κB consensus oligonucleotide (Promega, Madison, WI). Protein-oligonucleotide complexes were isolated by polyacrylamide gel electrophoresis, detected by autoradiography, and quantitated by densitometry using Scion ImagePC software (Scion, Frederick, MD).

Protocol 3: Bacterial Killing during *E. coli* Pneumonia

WT and TNFR-deficient mice were anesthetized as previously described, and 50 μl of *E. coli* suspended in saline as described earlier were instilled intratracheally. Subsequent plating demonstrated that the original inoculum instilled was 2.8×10^7 CFU. After 6 h, mice were killed by cervical dislocation, blood samples were collected from the inferior vena cava, and excised lungs were homogenized in cold sterile saline. Lung homogenates and blood samples were serially diluted and plated, and CFU were enumerated after overnight incubation at 37°C. Viable bacteria recovered from the lungs were expressed as a percentage of the original inoculum instilled.

Statistics

Data were presented as means ± standard error. WT and TNFR-deficient mice were compared by *t* test, and differences were considered significant when $P < 0.05$.

Results

Protocol 1: Acute Inflammation during *E. coli* Pneumonia

Histologic evidence of acute pneumonia, including emigrated neutrophils in the alveolar air spaces, was apparent 6 h after the intratracheal instillation of *E. coli* in both WT and TNFR-deficient mice (Figure 1). There were significantly more emigrated neutrophils in the alveolar air spaces of TNFR-deficient mice than in WT mice (Figure 2A). This increase in emigration did not result from peripheral blood neutrophilia, inasmuch as the numbers of circulating neutrophils did not differ between WT and TNFR-deficient mice either in the presence (Figure 2B) or absence ($0.8 \pm 0.1 \times 10^6$ and $0.8 \pm 0.2 \times 10^6$ neutrophils/ml in WT and TNFR-deficient mice, respectively; $n = 5$ mice/genotype) of *E. coli* pneumonia.

Neutrophil sequestration in the pulmonary capillaries was not compromised by TNFR deficiency. The quantity of neutrophils in the alveolar septae of WT and TNFR-deficient mice during *E. coli* pneumonia did not differ significantly (16 ± 2% and 20 ± 4% of alveolar septal volume, respectively; $n = 4$ mice/genotype).

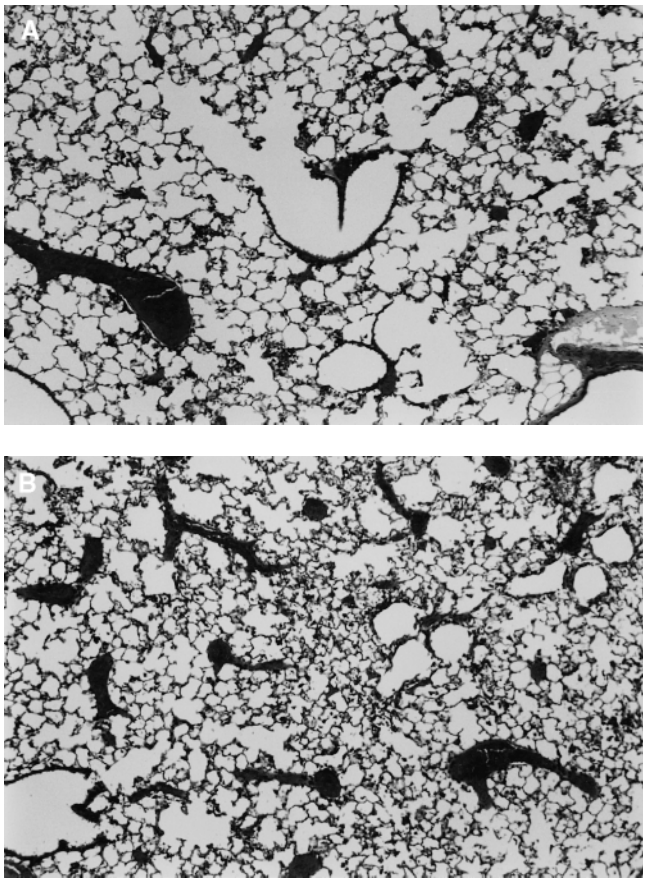

Figure 1. Histology of the lungs 6 h after the intratracheal instillation of *E. coli* to (*A*) WT and (*B*) TNFR-deficient mice. Emigrated neutrophils were apparent within the alveolar air spaces of parenchymal regions that received the instillate, as indicated by colloidal carbon within alveolar macrophages. Original magnification: ×100.

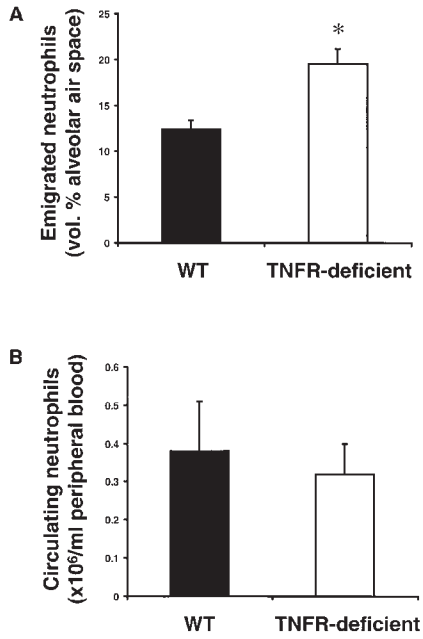

Figure 2. Neutrophils in WT and TNFR-deficient mice 6 h after intratracheal instillation of *E. coli.* (*A*) Neutrophil emigration. Emigrated neutrophils in the alveolar air spaces were quantitated using morphometric analysis and histologic sections. Data were pooled from two separate experiments involving a total of four mice in each group. *Asterisk* represents significant difference compared with value from WT mice. (*B*) Circulating neutrophils. Neutrophils were quantitated in peripheral blood samples collected from the inferior vena cava. Data were pooled from two separate experiments involving a total of four mice in each group. Values from WT and TNFR-deficient mice did not differ significantly.

The accumulation of extravascular albumin in the uninfected lungs of WT and TNFR-deficient mice did not differ significantly (33 ± 4 and 35 ± 3 μl per lung, respectively, over 6 h; $n = 5$ mice/genotype). The intratracheal instillation of *E. coli* resulted in increased extravasation of albumin, consistent with vascular leakage and pulmonary edema. The volumes of extravascular plasma equivalents accumulated within 6 h after instillation of *E. coli* did not differ between WT and TNFR-deficient mice (Figure 3).

An experiment was designed to compare neutrophil emigration at later time points. Five mice of each genotype received intratracheal instillations of *E. coli* as previously described. After 6 h, all mice were alive. However, by 24 h, four of the five TNFR-deficient mice had died. None of the five WT mice were dead after 24 h. Thus, TNFR deficiency resulted in increased mortality during *E. coli* pneumonia.

Protocol 2: NF-κB Translocation during *E. coli* Pneumonia

E. coli induced the nuclear translocation of NF-κB proteins in the lungs of WT mice (Figure 4). Similarly, NF-κB translocation was induced by *E. coli* in the lungs of TNFR-deficient mice (Figure 4). There were no significant differences in the quantities of NF-κB proteins in the nuclear fractions from lungs of WT and TNFR-deficient mice with or without *E. coli* pneumonia (Figure 4).

Protocol 3: Bacterial Killing during *E. coli* Pneumonia

By 6 h, WT mice demonstrated signs of effective killing of intrapulmonary bacteria, inasmuch as approximately half of the original inoculum was no longer viable (Figure 5). In contrast, over 100% of the original inoculum remained viable in the TNFR-deficient mice at this time point (Figure 5), significantly more than in WT mice. No bacterial CFU were recovered in 10-μl samples of venous blood from the five TNFR-deficient mice or from four of the five WT mice (two CFU of undetermined species were observed in a 10-μl blood sample from one WT mouse). Thus, the increased bacterial burden in the lungs of TNFR-deficient mice did not result in detectable bacteremia at this time.

Discussion

Bacterial clearance during *E. coli* pneumonia was significantly compromised and mortality was increased in TNFR-deficient mice compared with WT mice, suggesting that TNFR signaling is essential to effective pulmonary host defenses. Supporting this concept, soluble inhibitors of TNF-α compromise the killing of *Legionella*, *Streptococ-*

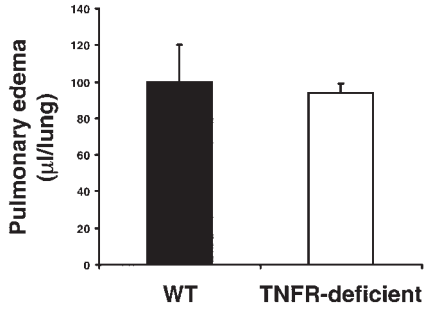

Figure 3. Accumulation of pulmonary edema fluid in WT and TNFR-deficient mice 6 h after intratracheal instillation of *E. coli*. Edema fluid was quantitated as volume of extravascular plasma equivalents per lung using [^{125}I]albumin and [^{51}Cr]RBC as radiotracers. Data were pooled from two separate experiments involving a total of four mice in each group. Values from WT and TNFR-deficient mice did not differ significantly.

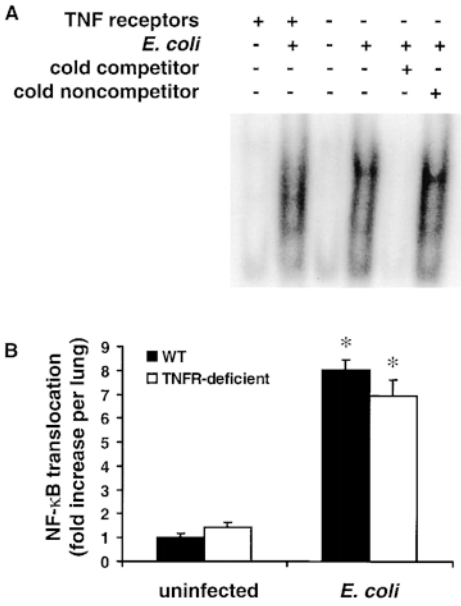

Figure 4. NF-κB translocation in WT and TNFR-deficient mice 3 h after intratracheal instillation of *E. coli*. NF-κB proteins in nuclear fractions from whole lungs were identified using electrophoretic mobility shift assays and quantitated by densitometric analysis. (*A*) Autoradiographic image depicting the region of the gel to which NF-κB proteins shift radiolabeled oligonucleotides containing NF-κB binding sequences. Each of the *first four lanes* shows NF-κB proteins isolated from single mice, with genotype and infection status indicated at the *top*. The *last two lanes* contain the same nuclear proteins and oligonucleotides as the fourth lane, but with excess cold competitor (nonradioactive oligonucleotides containing NF-κB binding sequences) or cold noncompetitor (nonradioactive oligonucleotides containing Sp1 binding sequences) in addition, as indicated. *E. coli* consistently induced the nuclear translocation of NF-κB proteins in both WT and TNFR-deficient mice; banding patterns did not differ consistently between genotypes. (*B*) Relative densitometric values of NF-κB binding activity in the nuclear fractions isolated from whole lungs. Data were pooled from two separate experiments involving a total of eight mice in the uninfected WT group and four mice in each of the other three groups. *Asterisks* represent significant effect of *E. coli* instillation. Values from WT and TNFR-deficient mice did not differ significantly.

cus, *Pseudomonas*, or *Klebsiella* in the lungs (35–38). Further, mutant mice deficient in TNFR1 are compromised in bacterial killing during systemic infections with *Listeria* or *Mycobacteria* (39–41). In contrast, mice deficient in either TNFR1 alone or in both TNFR1 and TNFR2 demonstrate enhanced, rather than reduced, elimination of aerosolized *Pseudomonas aeruginosa* from the lungs (42). Thus, during *E. coli* pneumonia as well as many (but not all) other settings of bacterial infection, TNF-α signaling is essential to effective antibacterial host defense.

Killing of gram-negative bacteria in the pulmonary air spaces requires both alveolar macrophages (43, 44) and neutrophils (45, 46). The deficiency of TNFRs may compromise the microbicidal functions of either or both cell types. Previous studies have demonstrated that TNF-α enhances the microbicidal functions of neutrophils *in vitro*. TNF-α increases the expression of opsonin receptors and pattern recognition receptors by neutrophils, including receptors for complement fragments (47), immunoglobulin (Ig)A (48), and formylmethionyl leucylphenylalanine (49), potentially augmenting the responses of these cells to microbes. Indeed, TNF-α stimulates increased phagocytosis of opsonized targets (48, 50) and increased elaboration of reactive oxygen intermediates in response to particulate or soluble stimuli (48, 49). Perhaps as a result of these combined effects, TNF-α increases the efficiency of bacterial killing by neutrophils *in vitro* (47, 48, 51). Macrophage microbicidal functions are also regulated by TNF-α (52–55). The present results suggest that TNFR-mediated stimulation of neutrophil and macrophage antibacterial activities is critical to acute host defenses during *E. coli* pneumonia.

In contrast to bacterial clearance, the acute inflammatory response was not compromised in TNFR-deficient mice compared with WT mice during *E. coli* pneumonia. Neutrophil emigration was actually increased in TNFR-deficient mice, to 167% of WT emigration. Pulmonary edema, as measured by extravasated albumin, was not affected by TNFR deficiency. Neutrophil sequestration in the alveolar septae was not decreased in TNFR-deficient mice compared with WT. NF-κB translocation in the lungs did not differ between the groups.

It is not clear from prior studies if and when TNF-α signaling may be required for neutrophil emigration during pneumonia. Soluble inhibitors of TNF-α do not affect neutrophil accumulation during 40 h of streptococcal pneumonia (38) or during 5 h of pneumonia induced by instillation of IL-1α (56). Soluble inhibitors of TNF-α do compromise neutrophil emigration during 4 h of IgG immune complex pneumonia (57) or during 48 (but not 24) h of *Klebsiella pneumoniae* pneumonia (37). Similarly, soluble inhibitors compromise emigration during 3 or 6 h of LPS pneumonia (27, 35), although different inhibitors provide different results (58) and the effects on neutrophil emigration observed at 6 h were not observed at 2, 4, or 12 h (27). TNFR1 deficiency does not compromise neutrophil emigration 24 h after intranasal insufflation of 100 μg LPS (26), but it does compromise emigration 24 h after pre-

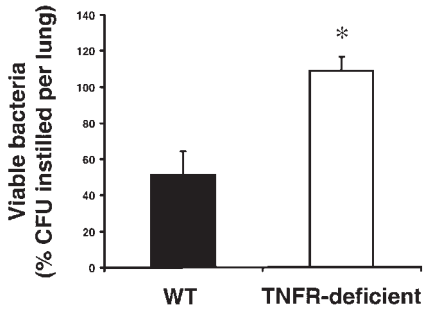

Figure 5. Antibacterial defenses in WT and TNFR-deficient mice 6 h after intratracheal instillation of *E. coli*. Viable bacteria were quantitated by enumerating viable CFU from homogenized lungs. Data were collected from a single experiment involving a total of five mice in each group. *Asterisk* represents significant difference compared with value from WT mice.

sumably lower doses of LPS were delivered to the lungs by aerosolization (42). Neutrophil emigration in response to aerosolized *P. aeruginosa* is not compromised by deficiency of TNFR1 alone or by the combined deficiency of TNFR1 and TNFR2, and in the former instance emigration is increased compared with WT (42). The previous results suggest that TNF-α may be essential to neutrophil emigration under a very limited set of as-yet-undefined circumstances. However, the present results indicate that neutrophil emigration during *E. coli* pneumonia can proceed in the absence of TNF-α signaling.

The observed increase in neutrophils within the alveolar air spaces of TNFR-deficient mice did not result from peripheral blood neutrophilia or an enhanced sequestration of neutrophils within the pulmonary capillaries. This increase may reflect increased neutrophil emigration, inasmuch as defective bacterial clearance due to the TNFR deficiency resulted in increased bacterial burdens in the lungs and likely increased stimulation for emigration. In addition, this increase in pulmonary neutrophils may reflect a decreased loss of emigrated neutrophils due to apoptosis, inasmuch as TNF-α hastens the progress of a subset of neutrophils to apoptosis (59). It is unclear how much such an effect of TNF-α on neutrophil apoptosis would contribute to increased neutrophil numbers in the lungs within only 6 h after bacterial instillation.

The present results suggest that NF-κB translocation occurs to a similar degree in the absence of TNFR signaling during *E. coli* pneumonia. TNF-α induces NF-κB translocation in many cell types, including macrophages, endothelial cells, epithelial cells, and fibroblasts (*see* References 60 and 61 for review), all of which were represented in the nuclear fractions from pneumonic lungs. It is impossible to determine from these studies whether specific cell types, such as pulmonary capillary endothelial cells, may have been selectively compromised in the translocation of NF-κB. However, it is clear that NF-κB translocation occurs in the absence of TNFR signaling during *E. coli* pneumonia in TNFR-deficient mice. NF-κB translocation may be elicited by *E. coli* products such as LPS, and by *E. coli*–induced proinflammatory factors other than TNF-α, such as IL-1 (62, 63), leukotriene B_4 (64), or H_2O_2 (65). Although the present data clearly demonstrate that proinflammatory signaling independent of the TNFRs can mediate the acute inflammatory response during bacterial pneumonia, it remains possible that mutant mice with lifelong deficiencies of the TNF-α signaling pathway may employ these alternative signaling pathways more readily than do nonmutant animals.

In conclusion, TNFR signaling is essential to efficient bacterial killing during *E. coli* pneumonia, but it is not required for neutrophil emigration during *E. coli* pneumonia in TNFR-deficient mice. Pulmonary edema accumulation and the nuclear translocation of the transcription factor NF-κB were not compromised by TNFR deficiency. These data suggest that the interruption of TNFR signaling compromises antibacterial host defense but does not decrease acute pulmonary inflammation during *E. coli* pneumonia in mice.

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