Roles for early response cytokines during *Escherichia coli* pneumonia revealed by mice with combined deficiencies of all signaling receptors for TNF and IL-1


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Mizgerd, Joseph P., Michal M. Lupa, Josephine Hjoberg, Joseph C. Vallone, Henry B. Warren, James P. Butler, and Eric S. Silverman. Roles for early response cytokines during *Escherichia coli* pneumonia revealed by mice with combined deficiencies of all signaling receptors for TNF and IL-1. Am J Physiol Lung Cell Mol Physiol 286: L1302–L1310, 2004. First published February 13, 2004; 10.1152/ajplung.00353.2003.—During infection, inflammation is essential for host defense, but it can injure tissues and compromise organ function. TNF-α and IL-1 (α and β) are early response cytokines that facilitate inflammation. To determine the roles of these cytokines with overlapping functions, we generated mice deficient in all of the three receptors mediating their effects (TNFR1, TNFR2, and IL-1RI). During *Escherichia coli* pneumonia, receptor deficiency decreased neutrophil recruitment and edema accumulation to half of the levels observed in wild-type mice. Thus these receptors contributed to maximal responses, but substantial inflammation progressed independently of them. Receptor deficiency compromised antibacterial efficacy for some infectious doses. Decreased ventilation during *E. coli* pneumonia was not affected by receptor deficiency. However, the loss of lung compliance during pneumonia was substantially attenuated by receptor deficiency. Thus during *E. coli* pneumonia in mice, the lack of signaling from TNF-α and IL-1 decreases inflammation and preserves lung compliance.

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TUMOR NECROSIS FACTOR-α (TNF-α) and interleukin-1 (IL-1α and IL-1β) are rapidly induced by receptors for microbial products. Every nucleated cell can express these cytokines, and all cells other than erythrocytes can respond to them. In vitro experiments, these cytokines induce gene expression (including chemokines and adhesion molecules) and increase permeability across microvascular endothelium (53, 66, 68). In vivo, the instillation or transgenic expression of these cytokines causes inflammation, including neutrophil accumulation and plasma extravasation (11, 17, 22, 23, 59). Thus these “early response cytokines” are characterized by rapid induction from diverse stimuli, widespread sites of synthesis and of action, and proinflammatory effects.

The overlapping patterns of gene expression and common inflammatory responses of TNF-α and IL-1 are mediated by interactions of these cytokines with distinct receptors. TNF-α binds TNFR1 and TNFR2, each of which induces intracellular signaling and gene expression (24, 64). IL-1α and IL-1β each bind IL-1RI, which induces intracellular signaling and gene expression, and IL-1RII, which functions as a decoy receptor incapable of signaling or gene induction (37, 56). Thus signaling induced by these cytokines is mediated by three receptors: TNFR1, TNFR2, and IL-1RI. A point of convergence for signaling pathways elicited by these cytokine receptors is the transcription factor NF-κB. Each of these three receptors induces IκB degradation, nuclear translocation of NF-κB, and transcription of κB-associated genes (24, 37, 64).

During infection, microbes and microbial products signal through pattern recognition receptors and induce expression of early response cytokines (7, 26, 29). In *Escherichia coli* pneumonia, acute inflammation (as measured by neutrophil recruitment and edema accumulation) is not inhibited by the interruption of TNF-α signaling via gene-targeted deficiency of both TNFR1 and TNFR2 (30). Similarly, neutrophil recruitment and edema accumulation are not inhibited by the loss of IL-1α and IL-1β signaling via the deficiency of IL-1RI (31). Thus none of these early response cytokine pathways is individually essential to the inflammatory responses to *E. coli* in the lungs. In contrast, both neutrophil recruitment and edema accumulation are decreased (but not eliminated) by the combined deficiency of TNFR1 and IL-1RI (31). These data indicate that inflammatory responses to *E. coli* in the lungs are mediated in part by early response cytokines, with TNFR1 or IL-1RI sufficient to compensate for the absence of the other receptor.

The studies with mice deficient in TNFR1 and IL-1RI (31) did not examine the functional significance of inflammation mediated by TNF-α and IL-1 during pneumonia. During infection, inflammatory responses can be beneficial (facilitating microbicidal activities) and/or detrimental (compromising organ functions). Furthermore, because TNFR2 remained uninterrupted in the studies of TNFR1/IL-1RI double mutant mice (31), these studies could not determine the net contributions of TNF-α and IL-1 signaling to the inflammatory responses to pulmonary infection with *E. coli*. TNF-α can use TNFR2 to activate macrophages (33) and endothelial cells (38). Transgene delivery of TNFR2 to mice can exacerbate inflammation and inflammatory pathology (3, 9, 18). The targeted mutation of TNFR2 decreases vascular permeability induced by TNF-α (10), host defenses against infection (46), and pathological responses to infection and inflammation (18, 40, 52, 54, 60). Thus to assess the significance of TNF-α and IL-1 signaling, TNFR2 signaling must be interrupted in addition to TNFR1

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and IL-1RI. Toward this goal, we generated triple mutant (TM) mice deficient in all three signaling receptors (TNFR1, TNFR2, and IL-1RI).

MATERIALS AND METHODS

Mice. Mice with targeted mutation of genes for TNFR1 and TNFR2 (39) were mated with mice with targeted mutation of genes for TNFR2 and IL-1RI (39). The progeny (F1) were bred together, and their progeny (F2) were genotyped by PCR amplification of DNA recovered from tail biopsies, to identify mice homozygous mutant for all three receptors. TM and wild-type (WT) mice of similar random hybrid genetic background (50–75% C57BL/6 and 25–50% 129/Sv genomes, predicted) were bred with mice of the same genotype and maintained under specific pathogen-free conditions in a full barrier facility. Consistent phenotypic differences between groups of WT and TM mice were attributed to the consistent genetic differences between groups resulting in the presence or absence of the three cytokine receptors. Mice were 6–8 wk of age at the time of experiments. All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals.

Histopathology. Excised lungs were fixed by intratracheal instillation of 10% formalin at 23 cmH₂O pressure. Brain, heart, liver, kidney, spleen, pancreas, thymus, skeletal muscle, salivary gland, esophagus, stomach, small intestine, cecum, large intestine, bone marrow (sternebrae), and nares were fixed by immersion in 10% formalin. Tissues were embedded in paraffin, and 8-μm sections were stained with hematoxylin and eosin and examined under light microscopy.

Intratracheal instillations. After anesthesia with ketamine hydrochloride (100 mg/kg im) and acepromazine maleate (5 mg/kg im), the trachea was surgically exposed, and a total volume of 50 μl was instilled via angiocatheter through the trachea to the left bronchus (31). Rcombinant murine TNF-α or IL-1β (R&D Systems, Minneapolis, MN) were instilled at 100 ng/mouse; vehicle was 1% bovine serum albumin in sterile saline. E. coli LPS (serotype 055:B5 from Sigma Chemical, St. Louis, MO) was instilled intratracheally at 100 μg/mouse. E. coli (strain 19138 from American Type Culture Collection, Manassas, VA) was instilled intratracheally at 10⁶ colony-forming units (CFU)/mouse, unless otherwise indicated (in bacterial clearance studies); concentration was determined by optical density and subsequent colony counting. Instillates contained colloidal carbon (1%) to mark deposition. At the end of experiments, mice were killed by inhalation of an overdose of halothane or by injection of an overdose of pentobarbital sodium.

NF-κB translocation. NF-κB translocation was measured in the lungs of mice by electrophoretic mobility shift assays, as previously described (30, 31). Lung lobes were excised 1 h after intratracheal instillation of vehicle or recombinant cytokine and snap-frozen in liquid nitrogen. Nuclear proteins were collected from frozen lung samples, and protein concentrations were measured by a bichinonic acid assay with bovine serum albumin as the standard. Nuclear proteins were incubated at 0.5 mg/ml with 3.5 nM [γ-32P]ATP-labeled NF-κB consensus oligonucleotide (Promega, Madison, WI). Protein-oligonucleotide complexes were separated from protein-free oligonucleotides by polyacrylamide gel electrophoresis and were detected by autoradiography.

Neutrophil recruitment. Mice were killed by halothane overdose 24 h after intratracheal instillation of LPS or E. coli. Peripheral blood samples were collected from the inferior vena cava. After erythrocyte lysis, leukocytes in peripheral blood samples were counted with a hemacytometer, and differential distributions were assessed in blood smears stained with Diff-Quick (Dade-Behring, Newark, DE). Lungs were excised and fixed by intratracheal instillation of 6% glutaraldehyde at a pressure of 23 cmH₂O. Emigrated neutrophils were quantified by morphometric analyses of histological lung sections (30, 31).

Investigators were blinded to the genotypes of the mice during morphometric examination.

Pulmonary edema. Plasma extravasation was quantified with radiotracers (30, 31). Anesthetized mice received intravenous injections of 125I-labeled human albumin (Malinckrodt Medical, Hazelwood, MO) 15 min before intratracheal instillations and of 51Cr-labeled murine erythrocytes 2 min before death, as markers for plasma and blood content, respectively. Mice were killed by halothane overdose 6 h after the intratracheal instillation of E. coli. The total volume of plasma equivalents in the lungs was derived from the 125I-albumin activity in the lungs and plasma. The volume of intravascular plasma in the lungs was calculated from the hematocrit (derived from the 125I-albumin activity in the blood and plasma) and the pulmonary blood volume (derived from the 51Cr-red blood cell activity in the lungs and blood). The volume of extravascular plasma equivalents in the lungs was calculated as the total volume of plasma equivalents minus the volume of intravascular plasma.

Infectious burden. Viable bacteria were quantified by colony counting (30). Serial dilutions from lungs collected 6, 24, or 48 h after infection were homogenized in sterile water and plated on blood agar. CFU were enumerated after incubation at 37°C.

Ventilation. Ventilation was assessed with a whole body plethysmograph designed for small rodents (Buxco Electronics, Troy, NY) before and 24 h after intratracheal instillation of E. coli. CO₂ accumulation was prevented by constant bias flow through the system. Unanesthetized and unrestrained mice were placed in plethysmograph chambers and allowed to equilibrate to the environment for several minutes. Pressure fluctuations were measured in plethysmograph reference chambers and converted to ventilatory parameters using Buxco software. Ventilation was measured continuously on a breath-by-breath basis, and average values for each parameter were calculated over a 10-min period for every mouse.

Pulmonary mechanics. Pulmonary mechanics after 24 h of E. coli pneumonia were assessed with a module 1 flexiVent rodent ventilator (SCIREQ; Montreal, PQ, Canada). After a lethal overdose of pentobarbital anesthesia, a 20-gauge tracheostomy tube was placed, a median sternotomy was performed, and the right main bronchus was ligated. The isolated left lung was ventilated with a tidal volume of 0.3 ml, a frequency of 2.5 Hz, and a positive end-expiratory pressure of 3 cmH₂O. Dynamic compliance was measured with a single-compartment linear model (45) during the delivery of a 0.3-ml sinusoidal wave form lasting 1 s. Quasistatic pressure-volume relationships over a 0.3-ml volume were measured using a 16-s, 7 step-up, 7 step-down volume signal. Quasistatic compliance was calculated post hoc as the slope between the points of stasis immediately greater than and less than 5 cmH₂O on the descending arm of the pressure-volume loop. Data were collected from the fourth set of identical maneuvers on each mouse to ensure similar volume history and stable measurements.

Statistics. Expected and observed frequencies were compared by the χ² test for independence. Normal distributions were tested with the Shapiro-Wilk W-test. Normal data were expressed as means ± SE. Data from groups of mice were compared by Student’s t-test, Mann-Whitney U-test, or ANOVA with Newman-Keuls post hoc test. Groups were considered significantly different when P < 0.05.

RESULTS

Generation of TM mice unresponsive to TNF or IL-1. The murine genes for TNFR1 (Tnfrsf1a), TNFR2 (Tnfrsf1b), and IL-1RI (Il1r1) reside on different chromosomes (6, 4, and 1, respectively). Assuming independent assortment of chromosomes, we endeavored to derive mice with mutations in all three genes by use of a selective breeding strategy. Matings of TNFR1/TNFR2 and TNFR2/IL-1RI mice produced F1 progeny of the expected genotype, heterozygous for both TNFR1
and IL-1RI and homozygous mutant for TNFR2 (Fig. 1A). Interbreeding the F1 progeny resulted in births of all nine possible genotypes (data not shown), including TM mice with homozygous mutations in all three genes (Fig. 1A). Genotypes of 121 progeny born to parents heterozygous for at least one of these three genes demonstrated that TM mice were born in the frequency predicted by Hardy-Weinberg equilibrium (data not shown). Thus the combined deficiency of these receptors did not confer a selective disadvantage during gestation. At 6 wk of age, TM mice demonstrated no gross evidence of disease, and the histology of lung and other tissues examined was unremarkable (data not shown). TM mice were fertile, and populations were maintained by breeding male and female TM mice.

To determine whether mutation of these receptors ablated responses to TNF and IL-1 in the lungs, recombinant murine cytokines were instilled intratracheally to WT and TM mice. TNF-α and IL-1β each induced the nuclear translocation of NF-κB in the lungs of WT mice (Fig. 1B). In contrast, neither of these cytokines induced the nuclear translocation of NF-κB in the lungs of TM mice (Fig. 1B). Thus TM mice were unresponsive to these cytokines in the lungs.

Acute inflammation in the lungs of mice unresponsive to TNF or IL-1. Before intratracheal instillations, TM mice demonstrated no differences in the numbers of neutrophils (Fig. 2A) or total leukocytes (data not shown) circulating in the peripheral blood. No neutrophils were observed in the alveolar air spaces of either WT or TM mice that did not receive instillations (Fig. 2B). The volume of extravascular plasma in the lungs did not differ in WT and TM mice without experimental pneumonias (Fig. 3). Thus baseline values for neutrophil recruitment in the lungs of WT and TM mice (Fig. 2C) were not affected by triple receptor deficiency.

The intratracheal instillation of E. coli LPS induced neutrophil recruitment in the lungs of WT and TM mice (Fig. 2C). The numbers of neutrophils in the venous blood being delivered to the lungs did not differ between WT and TM mice (Fig. 2A). In contrast, there were significantly fewer neutrophils in the alveolar air spaces of TM mice than in WT mice (Fig. 2, B and C). Thus, although neutrophil delivery to the lungs did not differ in WT and TM mice without experimental pneumonias (Fig. 3).
differ between genotypes, neutrophil emigration in the lungs was compromised by the triple receptor deficiency.

To determine whether neutrophil emigration elicited by a more complex bacterial stimulus than LPS was dependent on TNF and IL-1 receptors, living E. coli were instilled intratracheally to WT and TM mice. Similar to LPS, emigrated neutrophils were reduced but not eliminated by the absence of TNF and IL-1 receptors (Fig. 2B). The numbers of neutrophils being delivered to the lungs in venous blood did not differ between WT and TM mice (Fig. 2A). We quantified neutrophils in the alveolar septae by morphometry. E. coli pneumonia caused significant neutrophil sequestration in both WT (0.069 ± 0.007 and 0.004 ± 0.001 volume fractions of the lung, with and without pneumonia; P < 0.05) and TM mice (0.058 ± 0.007 and 0.002 ± 0.001 volume fractions of the lung, with and without pneumonia; P < 0.05). There were no significant effects of genotype on neutrophils in the septae, with or without pneumonia. Decreased numbers of neutrophils in the alveolar air spaces, but not in the venous blood or alveolar septae, indicate that neutrophil migration from the pulmonary capillaries to the alveolar air spaces was compromised by receptor deficiency. Receptors for TNF-α and IL-1 contributed approximately half of the neutrophil recruitment elicited by either E. coli LPS or living E. coli in the lungs.

In addition to neutrophil recruitment, the inflammatory response to bacteria in the lungs includes plasma extravasation, resulting in pulmonary edema. E. coli increased the accumulation of extravascular plasma in both WT and TM mice (Fig. 3). However, significantly less extravascular plasma accumulated in the lungs of TM mice with E. coli pneumonia compared with WT (Fig. 3). Thus maximal edema accumulation required signaling from these receptors. These studies demonstrate that TNF and IL-1 receptors make important contributions to inflammation elicited by intrapulmonary E. coli, but that some neutrophils and edema can accumulate in the lungs despite the absence of all three signaling receptors for these cytokines.

**Antibacterial host defenses of mice unresponsive to TNF or IL-1.** Inflammation, including the recruitment of neutrophils and plasma proteins from the blood, is essential to controlling bacterial infection in the lungs (58, 69). Figures 2 and 3 indicate that inflammation evoked by LPS or E. coli was decreased by the absence of receptors for TNF and IL-1. Therefore, we tested whether the combined deficiency of these three receptors compromised the ability of mice to control bacterial growth in the lungs.

After infection with a high dose of bacteria (730,000 CFU), similar to the above studies of acute inflammation, mice prevented the accumulation of bacteria in the lungs through 24 h, but there was a log increase in living bacteria between 24 and 48 h (Table 1). There were no significant differences between WT and TM mice at either time point (Table 1). Thus at this dose of bacteria, bacteria multiplied in the lungs regardless of whether or not mice had receptors for TNF-α and IL-1.

To further assess pulmonary host defenses, we infected WT and TM mice with lower doses of E. coli. After infection with lower doses, both genotypes reduced bacterial burdens by >98% over 48 h (Table 1). At the lowest dose, there were no significant differences between genotypes in bacterial clearance (Table 1). However, at the intermediate dose, there were fourfold more bacteria in TM lungs compared with WT after 24 h and 10-fold more bacteria in TM mice compared with WT after 48 h (Table 1). Thus, during E. coli infection of mouse lungs, a window of infectious dose requires these cytokine receptors for optimal antibacterial efficacy.

**Ventilation and lung mechanics in mice unresponsive to TNF or IL-1.** Inflammation, including neutrophil emigration and plasma extravasation, can impair lung function (25, 65). In rodents with bacterial pneumonia, the severity of infection associates with increased inflammation, decreased minute ventilation, and decreased lung compliance (8, 48). Therefore, we assessed whether these cytokine receptors contributed to changes in ventilation or compliance during pneumonia.

In the absence of pneumonia, tidal volume (Fig. 4A), respiratory rate (Fig. 4B), and minute ventilation (105 ± 8 and 120 ± 18 ml/min in WT and TM mice, respectively) did not differ between WT and TM mice. Furthermore, there were no significant differences between uninoculated WT and TM mice in peak inspiratory flow, peak expiratory flow, time of inspiration, end inspiratory pause, time of expiration, or end expiratory pause (Table 2 and data not shown). After 24 h of E. coli infection, tidal volume and respiratory rate increased, and minute ventilation decreased in experimental mice, compared with uninoculated controls (Fig. 4). There were no significant differences in minute ventilation between WT and TM mice (Table 2). Therefore, mice with the absence of TNF-α and IL-1 receptors required higher respiratory rates to achieve comparable minute ventilation.

### Table 1. Living bacteria recovered from WT and TM lungs during Escherichia coli pneumonia

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>WT CFU/mouse</th>
<th>TM CFU/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>18,000</td>
<td>730,000</td>
</tr>
<tr>
<td>18,000</td>
<td>36,000</td>
<td>32,000</td>
</tr>
<tr>
<td>130,000</td>
<td>140,000</td>
<td>140,000</td>
</tr>
<tr>
<td>370,000</td>
<td>400,000</td>
<td>400,000</td>
</tr>
</tbody>
</table>

Wild type (WT) and triple mutant (TM) mice deficient in all signaling receptors for TNF-α and IL-1 were infected with E. coli in the left lung lobe, and living bacteria were quantified after 24 or 48 hours. Data did not fit a normal distribution, so living bacteria recovered from the lungs were presented as median CFU/mouse with first and third quartiles indicated within parentheses. *P < 0.05 compared with WT (Mann-Whitney U-test, n = 8 mice/group).
infection in the left lung lobe, the tidal volumes did not consistently or significantly change for either WT or TM mice (Fig. 4A). However, respiratory rate significantly decreased for both genotypes during E. coli pneumonia (Fig. 4B). Decreased respiratory rate resulted from increased time of inspiration, time of expiration, and pause after expiration (Table 2). Respiratory rates and times of inspiration, expiration, and pauses did not differ between WT and TM mice with pneumonia (Table 2). The minute ventilations were decreased due to the diminished respiratory rates during pneumonia, but they did not differ between genotypes (56 ± 6 and 64 ± 18 ml/min in WT and TM mice, respectively). Thus these cytokine receptors were not essential to the altered ventilation induced by E. coli infection of the lungs.

Dynamic compliance did not significantly differ in uninfected WT and TM mice (Fig. 5A). E. coli infection decreased the dynamic compliance of both WT and TM lungs, but pneumonic lung from TM mice remained significantly more compliant than pneumonic lung from WT mice (Fig. 5A). Quasistatic pressure-volume loops also indicated that pneumonic lung from TM mice was more compliant than pneumonic lung from WT mice at any given pressure or volume examined (Fig. 5B). E. coli infection decreased the quasistatic compliance at 5 cmH2O pressure of lung tissue from WT mice (15.6 ± 0.6 and 12.0 ± 0.6 μl/cmH2O in nonpneumonic and pneumonic tissues, respectively; P < 0.05) but not from TM mice (17.4 ± 0.4 and 16.5 ± 2.1 μl/cmH2O, respectively; not statistically significant). Thus receptors for TNF and IL-1 contributed to the loss of compliance during E. coli pneumonia.

DISCUSSION

Mice deficient in TNFR1, TNFR2, and IL-1RI were generated to assess the net contribution of these receptor pathways to inflammation during bacterial pneumonia. The inflammatory response to E. coli in the lungs was significantly decreased, but

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**Table 2. Effect of E. coli pneumonia on respiratory cycle in WT and TM mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of inspiration, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nothing</td>
<td>48 ± 1</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>E. coli</td>
<td>68 ± 6*</td>
<td>73 ± 6*</td>
</tr>
<tr>
<td>End inspiratory pause, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nothing</td>
<td>5 ± 0.4</td>
<td>5 ± 0.3</td>
</tr>
<tr>
<td>E. coli</td>
<td>5 ± 0.4</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>Time of expiration, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nothing</td>
<td>83 ± 2</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>E. coli</td>
<td>161 ± 23*</td>
<td>215 ± 32*</td>
</tr>
<tr>
<td>End expiratory pause, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nothing</td>
<td>13 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>E. coli</td>
<td>81 ± 20*</td>
<td>111 ± 33*</td>
</tr>
</tbody>
</table>

WT and TM mice deficient in all signaling receptors for TNF-α and IL-1 were infected with E. coli in the left lung lobe, and ventilatory parameters were measured using Buxco plethysmography after 24 h. Values are means ± SE. *P < 0.05 compared with Nothing.
not eliminated, by the combined deficiency of all three signaling receptors for TNF-α and IL-1. These data conclusively demonstrate for the first time that pathways for cellular activation independent of these receptors can mediate neutrophil recruitment and edema accumulation during E. coli pneumonia. However, signaling from TNF or IL-1 cytokines is required for maximal inflammation.

We observed similar decreases in neutrophil recruitment in TM mice after the instillation of E. coli or of E. coli LPS. Previous studies demonstrate decreased neutrophil recruitment in mice deficient in both TNFR1 and IL-1RI in response to E. coli (31), but other studies observed no such defect after aerosolized LPS (16). Mice with targeted mutations of TNF-α and IL-1RI also showed no decrease in neutrophil emigration elicited by aerosolized LPS (32). The roles played by these cytokines may be influenced by aspects of design that differ across these studies, such as means of delivering stimulus, time for response, or methods of quantifying neutrophils. The present experiments, using identical study designs for LPS-induced inflammation and for E. coli pneumonia, demonstrate neutrophil recruitment elicited by LPS or E. coli to have similar requirements for these cytokine pathways.

Comparing the present studies with previous studies of E. coli pneumonia in mice with interruptions of only some of these three receptors provides no compelling evidence for a unique role of TNFRII in these innate immune responses. The combined deficiency of both TNFR1 and TNFR2 is not sufficient to decrease neutrophil recruitment or plasma extravasation during E. coli pneumonia (30). However, the combined deficiency of TNFR1 and IL1-RI decreases neutrophil recruitment to approximately half of WT levels (31). Similarly, the deficiency of TNFR1 and IL1-RI plus TNFR2 in TM mice decreased neutrophil recruitment to approximately half of WT levels in the present studies. Furthermore, the increase in extravascular plasma volume per lung during E. coli pneumonia was approximately a third of WT levels in TNFR1/IL1-RI mutant mice (31) and in TM mice in the present studies. These similar effects of TNFR1/IL1-RI deficiency and TNFR1/TNFR2/IL1-RI deficiency on neutrophil recruitment and plasma extravasation do not suggest a prominent role for TNFR2. However, the present studies with TM mice differed in several regards from previous studies of E. coli pneumonia in TNFR1/TNFR2 and TNFR1/IL1-RI mice, including the dose and strain of E. coli used for infection. These differences limit the chances that results collected over these three different studies would identify unique roles for TNFR2. Thus TNFR2 may have subtle roles in regulating innate immunity to E. coli in the lungs not evident in these studies.

These cytokine receptors activate intracellular signaling cascades that amplify pulmonary inflammation. NF-κB RelA is essential to neutrophil recruitment in response to E. coli LPS in the lungs (4). The present studies indicate that the nuclear translocation of NF-κB in the lungs is induced by these cytokines and dependent on these receptors, identifying insufficient NF-κB activation as one potential mechanism for decreased neutrophil recruitment in the absence of TNF-α and IL-1 signaling. Other intracellular signaling activities induced by TNF and IL-1 receptors and essential for acute pulmonary inflammation induced by bacterial stimuli in the blood or lungs include phosphorylation of proteins by p38 MAP kinase (35), arachidonic acid elaboration by cytosolic phospholipase A2 (34), and generation of reactive oxygen species by NADPH oxidase (12, 15). Extracellular signaling mediators induced by these receptors, regulating neutrophil recruitment to bacterial stimuli in the lungs, include the chemokines KC and macrophage inflammatory protein-2 (29), the adhesion molecule ICAM-1 (29), the cytokine IL-6 (21, 44), and the mediator high-mobility group box 1 (1). These interdependent intracellular and extracellular responses to TNF and IL-1 receptor ligation form an integrated network of signaling events that regulates acute inflammatory responses to bacteria in the lungs.

Our study showed that receptors for TNF-α and IL-1, and the augmented inflammation mediated by them, were not necessary for controlling E. coli growth through 24 h. However, after 48 h, a modest but significant defect in E. coli clearance was observed for a window of infectious dose. Therefore, maximal host defense against this bacteria in the lungs requires these cytokine pathways. During Streptococcus pneumoniae pneumonia, the interruption of TNF-α signaling (61) or of IL-1RI signaling (43) increases CFU/lung, suggesting that each of these cytokine pathways independently promotes bacterial killing during pneumonia. Interrupting TNF-α signaling can also increase bacterial burdens during pneumonias caused by E. coli (20), Pseudomonas aeruginosa (20), Klebsiella pneumoniae (19), and Legionella pneumophila (5) infections. However, interruption of TNF-α signaling (20, 57) or of IL-1RI signaling (51) can decrease intrapulmonary CFU during some lung infections. The number of living bacteria in the lungs is a complex variable that depends on multiple factors that modulate the antibacterial effects of innate immune responses and the suitability of the local environment for microbial growth. TNF-α and IL-1 affect many of these factors, with diverse and often competing effects on bacterial burden (28). For example, TNF-α and IL-1 can activate macrophages and neutrophils (55), which increases the killing of bacteria and decreases bacterial burden. TNF-α can cause cell death (2), which makes nutrients available to bacteria and increases bacterial burdens. TNF-α and IL-1 can also directly increase the rate of bacterial multiplication (20, 27), which increases bacterial burden. Because of this complexity, interrupting TNF-α and IL-1 signaling affects bacterial burdens in often unpredictable ways. The TM mice had modest defects in bacterial burdens in their lungs in the present experiments. The fact that interrupting these cytokine-receptor pathways often results in greater degrees of infection is an important limitation when considering such avenues as potential therapeutic and prophylactic approaches for patients with pneumonia (28).

E. coli pneumonia decreased the respiratory rate of mice without affecting tidal volume. Pneumococcal pneumonia induces similar ventilatory changes in mice (8). These ventilatory changes during E. coli or S. pneumoniae pneumonia in mice decrease minute ventilation to about half the levels of nonpneumonic mice. The decrease in minute ventilation suggests that mice are either hypoventilating or reducing their net metabolic rate during bacterial pneumonia. To our knowledge, the mechanisms for this decreased ventilation during pneumonia are unknown, and previous studies have not determined whether interrupting cytokine signaling affects ventilatory responses to pneumonia. Because ventilation of TM mice was reduced similar to WT mice during E. coli pneumonia, we conclude that this decrease in ventilation does not depend on TNF or IL-1 receptors.
For human patients with acute respiratory distress syndrome, decreased lung compliance significantly increases the risk of death (36). The compliance of rodent lungs exposed to E. coli LPS or E. coli decreases as expression of TNF-α and IL-1β, neutrophil recruitment, and plasma extravasation increase (14, 48). During E. coli pneumonia, the degree of infection (CFU/lung) correlates with both inflammation and loss of compliance, and E. coli products can directly interfere with surfactant function (48), suggesting that the bacterial burden may determine the compliance loss. However, in mice with *Pneumocystis carinii* pneumonia, the loss of compliance correlates with the degree of inflammation and not with the degree of infection, suggesting that the inflammatory process may be responsible for compliance loss (67). Thus both microbial products and inflammatory mediators may contribute to decreased compliance during pneumonia.

The present studies demonstrate that, during *E. coli* pneumonia, dynamic and quasistatic compliance decreased more in WT mice than in receptor-deficient mice. Because all mice were infected with the same bacteria and bacterial burdens did not differ between genotypes at this time point, the preservation of pulmonary compliance was not secondary to different microbial products between genotypes. The attenuated inflammation due to receptor deficiency may have contributed to preservation of lung compliance. Inflammation interferes with lung surfactant (e.g., via neutrophil-derived products such as proteases or reactive oxygen species and via dilution and disruption from edema fluid). In addition, pulmonary edema results in polymerized fibrin within alveoli and thickened septal walls. Furthermore, receptors for TNF-α and IL-1 may contribute more directly to decreased lung compliance. TNF-α, IL-1α, and IL-1β each signal to alveolar epithelial cells and alter surfactant composition and function (6, 13, 41, 49, 50, 62, 63), suggesting that the preservation of compliance in TM mice may result from the loss of signaling from TNF-α and IL-1 to alveolar epithelial cells.

To our knowledge, no previous studies have determined whether interrupting any or all of these three cytokine-receptor pathways, by genetic deficiency or other means, affects lung compliance during pneumonia. It will be important for future studies to determine whether any of these three receptors have unique roles in this physiological response and whether the acute interruption of these cytokine-receptor pathways has similar effects as the lifelong deficiency of these cytokine receptors. Furthermore, the present studies of compliance included one dose of one strain of bacteria. Increased doses of bacteria and infections with other types of bacteria may overcompensate for the requirement for these cytokine-receptor pathways in decreasing lung compliance during pneumonia. Because decreased compliance is a significant risk factor for death during acute respiratory distress syndrome (36), this preservation of lung compliance suggests an improved prognosis during pneumonia. If the loss of compliance actively contributes to morbidity and mortality, if interrupting these cytokine pathways during clinical pneumonias in humans has similar effects as during experimental pneumonias in mice, and if the roles of these cytokine-receptor pathways in decreasing lung compliance apply to infections with other bacteria and with greater doses of bacteria, then interrupting cytokine signaling could have therapeutic value for patients with severe pneumonia.

Because interruption of either TNF signaling or IL-1 signaling alone fails to decrease inflammatory responses to *E. coli* in mouse lungs (30, 31), whereas interruption of TNF-α and IL-1 together decreases neutrophil recruitment and diminishes pulmonary edema, the combined inhibition of these cytokines may prove superior to single cytokine inhibition for limiting inflammation in patients with pneumonia. Combinations of soluble cytokine inhibitors have been demonstrated to have additive or synergistic effects in decreasing inflammation during other types of experimental rodent challenges with bacteria or bacterial products (42, 47). Although caution is warranted due to the role of cytokines in bacterial clearance, the differences between experimental infections of rodents and clinical infections of humans, and the limited doses and types of bacteria so far examined, the present data suggest that interruption of cytokine signaling can preserve lung compliance during pneumonia.

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