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Roles of Interleukin-6 in Activation of STAT Proteins and Recruitment of Neutrophils during *Escherichia coli* Pneumonia

Matthew R. Jones, **Lee J. Quinton**, **Benjamin T. Simms**, **Michal M. Lupa**, **Mariya S. Kogan**, and **Joseph P. Mizgerd**
Physiology Program, Harvard School of Public Health, Boston, Massachusetts

Abstract

Interleukin (IL)–6 concentrations are positively associated with the severity of pneumonia, and this cytokine is essential to surviving experimental pneumococcal pneumonia. The role that IL-6 plays during pneumonia and its impact during gram-negative bacterial pneumonia remain to be determined. During *Escherichia coli* pneumonia, IL-6–deficient mice had increased bacterial burdens in their lungs, indicating compromised host defenses. Decreased neutrophil counts in alveolar air spaces, despite normal blood neutrophil counts and survival of emigrated neutrophils, suggested that defective neutrophil recruitment was responsible for exacerbating the infection. Neutrophil recruitment requires nuclear factor (NF)–κB, but IL-6 was neither sufficient nor essential to induce NF-κB–mediated gene expression in the lungs. In contrast, IL-6 induced the phosphorylation of signal transducer and activator of transcription (STAT) 1 and STAT3 in the lungs, and STAT1 and STAT3 phosphorylation during *E. coli* pneumonia was decreased by IL-6 deficiency. Thus, IL-6 plays essential roles in activating STAT transcription factors, enhancing neutrophil recruitment, and decreasing bacterial burdens during *E. coli* pneumonia.

Lower respiratory tract infections are the leading cause of disability-adjusted life years lost worldwide [1] and are the leading cause of hospitalizations and deaths due to infection in the United States [2,3]. Gram-negative bacterial rods are common causes of pneumonia in hospitals and nursing homes [4,5] and have especially high mortality rates [6,7].

The cytokine interleukin (IL)–6 is induced by a wide variety of infections. During community-acquired pneumonia, IL-6 concentrations in patients' blood and bronchoalveolar lavage (BAL) fluid are positively associated with the severity of disease [8–10]. During experimental *Streptococcus pneumoniae* infection in mice, IL-6 deficiency due to gene targeting increases bacterial burdens and mortality [11]. Thus, IL-6 improves defenses against *S. pneumoniae* in the lungs, although the responses regulated by IL-6 that are essential to the antipneumococcal host defenses remain to be determined.

Neutrophil recruitment is essential to effective host defenses against bacteria, particularly gram-negative rods, in the lungs [12,13]. Maximal neutrophil recruitment in the lungs requires the induction of IL-6 in response to some purified bacterial products, such as peptidoglycan or pneumolysin [14,15], but not to others, such as lipoteichoic acid or lipopolysaccharide [15, 16]. The roles that IL-6 plays in modulating neutrophil recruitment elicited by living bacteria in the lungs have yet to be identified. Furthermore, whether IL-6 impacts the host defenses

Reprints or correspondence: Dr. Joseph P. Mizgerd, Physiology Program, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115 (E-mail: jmizgerd@hsph.harvard.edu)..

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against gram-negative bacteria in the lungs remains to be determined. We hypothesized that IL-6 makes essential contributions to the host defenses during *E. coli* pneumonia, and we tested this hypothesis using IL-6–deficient mice.

MATERIALS AND METHODS

Mice

IL-6–deficient mice [17] were on a C57BL/6 background (Jackson Laboratories); triple mutant (TM) mice [18] deficient in tumor necrosis factor (TNF) receptors 1 and 2 and the type I IL-1 receptor were on a C57BL/6x129/Sv random hybrid background; and controls were wild-type *(wt)* mice with a background matched to mutants. Experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals.

Intratracheal instillations

Mice were anesthetized by the intramuscular injection of ketamine hydrochloride (100 mg/kg), acepromazine maleate (5 mg/kg), and atropine (0.1 mg/kg). The trachea was surgically exposed, and a total volume of 50 μL was instilled via an angiocatheter that was inserted through the trachea and into the left bronchus. Recombinant murine (rm) IL-6 (Endogen) was instilled at a dose of 0, 50, or 500 ng/mouse in sterile saline with 1% bovine serum albumin. *E. coli* (strain 19138; American Type Culture Collection) was instilled intratracheally at a dose of 10^6 cfu/mouse. Colloidal carbon (1%) was included in the instillate, to indicate deposition. At the conclusion of each experiment, mice were killed by halothane inhalation.

mRNA expression

RNA was extracted with Trizol (Life Technologies), column purified (Qiagen), and treated with DNase. For IL-6, reverse transcription (RT) was performed with 2 μg of RNA, random hexamers (Life Technologies), rRNasin ribonuclease inhibitor (Promega), and M-MLV reverse transcriptase (Promega). Real-time polymerase chain reaction (PCR) was performed using iQ SYBR Green Supermix (Biorad) and the primer set forward, 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′, and reverse, 5′-CACTAGGTTTGCCGAGTAGATCTC-3′. The primer set forward, 5′-AGAGGGAAATCGTGCGTGAC-3′, and reverse, 5′-CAATAGTGATGACCTGGCCGT-3′, was used to amplify β-actin cDNA. Copy numbers of IL-6 and β-bactin were calculated from standard curves generated from plasmid clones, and IL-6 mRNA content was normalized to that of β-actin. Intercellular adhesion molecule (ICAM)–1 RT and real-time RT-PCR was performed using the iScript One-Step RT-PCR Kit for Probes (Biorad). The primer set for amplification of ICAM-1 was forward, 5′-CACCATGCCTTAGCAGCTGAACAA-3′, and reverse, 5′-CGACCCTTCTAAGGAGTTGGAACA-3′. The primer set for amplification of 18s rRNA was forward, 5′-ATTCGAACGTCTGCCCTATCA-3′, and reverse, 5′-GTCACCCGTGGTCACCATG-3′. The probes for ICAM-1 and 18s rRNA were 5′-TCATGGTCCCAGGCGGCTCCACCTCAAAGA-3′ and 5′-TCGATGGTAGTCGCCGTGCCTACC-3′, respectively, and contained 6-FAM at the 5′ end and Black Hole Quencher–1 at the 3′ end. ICAM-1 fold induction (compared with baseline levels) [19] was normalized to 18s rRNA within each sample. Real-time RT-PCR reactions were performed using the iCycler iQ Real-Time PCR detection system (Biorad).

Cytokine concentrations

Cytokine concentrations were measured by ELISA (R&D Systems) in BAL fluid and lung homogenates collected at indicated times after *E. coli* infection. For BAL fluid, the trachea was cannulated, lungs were lavaged 12 times with 0.8−1.0-mL volumes of PBS, the lavageates were pooled, and cells were removed by centrifugation. For lung homogenates, excised lungs

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were ground using a sterile glass Duall homogenizer in a buffered salts solution containing 0.5% Triton X-100 and protease inhibitors.

Bacterial burdens

Lung homogenates were serially diluted in ice-cold sterile water, and aliquots were spread on 5% sheep blood–agar plates. After 18−24 h of incubation at 37°C, colonies were counted, and data for viable bacteria were expressed as colony-forming units per lung.

Neutrophil recruitment

Lungs and blood were collected 24 h after *E. coli* infection. Peripheral blood was drawn from the inferior vena cava. After erythrocyte lysis, leukocytes were counted using a hemacytometer, and differential distributions in stained blood smears were determined. Excised lungs were fixed by the intratracheal instillation of 6% glutaraldehyde at 23 cm H_2O pressure. Neutrophils in alveolar air spaces were quantified by morphometric analyses of histological lung sections [18,20,21]. For morphometric examinations, investigators were blinded to the identities of mice.

Neutrophil survival

Cells from BAL fluid were collected [22] from *wt* and IL-6–deficient mice 24 h after *E. coli* infection. Cell counts and viability were determined using a hemacytometer and trypan blue dye (Sigma), and morphological features were assessed using cytocentrifuge slide preparations. Preparations were 83%–96% neutrophils with an initial viability of 91%–96%. Cells were suspended at a concentration of 10^6 cells/mL in a buffered salt solution containing divalent cations, and 200-μL samples were cultured in 96-well ultra-low-attachment plates (Corning) at 37°C in 5% CO_2. Samples were withdrawn at indicated time points for assessment using a hemacytometer with trypan blue and a cytocentrifuge with Diff-Quick (Dade Behring).

Assessment of inhibitor of NF-κB (IκB) and signal transducer and activator of transcription (STAT) proteins by immunoblotting

IκB content and STAT1 and STAT3 phosphor-ylation were analyzed by immunoblotting. Excised lungs were homogenized in lysis buffer (buffered saline solution containing NP-40 (Calbiochem), sodium deoxycholate, SDS, sodium orthovanadate, and protease inhibitors) using a Kinematica Polytron, and total protein concentrations were measured using a bicinchoninic acid assay. Proteins were separated on 4%–12% gradient gels by SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene fluoride membranes. Membranes were probed with the following polyclonal antibodies: sc-371 against IκB-α (Santa Cruz Biotechnology), sc-945 against IκB-β (Santa Cruz Biotechnology), 9172 against STAT1 (Cell Signaling Technology), 9171 against STAT1 phosphorylated on tyrosine 701 (Cell Signaling Technology), 9132 against STAT3 (Cell Signaling Technology), or 9131 against STAT3 phosphorylated on tyrosine 705 (Cell Signaling Technology). After washing, membrane-bound primary antibodies were detected on autoradiographic film by horseradish peroxidase–conjugated secondary antibodies and the ECL Plus chemiluminescent system (Amersham Pharmacia Biotech).

Statistics

Data are expressed as means ± SEs. Data in groups of 2 were compared using Student's *t* test. Sets of >12 groups were compared using factorial analysis of variance with post hoc tests as indicated. If data failed Levene's test for homogeneity of variances, they were log transformed. Differences were considered to be statistically significant if $P < .05$.

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RESULTS

Expression of IL-6 during E. coli *pneumonia*

IL-6 was induced within 1 h of *E. coli* infection (figure 1*A*). Such rapid kinetics suggested that IL-6 transcription may result from signals generated by receptors for bacterial products. IL-6 mRNA concentrations peaked 6 h after infection but remained increased throughout the 24-h period that was examined (figure 1*A*). At later time points, IL-6 expression could be induced from a combination of receptors for bacterial products or host cytokines. IL-6 protein concentrations were measured in lung homogenates during *E. coli* pneumonia, and TM mice were used to determine the requirements of signaling from receptors for TNF-α and IL-1 (α and β). IL-6 concentrations were increased after infection, and concentrations in TM mice did not significantly differ from those in *wt* mice (figure 1*B*). Therefore, IL-6 expression during *E. coli* pneumonia does not require signaling from receptors for TNF-α and IL-1.

Bacterial burden

To determine whether IL-6 deficiency impacted innate host defenses against intrapulmonary *E. coli*, living bacteria were quantified in lungs from *wt* and IL-6–deficient mice. In both genotypes, bacterial burdens were decreased over time, but IL-6–deficient mice were less effective than *wt* mice at decreasing bacterial burdens (figure 2*A*). The differences between the mice were statistically significant by 24 h, when IL-6–deficient mice already had several-fold more bacteria in their lungs than did *wt* mice. By 48 h, *wt* mice had eliminated 99.9% of the bacterial burden. IL-6–deficient mice were also controlling the infection for 48 h, but they had hundreds-fold more bacteria in their lungs than did *wt* mice. Bacteremia was never detected in *wt* mice (data not shown) but was occasionally observed in IL-6–deficient mice (20%–25% of mice at either time point after infection). Thus, IL-6 deficiency compromises antibacterial host defenses during *E. coli* pneumonia.

Neutrophil recruitment

Neutrophils are essential to clearing gram-negative bacterial rods from the lungs [12,13]. *E. coli* infection induced neutrophil recruitment in both *wt* and IL-6– deficient mice (figure 2*B*), demonstrating that some neutrophils emigrate in the absence of IL-6. However, only one-half as many neutrophils were present in alveolar air spaces of IL-6–deficient mice, compared with those of *wt* mice (figure 2*C*). These data indicate that IL-6 is essential to maximal neutrophil accumulation in alveolar air spaces during *E. coli* pneumonia.

Because IL-6 can promote granulopoiesis and neutrophil mobilization from the bone marrow [23–25], we determined whether IL-6 deficiency decreased blood neutrophil counts during *E. coli* pneumonia. Circulating neutrophil counts did not significantly differ between genotypes, in mice with or without *E. coli* pneumonia (figure 3*A*). These data suggest that roles of IL-6 in promoting granulopoiesis and mobilizing neutrophils from the bone marrow were unlikely to be responsible for the decrease in emigrated neutrophils during *E. coli* pneumonia.

Because IL-6 can prolong the life span of neutrophils in vitro [26,27], we determined whether the life span of emigrated neutrophils was altered by IL-6 deficiency. After *E. coli* infection, neutrophils cultured from *wt* and IL-6–deficient mice developed pyknosis and karyorhexis during a similar time frame (data not shown). Loss of plasma membrane integrity, as measured by trypan blue exclusion, did not differ between genotypes (figure 3*B*). These data suggest that the life span of neutrophils that emigrated during *E. coli* pneumonia was not shortened by IL-6 deficiency. Decreased neutrophil counts in alveolar air spaces, coupled with normal blood neutrophil counts and normal survival of emigrated neutrophils, suggest that IL-6 was necessary for maximal recruitment of neutrophils from the pulmonary capillaries to alveolar air spaces during *E. coli* pneumonia.

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NF-κB–regulated genes essential to neutrophil recruitment

ICAM-1, KC, and macrophage inflammatory protein (MIP)–2 are necessary for neutrophil recruitment [13,28–32] and are regulated by NF-κB [20,21,33–36] in response to gram-negative stimuli in the lungs. Because IL-6 can activate NF-κB in vitro [37], we determined whether IL-6 contributed to expression of these genes or NF-κB activity during *E. coli* pneumonia.

ICAM-1 mRNA concentrations were increased 6 h after *E. coli* infection, with no significant differences between genotypes (figure 4*A*). By 24 h, ICAM-1 mRNA concentrations were greater in IL-6–deficient mice than in *wt* mice (figure 4*A*). Thus, IL-6 was not essential for induction of ICAM-1. Concentrations of KC and MIP-2, which are particularly important in BAL fluid [38], peaked 6 h after *E. coli* infection [20]. KC and MIP-2 concentrations did not differ between genotypes (figure 4*B*). Circulating KC also enhances neutrophil recruitment [39]. However, circulating KC concentrations did not significantly differ between *wt* and IL-6–deficient mice 6 h after infection (870 ± 84 vs. 1899 ± 469 pg/mL). Thus, none of these mediators required IL-6 during *E. coli* pneumonia.

NF-κB activation by lipopolysaccharide in the lungs is mediated by the degradation of IκB-α and IκB-β [40]. Similarly, *E. coli* infection decreased concentrations of both IκB-α and IκB-β (figure 4*C*) in mice. IL-6 deficiency did not alter the IκB protein content in the lungs during *E. coli* pneumonia (figure 4*C*), suggesting that IL-6 was not necessary for IκB degradation. Furthermore, IκB-α and IκB-β were unaffected by the instillation of up to 500 ng of rmIL-6 (figure 4*D*), suggesting that IL-6 is not sufficient to induce the degradation of lung IκB proteins. Combined with the NF-κB–dependent gene expression data given above, these results argue against regulation of NF-κB by IL-6 during *E. coli* pneumonia.

Other gene products regulating neutrophil recruitment in the lungs

We examined whether other cytokines relevant to acute neutrophil recruitment during bacterial pneumonia were dependent on IL-6. The cytokine IL-17 is necessary for neutrophil recruitment during gram-negative bacterial pneumonia [41,42]. Because IL-17 expression is delayed during pneumonia and requires multiple upstream cytokines [43,44], we measured IL-17 concentrations in lung homogenates collected 24 h after *E. coli* infection. Compared with lungs from *wt* mice, lungs from IL-6–deficient mice had increased IL-17 concentrations (table 1). Maximal expression of lipopolysaccharide-induced CXC chemokine (LIX), which contributes to neutrophil recruitment in the lungs [45], requires IL-6 under some conditions [46]. Because LIX expression during *E. coli* pneumonia has not been characterized, we measured LIX concentrations 6 and 24 h after *E. coli* infection. LIX concentrations were unchanged (at 6 h) or increased (at 24 h) by IL-6 deficiency (table 1). Altogether, these data indicate that decreased neutrophil recruitment in IL-6–deficient mice with *E. coli* pneumonia does not result from decreased concentrations of LIX or IL-17.

STAT1 and STAT3 phosphorylation

IL-6 activates the transcription factors STAT1 and STAT3 in many cells [47]. To determine whether IL-6 activates STAT1 and STAT3 in the lungs, rmIL-6 was intratracheally instilled, and tyrosine phosphorylation, which is essential for the transcriptional activity of these factors [48], was assessed by immunoblotting. Both STAT1 and STAT3 were phosphorylated in the lungs in response to IL-6 in a dose-dependent fashion (figure 5*A*), indicating that IL-6 in the lungs was sufficient for phosphorylation of these transcription factors.

STAT1 and STAT3 were also activated in the lungs during *E. coli* pneumonia (figure 5*B*). Phosphorylation of both factors peaked 6 h after infection (figure 5*B*), which was concomitant with peak concentrations of IL-6 mRNA and protein (figure 1). At the 6-h time point,

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concentrations of phosphorylated STAT1 and phosphorylated STAT3 were decreased in the lungs from IL-6–deficient mice, compared with those from *wt* mice (figure 5*C*). Concentrations of phosphorylated STAT3 and STAT1 were similarly decreased 15 h after infection (data not shown). At the 2- and 24-h time points, there were lower phosphorylated STAT protein concentrations and no discernible differences between genotypes (data not shown). These data demonstrate that maximal STAT activation required IL-6 during *E. coli* pneumonia. In contrast to the phospho-specific signals, the total concentrations of STAT1 and STAT3 were unaffected by rmIL-6, *E. coli* infection, and/or IL-6 deficiency (figure 5*A*–5*C*), indicating that phosphorylation, rather than expression, is the level of regulation. These data demonstrate that activation of STAT1 and STAT3 was partially dependent on IL-6 during *E. coli* pneumonia, and peak STAT concentrations that occurred 6−15 h after infection required IL-6.

Effects of exogenous IL-6 during E. coli *pneumonia*

To determine whether exogenous IL-6 was sufficient to restore responses of IL-6–deficient mice during pneumonia, rmIL-6 or vehicle was instilled with the bacteria. A single 100-ng dose of IL-6 per mouse, which was sufficient to cause the phosphorylation of STAT1 and STAT3 in the lungs (figure 5*A*), was able to restore neutrophil recruitment in IL-6–deficient mice with *E. coli* pneumonia (table 2). These results indicate an acute role for this cytokine early during the course of *E. coli* pneumonia. In contrast, including 100 ng of rmIL-6 with *E. coli* was not sufficient to restore bacterial clearance in IL-6–deficient mice (table 2). Optimal host defenses against *E. coli* in the lungs may require a more prolonged or coordinated signaling from IL-6 than was achieved by instilling a single bolus of this cytokine at the time of infection.

DISCUSSION

IL-6 concentrations are positively associated with the severity of disease in patients with pneumonia [8–10]. IL-6 is essential to overcoming *S. pneumoniae* infection in the lungs [11] or *E. coli* infection or polymicrobial sepsis in other tissues [49–51]. The results of the present study indicate that bacterial clearance is compromised by IL-6 deficiency during *E. coli* pneumonia, and this cytokine directs host responses to this infection by activating STAT transcription factors and enhancing neutrophil recruitment.

IL-6 plays a complex role in inflammation, because it can both promote and limit neutrophil emigration [14–16]. To our knowledge, the roles of IL-6 in neutrophil recruitment elicited by *E. coli* or other living bacteria in the lungs have yet to be determined. In the present study, neutrophil recruitment during *E. coli* pneumonia in C57BL/6 mice became detectable by 6 h, reached peak concentrations by 24 h, and was maintained to at least 48 h after infection. During this period, bacterial burdens decreased in both *wt* and IL-6–deficient mice but did so less effectively in the absence of IL-6. We observed the neutrophil recruitment elicited during *E. coli* pneumonia to be decreased by IL-6 deficiency, indicating that neutrophil recruitment during this infection depends, in part, on IL-6. Neutrophils are needed to kill gram-negative bacteria in the lungs [12,13], so decreased neutrophil recruitment may contribute to the increased bacterial burdens of IL-6–deficient mice with *E. coli* pneumonia. The restoration of neutrophil recruitment, but not bacterial clearance, by a bolus of exogenous IL-6 delivered at the time of infection suggests that optimal host defenses require not only the presence of neutrophils but also prolonged IL-6 signaling. The enhancement of neutrophil bactericidal functions after transepithelial migration requires IL-6 signaling [52], suggesting that there may have been defects in bacterial killing by neutrophils in alveolar air spaces of IL-6– deficient mice. Therefore, *E. coli* clearance may be compromised in IL-6–deficient mice because of a combination of decreased neutrophil recruitment and suboptimal activation of recruited neutrophils.

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Gene expression and neutrophil recruitment elicited by gram-negative bacterial stimuli in the lungs are mediated by NF-κB [20,21,33,34,36]. IL-6 transcription is regulated by NF-κB [53, 54]. NF-κB can be activated by receptors for bacterial products or by endogenous factors such as cytokines. To determine the importance of receptors for the early response cytokines IL-1 (α and β) and TNF-α in IL-6 expression during *E. coli* pneumonia, we measured IL-6 concentrations in lungs from TM mice and found that IL-6 did not require signaling from these cytokines. IL-6 activated NF-κB in an intestinal epithelial cell line in vitro [37], suggesting the possibility that IL-6 is upstream of NF-κB activation during pneumonia. However, data in the present study demonstrate that IL-6 in alveolar air spaces is not sufficient to activate NF-κB in the lungs. NF-κB could be activated by IL-6 in the interstitial or vascular compartments of the lungs or by IL-6:soluble IL-6 receptor complexes formed during pneumonia. However, IκB degradation and the expression of NF-κB–dependent chemokines (ICAM-1, KC, and MIP-2) were unaffected by IL-6 deficiency during *E. coli* pneumonia, suggesting that IL-6 is not necessary for NF-κB activation during pneumonia. These data cannot preclude a role for IL-6 in activating NF-κB during *E. coli* pneumonia, but, altogether, they suggest that IL-6 is more likely to be downstream, rather than upstream, of NF-κB during this infection.

Because NF-κB was not affected by instillation of rmIL-6 or IL-6 deficiency, we considered other transcription factors that might be regulated by IL-6. The STAT family of transcription factors is regulated by cytokines [55]. STAT1 and STAT3 can be activated by IL-6 [47], but, to our knowledge, there is little or no evidence indicating that IL-6 activates other STAT proteins. Therefore, we examined STAT1 and STAT3 in the lungs. The present study demonstrates that STAT1 and STAT3 transcription factors are activated during *E. coli* pneumonia. In contrast to NF-κB, rmIL-6 activated STAT1 and STAT3 in the lungs. Furthermore, STAT activation after *E. coli* infection was partially dependent on endogenous IL-6. Therefore, IL-6 functions to activate these 2 STAT transcription factors during *E. coli* pneumonia.

The roles that STAT proteins play during pneumonia are an important focus for future research. STAT1 and STAT3 can regulate cell migration [56–59], suggesting that IL-6 activation of STAT1 and STAT3 in neutrophils may contribute to neutrophil emigration during pneumonia. Furthermore, STAT1 and STAT3 regulate the expression of extracellular mediators that may contribute to neutrophil recruitment, including CC and CXC chemokines [60–62], adhesion molecules [61,62], S100 proteins [63], acute-phase response proteins [64,65], and complement proteins [63]. Therefore, IL-6 activation of STATs may contribute to neutrophil recruitment during pneumonia by inducing genes in neutrophils (mediating migration) or by inducing genes in lung parenchymal cells (guiding migration). STAT3 also has antiapoptotic functions [66]. Loss of STAT3 function in the lungs [67] or other tissues [66] predisposes mice to premature death, perhaps because of excessive cell death, when challenged. Therefore, IL-6 activation of STAT3 may be protective during pneumonia by both facilitating the host defenses and preventing inflammatory injury.

Data in the present study demonstrate that IL-6 plays several roles during *E. coli* pneumonia: activating STAT1 and STAT3, facilitating neutrophil recruitment, and improving bacterial clearance. These findings suggest a causal relationship between IL-6–induced STAT activation and neutrophil-dependent killing of bacteria in the lungs, although the findings must be tested with further studies. It is also likely that IL-6 regulates transcription factors other than STAT1 and STAT3 and that they contribute to the phenotype of IL-6–deficient mice with *E. coli* pneumonia. Because data in the present study show that IL-6 facilitates both STAT activation and pulmonary host defenses, manipulating IL-6 and STAT signaling pathways deserves further consideration in the modification of innate immunity and protection of the lungs during bacterial pneumonia.

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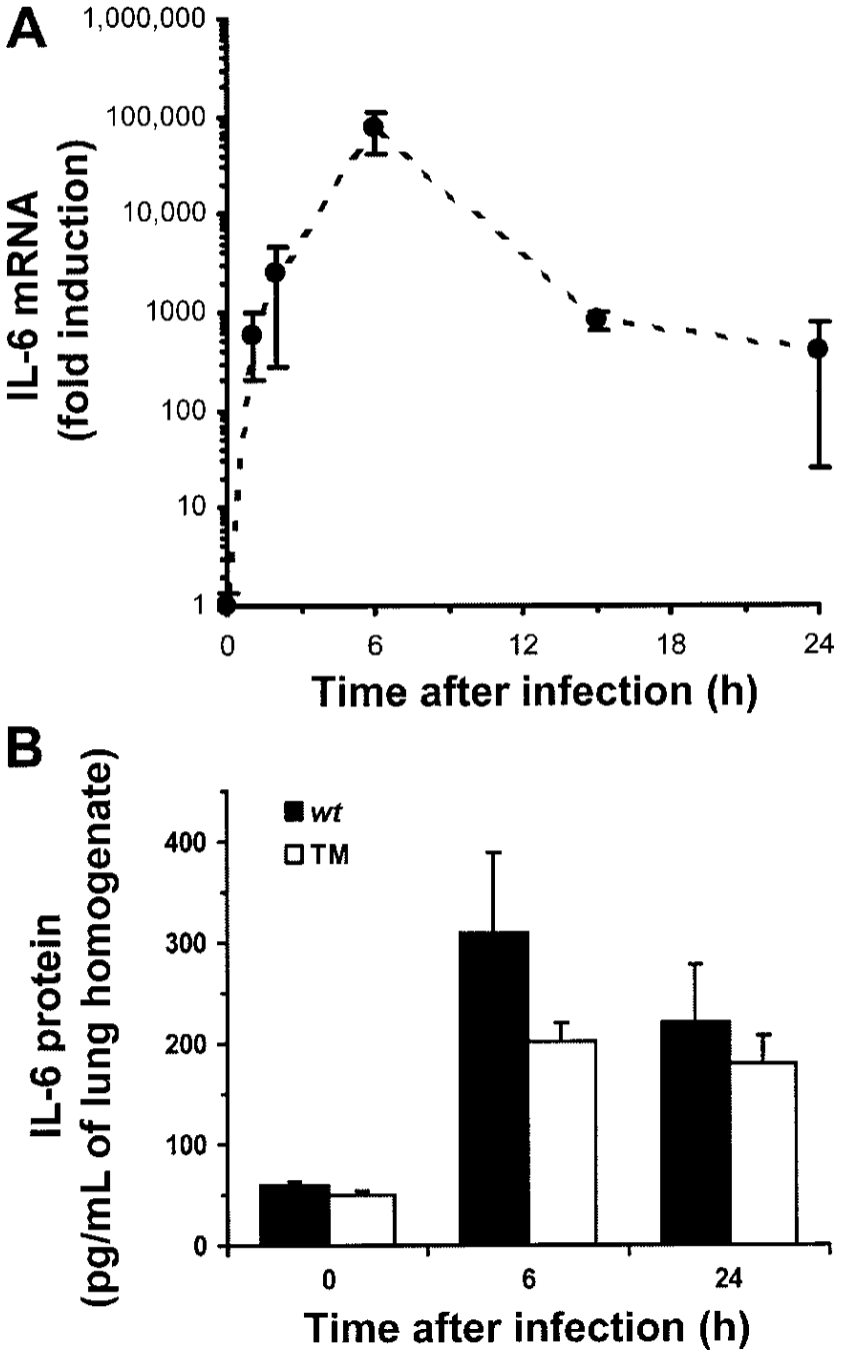

Figure 1.

Interleukin (IL)–6 in the lungs during *Escherichia coli* pneumonia. *A*, IL-6 mRNA in the lungs. IL-6 mRNA increased within 1 h of infection and remained increased through 24 h after infection. IL-6 and β-actin mRNA were measured in lung homogenates using quantitative reverse-transcription polymerase chain reaction. IL-6 transcripts were normalized to β-actin transcripts for each sample. The effect of time on IL-6 mRNA was statistically significant (P<.05, 1-way analysis of variance [ANOVA]). *B*, IL-6 protein in the lungs. IL-6 protein increased during infection of either wild-type mice (n = 5 mice/group) or triple mutant (TM) mice (n = 5 mice in the 0-h group, n = 4 mice in the 6-h group, and n = 6 mice in the 24-h group) lacking signaling receptors for tumor necrosis factor–α and IL-1. IL-6 concentrations

in lung homogenates were determined using ELISA. There was a statistically significant effect of time ($P<.05$, 2-way ANOVA) but not of genotype.

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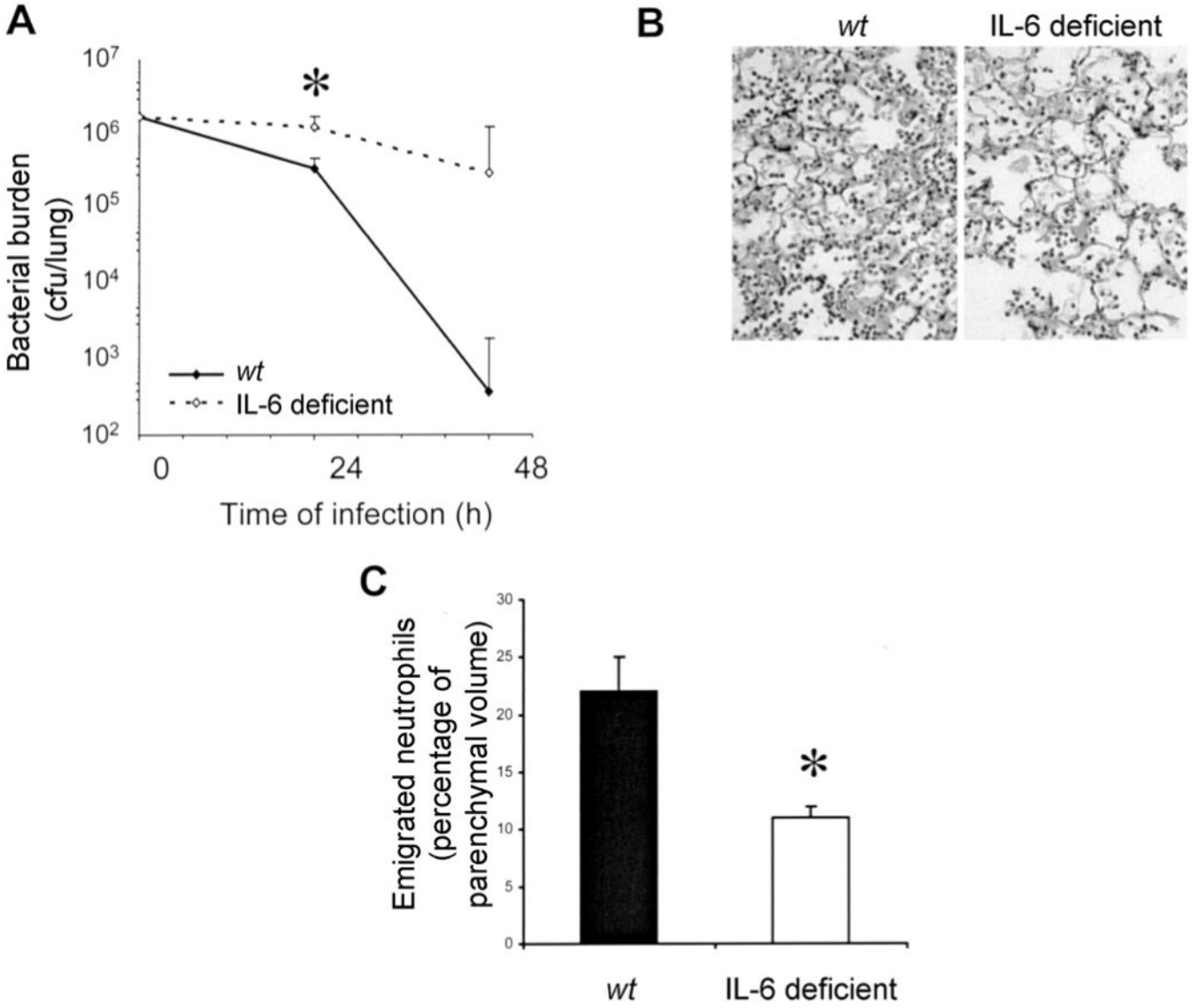

Figure 2.

Effects of interleukin (IL)–6 deficiency on neutrophil accumulation and bacterial clearance during *Escherichia coli* pneumonia. *A*, Bacterial clearance and IL-6 deficiency. Living bacteria were quantified in lung homogenates collected from wild-type *(wt)* and IL-6–deficient mice 24 h after infection (n = 5−9 mice/group). The effect of time on colony-forming units was statistically significant (P<.05 , 2-way analysis of variance [ANOVA]), and so was the effect of genotype (denoted by asterisk; P<.05, 2-way ANOVA). *B*, Effect of IL-6 deficiency on accumulation of neutrophils in alveolar air spaces. Representative images are shown of pulmonary parenchyma from lungs of *wt* and IL-6–deficient mice. Left lung lobes were collected 24 h after infection, fixed by the instillation of 6% glutaraldehyde at 23 cm H_2O pressure, and embedded in paraffin, and 5-mm sections were stained with hematoxylin-eosin. Histological analyses revealed acute inflammation with a diffuse and patchy distribution throughout the left lung lobe parenchyma. Polymerized proteinaceous material was prominent in alveolar air spaces. Emigrated neutrophils were present in alveolar air spaces, with more apparent in lungs from *wt* mice than in those from IL-6–deficient mice. *C*, Emigrated neutrophils in alveolar air spaces of IL-6–deficient mice. Emigrated neutrophils in lung

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sections (n = 7 mice/group) 24 h after infection, as in panel *B,* were quantified using standard point-counting morphometric techniques. The relative volumes of the parenchymal regions occupied by emigrated neutrophils were calculated by investigators blinded to the identities of the mice and were expressed as a percentage of the total parenchymal region volume (including both tissue and air spaces). *P<.05, Student's t test.

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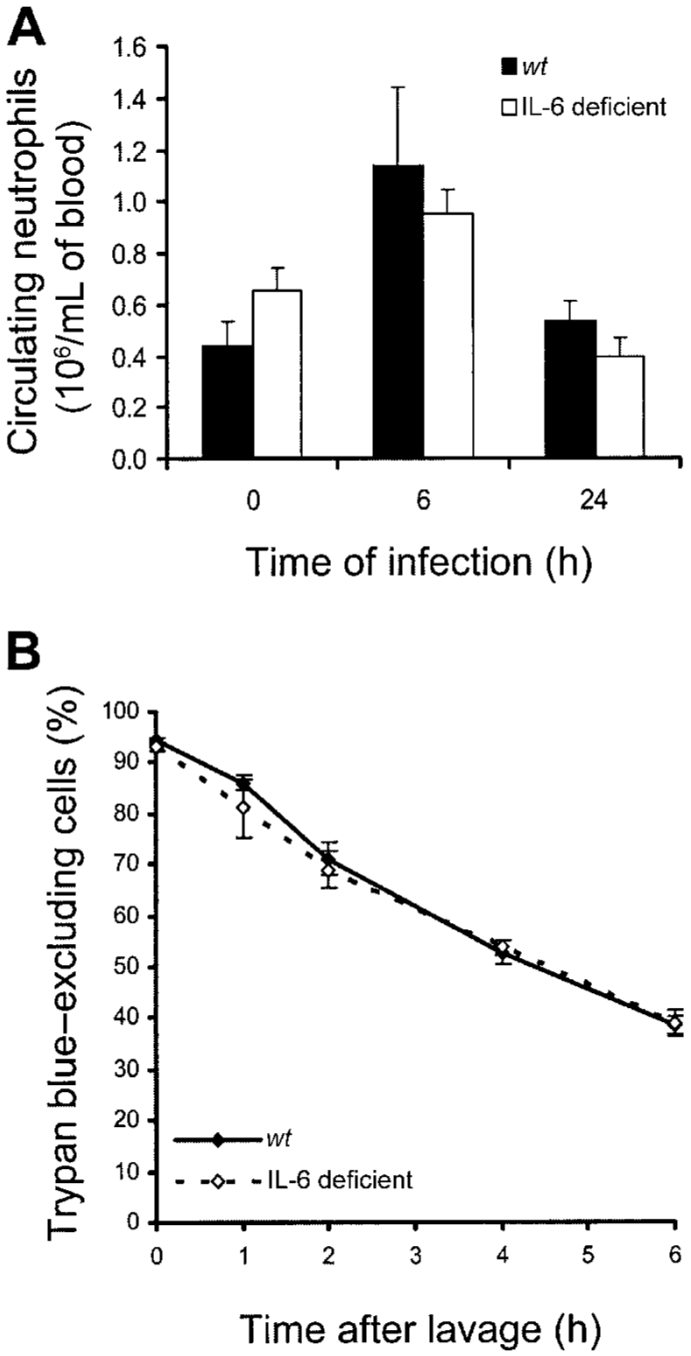

Figure 3.

Effect of interleukin (IL)–6 deficiency on blood neutrophils and survival of emigrated neutrophils. *A*, Neutrophils in venous blood from wild-type *(wt)* and IL-6–deficient mice with *Escherichia coli* pneumonia. Neutrophils were quantified in venous blood samples from mice 0, 6, or 24 h after infection. There was a significant effect of time ($P<.05$, 2-way analysis of variance [ANOVA]) but not of genotype on blood neutrophil counts. *B*, IL-6 deficiency and the life span of emigrated neutrophils. Emigrated neutrophils were collected from alveolar air spaces of *wt* and IL-6–deficient mice by bronchoalveolar lavage 24 h after infection and were cultured in vitro for the designated time. Viability was assessed using trypan blue exclusion,

with neutrophils collected from 4 mice/group. The viability of emigrated neutrophils decreased over time but did not differ between genotypes (2-way ANOVA).

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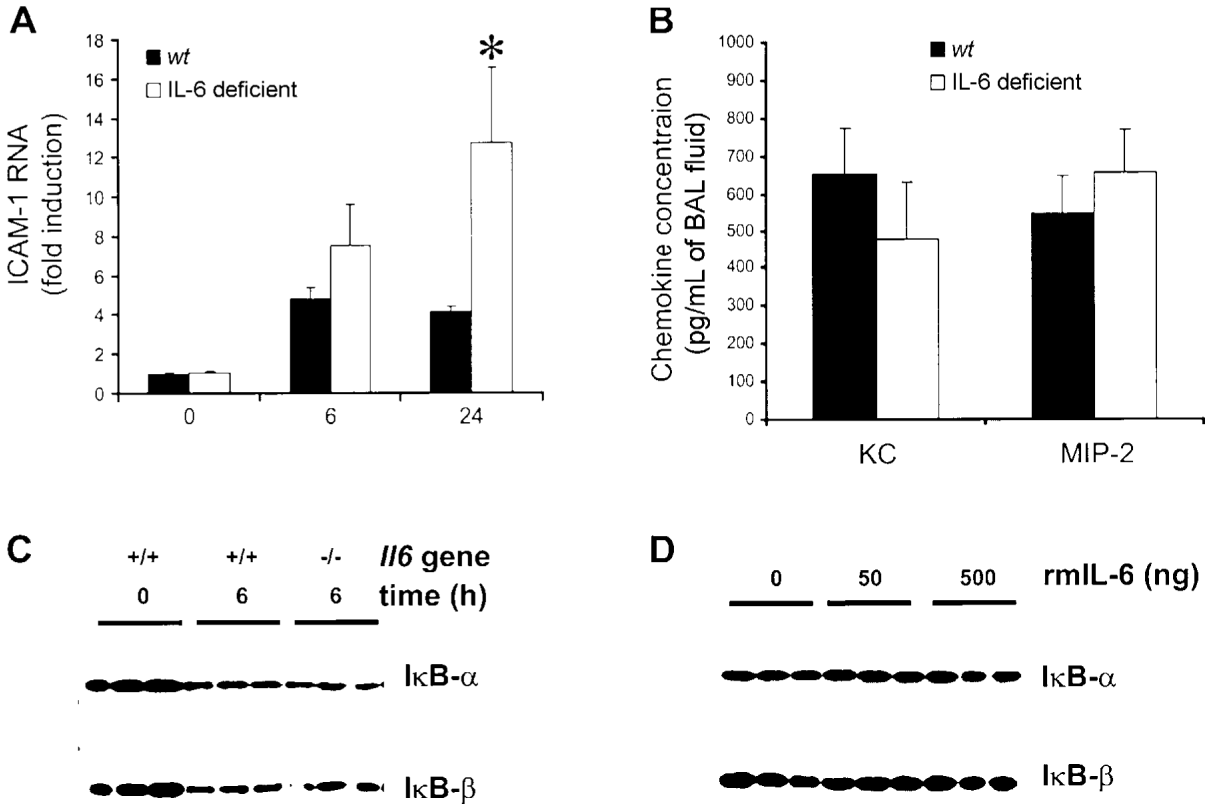

Figure 4.

Interleukin (IL)–6 and NF-κB–mediated gene expression during *Escherichia coli* pneumonia. *A*, Intercellular adhesion molecule (ICAM)–1 RNA concentrations. Steady-state concentrations of ICAM-1 RNA did not differ between wild-type *(wt)* and IL-6–deficient mice. Lungs were collected from 10 mice/genotype at 6 h or from 3 mice/genotype at 0 and 24 h after infection. ICAM-1 transcripts were measured by quantitative reverse-transcription polymerase chain reaction, normalized to 18s rRNA, and then expressed as the fold induction from 0-h levels, which did not differ between genotypes. Both time and genotype had significant effects (denoted by asterisk; $P<.05$, 2-way analysis of variance and Bonferroni post hoc test). *B*, Concentrations of the NF-κB–dependent chemokines KC and macrophage inflammatory protein (MIP)–2. Chemokine concentrations did not differ between *wt* and IL-6–deficient mice ($n = 5–6$ mice/group). Lungs were collected 6 h after infection, and chemokine concentrations in bronchoalveolar lavage fluid (BAL) were determined using ELISA. There was no significant effect of genotype (Student's *t* test). *C*, Inhibitor of NF-κB (IκB) content in the lungs. IκB content decreased during *E. coli* pneumonia but did not differ between genotypes. The IκB content was assessed by immunoblotting at the indicated time after infection. Each lane contains protein from an individual mouse that was either homozygous (+/+) or mutant (−/−) for the *Il6* gene. *D*, IκB content in the lungs and IL-6. IκB content was not affected by the intratracheal instillation of recombinant murine (rm) IL-6. IκB content in *wt* mice that received the indicated dose of rmIL-6 was assessed by immunoblotting. Each lane contains protein from an individual mouse.

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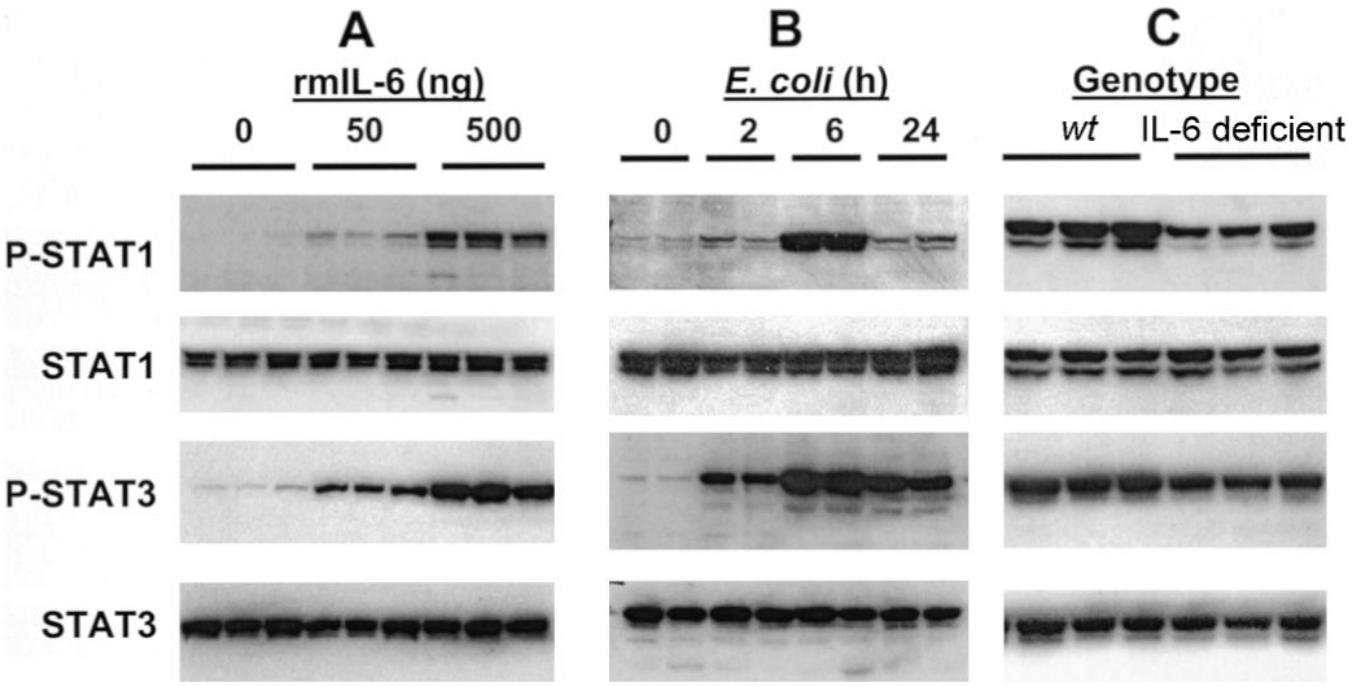

Figure 5.

Interleukin (IL)–6 and activation of signal transducer and activator of transcription (STAT) proteins in the lungs during *Escherichia coli* pneumonia. Concentrations of tyrosine-phosphorylated (P) and total STAT proteins in the lungs were assessed by immunoblotting. Each lane contains protein from an individual mouse. *A*, IL-6 and phosphorylation of STAT1 and STAT3 in the lungs. Recombinant murine (rm) IL-6 was instilled intratracheally to wild-type *(wt)* mice at the indicated dose per mouse, and lungs were collected 1 h later. The instillation of rmIL-6 was sufficient to induce phosphorylation. *B*, Phosphorylation of STAT1 and STAT3 during *E. coli* pneumonia. Lungs were collected from *wt* mice at the indicated time after infection, and phosphorylation was assessed. *C*, IL-6 deficiency and STAT1 and STAT3 phosphorylation. Lungs were collected from *wt* and IL-6–deficient mice 6 h after infection, and phosphorylation was assessed. IL-6 deficiency decreased phosphorylation.

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Table 1

Cytokine concentrations in the lungs.

Cytokine, time point, sample	*wt* mice	IL-6–deficient mice	P
IL-17, 24 h, lung	15 ± 2	67 ± 11	<.01
LIX, 6 h, BAL fluid	87 ± 12	122 ± 22	.2
LIX, 24 h, lung	550 ± 31	860 ± 56	<.01

NOTE. Cytokine concentrations in bronchoalveolar lavage (BAL) fluid or lung homogenates collected 6 or 24 h after the intratracheal instillation of *Escherichia coli* were measured by ELISA. Data are expressed in picograms per milliliter and are the means ± SE from 5−6 mice/group. IL, interleukin; LIX, lipopolysaccharide-induced CXC chemokine; *wt*, wild-type.

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Table 2

Effect of interleukin (IL)–6 on neutrophils and *Escherichia coli*.

	***wt* mice and vehicle**	**IL-6–deficient mice and vehicle**	**IL-6–deficient mice and rmIL-6**	***P*[a]**
Neutrophils	17 ± 2	10 ± 2	17 ± 4	.9
E. coli	36 ± 16	202 ± 51	257 ± 107	<.02

NOTE. Either vehicle or recombinant murine (rm) IL-6 at a dose of 100 ng/mouse was included with *E. coli* in the intratracheal instillates to wild-type *(wt)* or IL-6–deficient mice, and lungs were collected 24 h after infection. Emigrated neutrophils in alveolar air spaces are expressed as a percentage of the volume of alveolar parenchyma, and living bacteria are expressed as thousands of colony-forming units per lung. Data are the means ± SE from 3−4 mice/group (neutrophils) or 5−8 mice/group (bacteria). Analyses suggest that impaired neutrophil recruitment, but not bacterial clearance, in IL-6–deficient mice was restored by the administration of exogenous IL-6.

[a] *wt* mice and vehicle vs. IL-6–deficient mice and rmIL-6 (analysis of variance and Duncan's test).