Nuclear Factor-κB p50 Limits Inflammation and Prevents Lung Injury during *Escherichia coli* Pneumonia

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Inflammatory responses to infection must be precisely regulated to facilitate microbial killing while limiting host tissue damage. Many inflammatory genes are regulated by κB sites, and the p50 subunit of nuclear factor-κB suppresses the expression of κB-associated genes *in vitro*. We hypothesized that p50 is essential to prevent excessive inflammation and injury during infection. During pulmonary infection with *Escherichia coli*, the gene-targeted deficiency of p50 did not affect bacterial clearance from mouse lungs, but it resulted in increased expression of proinflammatory cytokines 6 to 24 hours after infection. This dysregulation exacerbated inflammation (neutrophil recruitment), respiratory distress (pulmonary edema and blood gas exchange impairment), and decompartmentalization (transit of protein and bacteria from the air spaces to the blood). We interpret these studies to indicate that endogenous p50 protects the host by curbing inflammatory responses to prevent injury, essential to survive pneumonia.

Keywords: acute lung injury; bacterial pneumonia; cytokines; neutrophil recruitment; transcription factors

Lung infections are responsible for the loss of more disability-adjusted life-years worldwide than any other disease (1, 2). The burden of this disease is disproportionately high in developing countries, but even in wealthy societies such as the United States the leading cause of death due to infection is respiratory infection (3).

Bacteria in the lungs induce the expression of multiple genes, determined by the combined actions of multi-subunit complexes recruited to the chromatin by DNA-binding proteins (4). Many genes elicited by bacteria in the lungs are regulated by κB sites in the DNA, which bind nuclear factor-κB (NF-κB) proteins (5). Gram-negative bacteria or LPS in the lungs of mice induces the nuclear translocation of NF-κB, including homodimers and heterodimers of RelA and p50 (6, 7).

RelA binds histone acetyl transferases, recruits coactivator complexes to κB sites, and induces gene expression (8, 9). RelA deficiency decreases the expression of chemokines and adhesion molecules induced by bacterial LPS in the lungs (10). Because chemokines and adhesion molecules mediate neutrophil emigration from the pulmonary capillaries (11), the inability to induce such gene expression compromises neutrophil recruitment and predisposes RelA-deficient mice to bacterial pneumonia (10).

In contrast to RelA, NF-κB p50 can either promote or suppress gene expression. p50 can use other nuclear proteins to recruit histone acetyl transferases and induce gene expression in at least some circumstances (12). However, in most cases, p50/p50 homodimers suppress gene expression. Facilitating p50 interactions with κB sites in the DNA decreases *in vitro* gene expression (13–17), and preventing p50 interactions with κB sites increases *in vitro* gene expression (16, 17). The suppression of gene expression by p50 is mediated in part by corepressor complexes containing histone deacetylases. p50 binds histone deacetylases directly (18) and via nuclear adapter proteins such as Bcl-3 (12). By these protein interactions, p50 recruits corepressor complexes to κB sites in the DNA and remodels the local chromatin to decrease expression of κB-associated genes (12, 18). Although mechanistic insight at the molecular level is emerging, the functional significance of p50-mediated inhibition of gene expression remains speculative.

Products of proinflammatory genes are dangerous, and their expression must be limited (19). Elevated levels of proinflammatory cytokines correlate with life-threatening inflammatory injuries, particularly septic shock and the acute respiratory distress syndrome (ARDS) (20–22). Exogenous proinflammatory cytokines can mimic the pathophysiology of these disease processes (23–27), and inhibition of the endogenous cytokines can prevent injury in experimental models of lung injury or sepsis (28–31). Thus, excessive levels of proinflammatory cytokines may be sufficient and necessary for inflammatory injury.

Many of the injurious proinflammatory genes are regulated by κB sites in the DNA (32). We postulated that an essential role for the suppression of κB-associated genes by p50 could be to limit the expression of dangerous genes to prevent inflammatory injury. This postulate was examined by testing the specific hypothesis that p50 deficiency increases pulmonary inflammation and injury during bacterial pneumonia, a common underlying cause of acute lung injury and ARDS (22). Some of the results of these studies have been previously reported in the form of an abstract (33).

METHODS

Mice

p50-deficient mice had targeted mutation of the *Nfkb1* gene (34). For all but one study, p50-deficient mice were on a random hybrid background (C57BL/6 × 129/Sv). For these studies, wild-type (WT) control mice were C57BL/6 × 129/Sv random hybrid generation F2 (Jackson Laboratory; Bar Harbor, ME) or F3 (progeny pf F2). A single study, as indicated, examined p50-deficient mice that had been backcrossed six generations (N6) onto the 129/Sv strain, compared with inbred 129/Sv mice. Mice were maintained under specific pathogen-free conditions in a full-barrier facility until experiments, at 6 to 10 weeks of age.

Intratracheal Instillations

After anesthesia with ketamine hydrochloride (100 mg/kg intramuscularly) and acepromazine maleate (5 mg/kg intramuscularly), the trachea was surgically exposed and 50 μl were instilled via an angiocatheter through the trachea to the left bronchus (30). Concentrations of *Escherichia coli* (strain 19138; American Type Culture Collection, Manassas,

VA) were determined by colony counting. Heat-killed *E. coli* contained no cfu. *E. coli* LPS (serotype 055:B5; Sigma Chemical, St. Louis, MO) was instilled at 100 µg/mouse. Instillates contained colloidal carbon (1%) to mark deposition. At the end of the experiments, mice were killed by inhalation of a lethal overdose of halothane.

Bacterial Viability

Viable bacteria were quantified by colony counting (6). Serial dilutions from blood or organs homogenized in sterile water were plated on blood agar, and cfu were enumerated after incubation at 37°C.

Cytokine Expression

Cytokine concentrations were quantified in bronchoalveolar lavage (BAL) fluid (30) and collected as detailed in the online supplement using ELISA (R&D Systems, Minneapolis, MN). The particular cytokines examined (KC, macrophage inflammatory protein-2 [MIP-2], tumor necrosis factor-α, interleukin [IL]-1β, and IL-6) were picked because (*1*) each of their genes contains functional κB sites (32), making them more likely to be directly influenced by loss of the NF-κB protein p50 and (*2*) each of these cytokines is associated with excessive lung injury in human and/or animal studies, making them more likely to be relevant to the physiologic results of p50 deficiency (30, 31, 35–37).

Emigrated Neutrophils

Neutrophil emigration was assessed in lungs fixed with 6% glutaraldehyde at 23 cm H_2O pressure 24 hours after instillation of 10^6 cfu *E. coli*. Neutrophils in alveolar air spaces and septae were quantified using morphometric analyses by investigators blinded to the genotypes of the mice (6). Circulating neutrophils were quantified using a hemacytometer and stained blood smears.

Extravasated Plasma

Anesthetized mice received intravenous injections of ^{125}I-labeled human albumin (Mallinckrodt Medical, Hazelwood, MO) 15 minutes before intratracheal instillations and of sodium chromate-51–labeled murine red blood cells 2 minutes before halothane overdose, as markers for plasma and blood content, respectively. The volume of extravascular plasma equivalents in the lungs was calculated (6, 30) as the total volume of plasma equivalents minus the volume of intravascular plasma (additional detail provided in online supplement).

Arterial Blood Gases

Blood was collected using dry heparanized syringes from the abdominal aorta in anesthetized mice. Blood gases were measured with a Ciba-Corning 248 Blood Gas Analyzer and corrected online for core body temperature recorded from a rectal probe. Body temperatures did not differ between genotypes (data not shown). Alveolar-arterial oxygen differences were calculated using the alveolar gas equation.

Protein Transit from Alveoli

Anesthetized mice received an intratracheal instillation of *E. coli* (10^6 cfu), ^{125}I-labeled human albumin (25 µg) as a radiotracer (38), and colloidal carbon (1%) in sterile saline. Control instillations had no bacteria. After 6 hours, mice were killed by halothane overdose and blood was withdrawn from the inferior vena cava. The volume concentration of intratracheal instillate in the peripheral blood (µl instillate/ml blood) was calculated as the cpm/ml blood divided by the cpm/µl instillate.

RESULTS

Mortality and Histopathology

Gram-negative enterobacteria, including *E. coli*, are common causes of pneumonia in hospitals and nursing homes (39, 40). When *E. coli* pneumonia was induced in mice by the intratracheal instillation of 10^6 cfu, most WT mice survived through 7 days

after infection (Figure 1A). Quantitative culture demonstrated 0.1% or less of the original inoculum remaining viable in the lungs from all mice surviving 7 days. In contrast, between 45 and 69 hours after infection with *E. coli*, all p50-deficient mice died (Figure 1A). Thus, a dose of bacteria that was effectively eliminated by WT mice was lethal to p50-deficient mice.

Postmortem tissues from p50-deficient mice exhibited consistent histopathology. The left lung lobe, into which the bacterial inoculum was instilled, demonstrated diffuse inflammation consistent with acute lung injury (Figure 1B). Emigrated leukocytes including neutrophils were evident in the air spaces of alveoli, in the perivascular interstitium, and in the air spaces and interstitium of the conducting airways. Diffuse pulmonary edema was indicated by polymerized fibrin deposition within alveolar air spaces and by expanded interstitial and lymphatic compartments. Focal hemorrhage and loss of architectural integrity were apparent. Bacterial rods, consistent with *E. coli*, were occasionally observed in the air spaces and were prevalent in the interstitial tissues and lymphatics surrounding large blood vessels and airways (Figure 1C). Right (noninstilled) lung lobes demonstrated less inflammation, although bacterial aggregates and inflammatory infiltrates were present focally (data not shown). Liver sections demonstrated partial depletion of glycogen stores and occasional fatty accumulations, consistent with metabolic stress, but few inflammatory cells (data not shown). Neither kidneys nor spleens demonstrated histologic evidence of local inflammation or injury (data not shown). Thus, the primary abnormalities apparent in histologic sections were in the lungs at the time of death of p50-deficient mice.
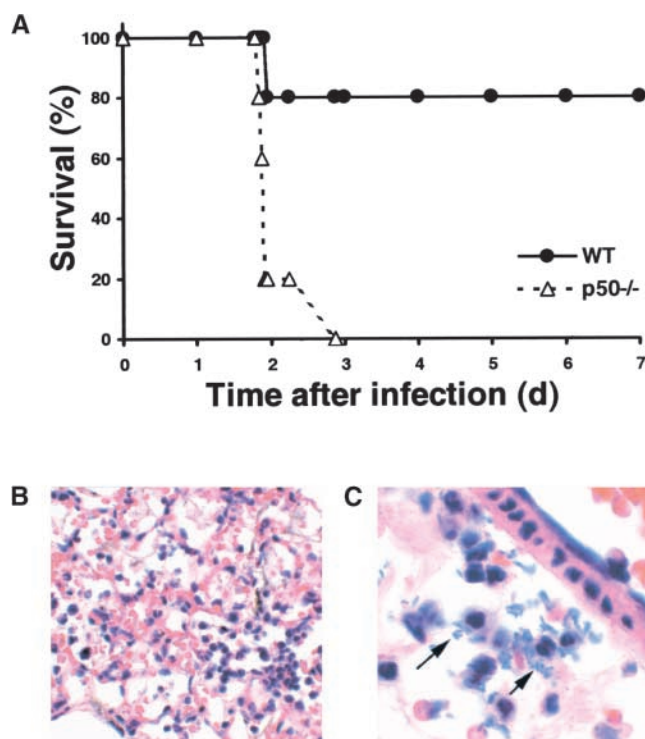

Figure 1. Mortality of p50-deficient mice during *Escherichia coli* pneumonia. (*A*) Survival through 7 days after intratracheal instillation of 10^6 cfu to five wild-type (WT) and five p50-deficient mice. (*B*) Low-power magnification of postmortem sample from the lungs of a p50-deficient mouse, demonstrating diffuse pulmonary inflammation consistent with acute lung injury. (*C*) Higher magnification of postmortem sample from the lungs of a p50-deficient mouse demonstrates bacterial rods, particularly in the peribronchial interstitial tissues.

Bacteria in the Lungs

Increased mortality during bacterial pneumonia may have resulted from a failure to control bacterial growth in the lungs or to prevent inflammatory injury to the lungs. To determine whether p50 deficiency compromised antibacterial host defenses in the lungs, living bacteria per lung were quantified by colony counting. When approximately 10^6 cfu/mouse were instilled, the dose that resulted in differential lethality in WT and p50-deficient mice (Figure 1A), both genotypes of mice decreased the number of living bacteria over a 24-hour period (Figure 2). There were no significant differences between genotypes. When 10^7 cfu/mouse were instilled, posing a more substantial challenge to innate immune host defenses, WT and p50-deficient mice prevented bacterial growth in the lungs for 6 hours, but bacteria proliferated to more than 20-fold the original inoculum by 24 hours (Figure 2). Again, there were no significant differences between genotypes. These data suggest that the clearance of intrapulmonary *E. coli* was not dependent on p50 through 24 hours.

To determine whether antibacterial host defenses required p50 at a later time point, WT and p50-deficient mice received a lower dose of *E. coli* (10^5 cfu), preventing the physiologic collapse and deaths of p50-deficient mice from confounding interpretations. After 48 hours, the median percentage of the original inoculum remaining viable did not significantly differ between WT and p50-deficient mice (5.0 and 3.1%, respectively). Altogether, these data indicate that p50-deficient mice did not differ from WT in constraining the growth of higher doses of *E. coli* in the lungs through 24 hours or in clearing lower doses of pulmonary *E. coli* through 48 hours. We cannot rule out the possibility that p50-deficient mice had a defect in clearance that was not revealed by these studies, but the data collected do not identify any effect of p50 deficiency on the pulmonary clearance of *E. coli*.

Expression of Cytokines

To determine how p50 deficiency affected the cytokine elaboration induced by bacteria in the lungs, cytokine concentrations were measured by ELISA in BAL fluids collected before and at several time points after infection with *E. coli*. There were no significant differences in cytokine concentrations before *E. coli* infection (Figure 3). Concentrations of KC, MIP-2, and tumor necrosis factor-α were each increased in the BAL fluids within 3 hours after infection (Figure 3). At this time, there were no significant differences in cytokine concentrations between
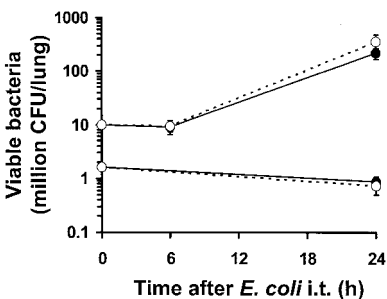
Figure 3. p50 deficiency alters cytokine levels during *Escherichia coli* pneumonia. Mice were infected by intratracheal instillation of 10^6 cfu *E. coli*, and bronchoalveolar lavage fluids (BALF) were collected from the lungs at designated time points. Concentrations (pg/ml) of macrophage inflammatory protein-2 (MIP-2), KC, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in BALF were quantified by ELISA. *Asterisks* indicate significant difference from wild-type (WT) values (two-way analysis of variance with Fisher's least significant difference test *post hoc*; n = 4 mice in each group at 0 hour, 4 mice in each group at 3 hours, 6 mice in each group at 6 hours, and 3 or 4 mice in each group at 24 hours).

Figure 2. Net efficacy of antibacterial host defenses in the lungs did not differ between wild-type (WT) and p50-deficient mice during *Escherichia coli* pneumonia. Mice were infected by intratracheal instillation of 10^6 or 10^7 cfu *E. coli*, as indicated. Lungs were collected and homogenized at the designated times, and the numbers of viable bacteria were quantified by colony counting. *Filled circles* represent WT mice (five or six mice at each time and dose), and *open circles* represent p50-deficient mice (four or five mice at each time and dose). WT and p50-deficient mice did not significantly differ by analysis of variance.
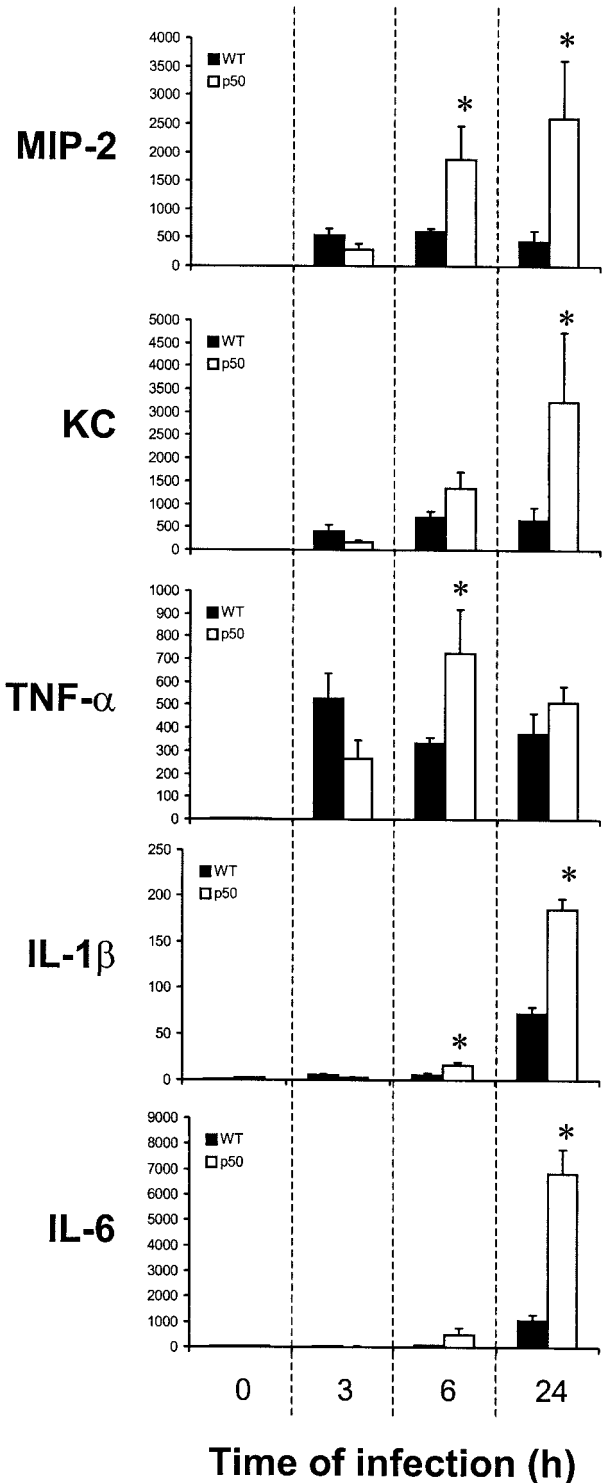
WT and p50-deficient mice (Figure 3), although there was a trend toward lower levels in the p50-deficient mice.

In contrast, 6 and 24 hours after infection, p50-deficient mice showed significantly elevated concentrations of cytokines in the BAL fluids compared with WT. After 6 hours of infection, concentrations of MIP-2, tumor necrosis factor-α, and IL-1β were significantly increased in the BAL fluids of p50-deficient mice compared with WT (Figure 3). After 24 hours, concentrations of MIP-2, KC, IL-1β, and IL-6 were significantly greater in the BAL fluids of p50-deficient mice compared with WT mice (Figure 3). These data suggest that, 6 to 24 hours after infection, p50 functions to limit the expression of proinflammatory cytokines during *E. coli* pneumonia.

Neutrophil Recruitment

Increased concentrations of cytokines in the lungs can exacerbate inflammation and injury. Emigrated neutrophils are a hallmark of acute inflammation and contribute to lung injury (41, 42). Neutrophil emigration was compared in WT and p50-deficient mice during *E. coli* pneumonia. In the absence of pulmonary infection, there were no neutrophils observed in the alveolar air spaces of WT or p50-deficient mice. Neutrophils did not significantly differ between WT and p50-deficient mice in the systemic venous blood ($354,000 \pm 48,000$ and $670,000 \pm 175,000$ neutrophils/ml, respectively) or the pulmonary capillaries (0.022 ± 0.013 and 0.037 ± 0.016 volume fraction of alveolar septae, respectively). *E. coli* induced neutrophil sequestration in the pulmonary capillaries of both WT and p50-deficient mice (0.241 ± 0.022 and 0.176 ± 0.034 volume fraction of alveolar septae, respectively), and the amount of sequestered neutrophils did not significantly differ between genotypes. *E. coli* in the lungs induced neutrophil emigration into the alveolar air spaces (Figure 4A). Significantly more emigrated neutrophils were observed in the lungs of p50-deficient mice compared with WT (Figure 4B). In addition, there were more neutrophils in the peripheral blood of pneumonic p50-deficient mice compared with WT (Figure 4C). Thus, neutrophil mobilization into the circulation and recruitment to the infected lung was increased by p50 deficiency.

Respiratory Distress

Acute lung injury and ARDS are defined by acute and diffuse inflammation in the lungs that results in pulmonary edema and blood gas exchange impairment (22). Pulmonary edema and arterial blood gases were compared in WT and p50-deficient mice. Although net bacterial survival in the lungs was not affected by p50 deficiency (Figure 2), the possibility that altered bacterial growth or metabolism could influence respiratory distress was formally excluded in these studies by using heat-killed *E. coli* as a stimulus. Heat-killed *E. coli* were instilled at the equivalent of 10^6 cfu/mouse, the dose of living bacteria that induced differential mortality (Figure 1A).

In the absence of bacterial stimuli, plasma extravasation did not differ between WT and p50-deficient mice (Figure 5A). Heat-killed *E. coli* increased the amount of extravascular plasma that accumulated in the lungs over a 6-hour period (Figure 5A). Heat-killed *E. coli* induced significantly more extravascular plasma accumulation in the lungs of p50-deficient mice compared with WT (Figure 5A). Thus, p50 deficiency resulted in greater pulmonary edema in response to nonviable bacterial stimuli in the lungs.

To determine whether excessive host responses due to p50 deficiency impaired pulmonary blood gas exchange, blood gases were measured in arterial blood samples from WT and p50-deficient mice after instillation of heat-killed *E. coli*. Arterial
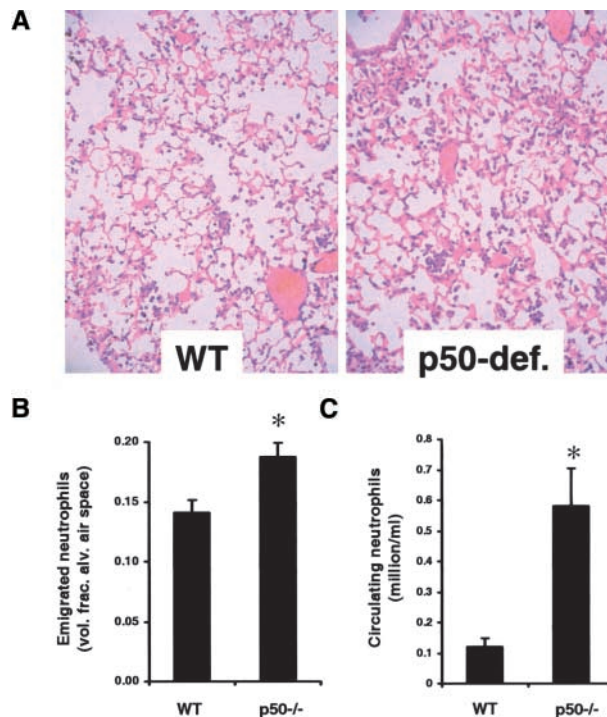

Figure 4. p50 deficiency increases neutrophil recruitment during *Escherichia coli* pneumonia. Lungs were fixed and venous blood collected 24 hours after the intratracheal instillation of 10^6 cfu *E. coli*. *Asterisks* indicate significant difference from wild type (WT) (Mann–Whitney *U* test; n = 6 mice in each group). (*A*) Histology of lungs, demonstrating emigrated neutrophils and polymerized fibrin in the alveolar air spaces. Lungs were fixed with glutaraldehyde, and 8-μm sections were stained with hematoxylin and eosin. (*B*) Emigrated neutrophils in the alveolar air spaces, quantified as volume fraction of the alveolar air space using morphometry. (*C*) Circulating neutrophils in the peripheral blood, quantified using a hemacytometer for leukocyte counts and stained blood smears for differential cell populations.

Pco_2 did not differ between genotypes (54.8 ± 3.8 and 55.0 ± 5.3 mm Hg in p50-deficient and WT mice, respectively), but the p50-deficient mice were more hypoxemic (Po_2 of 40.9 ± 5.4 and 58.7 ± 10.9 mm Hg in p50-deficient and WT mice, respectively). The alveolar–arterial oxygen gradient was worse in the p50-deficient mice compared with WT mice (Figure 5B), demonstrating that net pulmonary gas exchange was impaired to a greater degree in the p50-deficient mice. Thus, p50 deficiency increased respiratory distress in response to nonviable *E. coli* in the lungs, likely resulting from increased local inflammation and injury. Because these acute lung injury studies used heat-killed *E. coli*, we conclude that differential host response to the bacterial products (rather than different amounts or types of bacterial products) were responsible for the increased injury in p50-deficient mice.

Decompartmentalization

Respiratory distress is one important sequela of lung injury. Another important result of lung injury is decompartmentalization, in which the normally tight alveolar epithelial barrier is compromised and alveolar contents reach the circulating blood. This can exacerbate systemic inflammatory responses when proteins such as proinflammatory cytokines gain greater access to the circulation (38, 43). When bacteria gain access to the circulation, disseminated infection results (38, 44).

In the absence of bacterial stimuli, protein transit from the alveolar air spaces to the blood did not differ between WT and
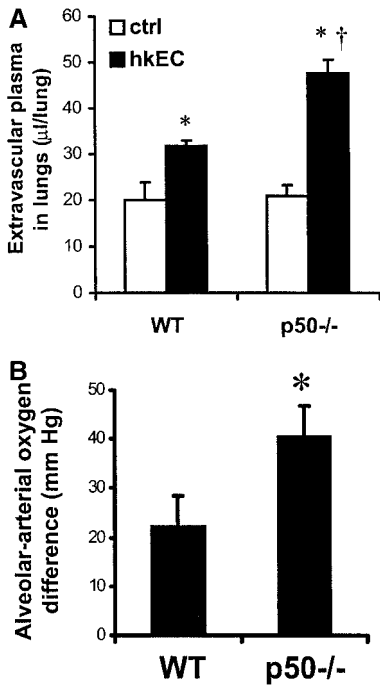
Figure 5. p50-deficiency increases acute lung injury elicited by heat-killed *Escherichia coli* (hkEC) in the lungs. (*A*) Pulmonary edema, quantified as volume of extravascular plasma in the lungs using radiotracers (^{125}I-albumin as a plasma marker and sodium chromate-51 murine red blood cells as a blood marker). The accumulation of extravascular plasma in the lungs was measured over a 6-hour period in mice that did (indicated as hkEC) or did not (indicated as ctrl) receive intratracheal instillation of hkEC at a dose equivalent to 10^6 cfu. *Asterisks* indicate significant difference from ctrl mice of that genotype and *dagger* indicates significant differences from wild-type (WT) values at that time point (two-way analysis of variance with the Fisher's least significant difference test *post hoc*; n = 5 mice in each ctrl group, and 6 or 7 mice in each hkEC group). (*B*) Pulmonary blood gas exchange impairment. Blood was collected from the abdominal aorta 24 hours after the intratracheal instillation of hkEC at a dose equivalent to 10^6 cfu. Blood gases were measured and corrected online for core body temperature, and alveolar-arterial oxygen differences were calculated from Pa_{O_2} and Pa_{CO_2} using the alveolar gas equation. *Asterisks* indicate significant difference from WT values (Mann–Whitney *U* test; n = 5 or 6 mice in each group).

p50-deficient mice (Figure 6A). *E. coli* increased the amount of protein transit to the blood (Figure 6A). Significantly, more protein from the alveolar air spaces was circulating in the blood of p50-deficient mice compared with WT mice after infection (Figure 6A). Thus, p50 deficiency increased decompartmentalization, as measured by transit of albumin from the alveolar space to the blood, during bacterial pneumonia.

To determine whether decompartmentalization led to disseminated infection, living bacteria were quantified in the extrapulmonary tissues after intrapulmonary infection with *E. coli*. Living bacteria were detected in at least one extrapulmonary tissue in three of eight WT mice and in all eight of eight p50-deficient mice (p < 0.01). Furthermore, greater numbers of bacteria were observed in every extrapulmonary tissue examined from p50-deficient mice compared with WT mice (Figure 6B). Thus, although intrapulmonary bacterial content was not different in p50-deficient mice compared with WT mice (Figure 2), p50 deficiency increased the frequency and severity of disseminated infection during bacterial pneumonia.

Influence of Genetic Background

The studies described previously here used mice with a random hybrid genetic background (C57BL/6 × 129/Sv). A subset of studies was repeated with p50-deficient mice that had been backcrossed onto a 129/Sv background for six generations, to better determine whether mutation in the gene for p50 was responsible for the phenotypic differences observed. A set of six 129/Sv mice and five p50-deficient mice on a 129/Sv background received intratracheal instillations of 10^6 cfu *E. coli*, as described pre-
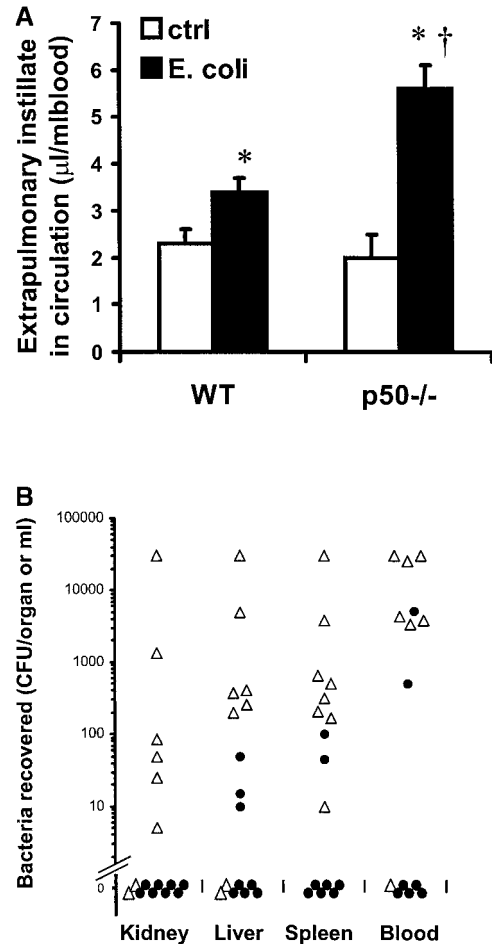

Figure 6. p50-deficiency exacerbates decompartmentalization during *Escherichia coli* pneumonia. (*A*) Transit of protein from the alveoli to the blood. Mice received intratracheal instillations of radiotracer (^{125}I-albumin) that did (indicated as *E. coli*) or did not (indicated as ctrl) also include 10^6 cfu *E. coli*. Venous blood was collected 6 hours later, and the blood concentration of intratracheally instilled protein was quantified using a γ counter. *Asterisks* indicate significant difference from ctrl values within genotype, and *dagger* indicates significant differences from wild-type (WT) values (two-way analysis of variance with the Fisher's least significant difference test *post hoc*; n = 3 or 4 mice in each group). (*B*) Dissemination of bacteria to extrapulmonary tissues. Mice received intratracheal instillations of 10^6 cfu *E. coli*. Venous blood samples and indicated organs were collected 24 hours later, and the numbers of viable bacteria per volume diluted blood or organ homogenate were quantified by colony counting. Each point represents the value from an individual mouse. Extrapulmonary infection was significantly more frequent in p50-deficient mice (*open triangles*) compared with WT (*closed circles*) (corrected Pearson χ^2 test; n = 7 or 8 mice per group). Significantly, more cfu were observed in p50-deficient mice compared with WT for each tissue (Mann–Whitney *U* test; n = 7 or 8 mice per group).

viously. BAL fluids and blood samples were collected after 24 hours of infection. IL-6 concentrations were measured in the BAL fluids, and living bacteria were quantified in the blood. IL-6 was significantly elevated in p50-deficient mice compared with WT mice (5,732 ± 1,164 and 2,331 ± 234 pg/ml, respectively; p < 0.05, Mann–Whitney *U* test). Only one of six WT mice (17%) was bacteremic, with 500 cfu/ml blood. In contrast, three of five p50-deficient mice (60%) were bacteremic (p < 0.05, corrected Pearson χ^2 test), with 1,100, 1,600, or 3,900 cfu/ml blood. Thus, p50 deficiency in a 129/Sv background increased

IL-6 expression and bacterial dissemination, similar to the observations made with pneumonic p50-deficient mice on the hybrid background (Figures 3 and 6B). These data suggest that mutation in the gene for p50 increased gene expression and exacerbated lung injury in mice that had been backcrossed six generations as well as in mice with random hybrid backgrounds.

DISCUSSION

During *E. coli* pneumonia, p50 deficiency increased mortality. p50 deficiency did not alter the numbers of living bacteria in the lungs at any time point or dose examined. However, p50 deficiency increased inflammation in the lungs, including elevated levels of cytokines and increased neutrophil recruitment. This increase in inflammation exacerbated pulmonary edema and blood gas exchange impairment, suggesting that respiratory distress may have contributed to the deaths of p50-deficient mice with pneumonia. In addition, p50 deficiency increased the leak of protein and bacteria from the lungs, causing bacteremia and multiple organ infection. Increased systemic infection may also have contributed to the mortality of p50-deficient mice with pneumonia. Because the absence of p50 exacerbates inflammation, lung injury, respiratory distress, and systemic infection, we conclude that p50 limits inflammatory injury during pneumonia.

p50 is a transcription factor that binds κB sequences in the DNA and regulates gene expression. The physiologic effects of p50 deficiency likely result from essential roles for p50 in regulating the expression of κB-associated genes. Although p50 is only one of many transcription factors that bind the promoter regions regulating the expression of these inflammatory genes, the absence of p50 was sufficient to alter the concentrations of κB-regulated cytokines in the lungs during *E. coli* pneumonia.

Within 2 hours after the intratracheal instillation of *E. coli* LPS to mice, the most prominent NF-κB complexes detected in electrophoretic mobility shift assays are p50/RelA heterodimers (7). The deficiency of RelA significantly decreases the early expression of multiple κB-associated genes induced by *E. coli* products in the lungs (10). p50 deficiency did not significantly decrease cytokine concentrations measured after 3 hours of *E. coli* pneumonia, although there was a tendency for diminished levels of cytokines that did not reach statistical significance. If p50 has any essential role within the first hours of infection, it may be as a partner with RelA in facilitating the earliest gene induction. However, neither p50 nor RelA completely abolishes this gene induction, suggesting that no homodimer or heterodimer of these two NF-κB proteins is absolutely essential for gene induction by gram-negative bacterial stimuli in the lungs. Transcription factors other than NF-κB, such as activator protein-1 (AP-1) or CCAAT/enhancer binding protein-β (C/EBP-β), may induce this gene expression.

By 6 hours after the intratracheal instillation of *E. coli* LPS to mice, p50/p50 homodimers increase dramatically within the nuclei of the lungs (7). p50/p50 homodimers decrease gene expression *in vitro* (12–18). At the 6- and 24-hour time points, concentrations of κB-regulated cytokines (including KC, MIP-2, tumor necrosis factor-α, IL-6, and IL-1β) were significantly increased in the lungs of p50-deficient mice compared with WT mice. Thus, we propose that p50/p50 homodimers have an essential role in limiting the later gene expression during *E. coli* pneumonia.

The excessive expression of κB-associated genes may be responsible for inflammatory injury to the lungs. Tumor necrosis factor-α and IL-1 are necessary and sufficient for injury in laboratory animal models of inflammation (23–30). Neutrophil-recruiting CXC chemokines such as human IL-8 and mouse KC and MIP-2 correlate with lung injury in human patients (35, 36) and are

essential to ventilator-induced lung injury in mice (31). IL-6 levels in the lungs significantly correlate with death due to ARDS (37) and prevention of excessive IL-6 during sepsis decreases mortality (45). Each of these inflammatory cytokines is regulated by κB sites (32) and was increased by p50 deficiency in the present studies. Thus, preventing the overexpression of these cytokines may be an essential function of p50 in preventing lung injury during pneumonia.

Alternatively, the lung injury of p50-deficient mice may be mediated by overwhelming inflammatory dysregulation due to overexpression of not one or several but many κB-regulated genes. Patients with sepsis and ARDS present with dysregulations of multiple and diverse inflammatory mediators (22, 46, 47). Thus far, these patients have proved relatively refractory to potential therapies interfering with individual molecules (22, 46, 47). To the degree that the lung injury of p50-deficient mice with pneumonia reflects this multifaceted pathogenesis, this experimental situation may prove especially appropriate for elucidating the integrated signaling networks contributing to acute lung injury.

In addition to an essential role for p50 in limiting the acute expression of proinflammatory cytokines, other factors may have contributed to excess inflammation in p50-deficient mice. First, physiologic changes due to the lifelong absence of p50 may affect phenotypic responses to infection. For example, p50-deficient mice have defective immunoglobulin class switching and decreased circulating immunoglobulins (34, 48, 49). Although specific antibodies were not likely elicited within the 6- to 24-hour time frame of the present experiments, acute inflammatory responses elicited by bacteria in the lungs could be affected by natural antibodies circulating before infection, which could differ in the WT and p50-deficient mice. Second, in addition to p50, the precursor protein p105 is absent in p50-deficient mice (Reference 34 and personal observations). p105 may have independent functions from p50 (50, 51), which could contribute to the prevention of inflammatory injury. Third, p50 may be essential to the expression of genes that are antiinflammatory during *E. coli* pneumonia. It has been suggested that NF-κB–dependent gene expression is essential to a negative feedback loop that limits the inflammatory process during carrageenin-induced pleurisy (52). The induction of antiinflammatory mediators could directly or indirectly depend on p50 function. Fourth, genetic variability could have contributed to differences between WT and p50-deficient mice because most of these studies used mice on a random hybrid background. However, the experiments that were conducted in mice that had been backcrossed six generations support the conclusion that p50 deficiency rather than other variations in the genome were responsible for the phenotypic effects observed.

The prevention of injury by endogenous p50 in mice with experimental pneumonias may have implications for human patients with or at risk for acute lung injury. The databases of the National Center for Biotechnology Information (Bethesda, MD) report dozens of polymorphisms in the human gene for p50, *Nfkb1*. If polymorphisms influence p50 expression or activity, a reasonable and testable hypothesis will be that such polymorphisms alter susceptibility to acute lung injury and ARDS. Therapeutically, increasing p50 or p50-like activity may be an avenue for preventing or treating acute lung injury. As the molecular mechanisms by which p50 functions to limit gene expression are further elucidated, it may be possible to rationally design drugs to mimic these activities of p50 in the lungs.

In addition to *E. coli* pneumonia, p50 is critical during infections of other organs with other bacteria. After peritoneal infection with *Streptococcus pneumoniae*, p50-deficient mice died more quickly than WT mice. During a similar peritoneal *S. pneumoniae*

infection, mice heterozygous for p50 as well as Bcl-3 genes had increased mortality and bacteremia compared with mice WT for p50 and heterozygous for Bcl-3 (53). During gastrointestinal *Helicobacter hepaticus* infection, p50-deficient mice developed worse lesions with elevated cytokine expression (54). Thus, p50 is protective during diverse bacterial infections.

The present studies identify NF-κB p50 as essential for preventing inflammatory lung injury during *E. coli* pneumonia, an important infection in hospitals and nursing homes (39, 40). The p50-mediated suppression of κB-associated genes may prevent inflammatory injury during other infections and may prevent acute lung injury resulting from noninfectious disorders such as aspiration, mechanical ventilation, and trauma. Finally, because p50 is ubiquitously expressed and activated by a wide variety of stresses, this newly identified tissue-protective antiinflammatory function of p50 may be broadly applicable and relevant to diseases unrelated to bacterial infections or lung injury.

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