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NF-κB p50 facilitates neutrophil accumulation during LPS-induced pulmonary inflammation

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Abstract

Background: Transcription factors have distinct functions in regulating immune responses. During *Escherichia coli* pneumonia, deficiency of NF-κB p50 increases gene expression and neutrophil recruitment, suggesting that p50 normally limits these innate immune responses. p50-deficient mice were used to determine how p50 regulates responses to a simpler, non-viable bacterial stimulus in the lungs, *E. coli* lipopolysaccharide (LPS).

Results: In contrast to previous results with living *E. coli*, neutrophil accumulation elicited by *E. coli* LPS in the lungs was decreased by p50 deficiency, to approximately 30% of wild type levels. Heat-killed *E. coli* induced neutrophil accumulation which was not decreased by p50 deficiency, demonstrating that bacterial growth and metabolism were not responsible for the different responses to bacteria and LPS. p50 deficiency increased the LPS-induced expression of κB-regulated genes essential to neutrophil recruitment, including KC, MIP-2, ICAM-1, and TNF-α suggesting that p50 normally limited this gene expression and that decreased neutrophil recruitment did not result from insufficient expression of these genes. Neutrophils were responsive to the chemokine KC in the peripheral blood of p50-deficient mice with or without LPS-induced pulmonary inflammation. Interleukin-6 (IL-6), previously demonstrated to decrease LPS-induced neutrophil recruitment in the lungs, was increased by p50 deficiency, but LPS-induced neutrophil recruitment was decreased by p50 deficiency even in IL-6 deficient mice.

Conclusion: p50 makes essential contributions to neutrophil accumulation elicited by LPS in the lungs. This p50-dependent pathway for neutrophil accumulation can be overcome by bacterial products other than LPS and does not require IL-6.

Background

The innate immune response to bacteria in the lungs requires the recruitment and activation of neutrophils, mediated by the coordinated expression of diverse genes (see [1] for review). In rodents, neutrophils recognize at least 2 chemokines (KC and macrophage inflammatory protein-2, MIP-2) that are synthesized *de novo* in response to gram-negative bacteria or LPS in the lungs, and each is independently essential to maximal neutrophil recruitment [2-4]. Neutrophils also recognize the adhesion molecule intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is constitutively expressed, but LPS and gram-negative bacteria in the lungs result in increased expression [5,6] and ICAM-1 is required for maximal neutrophil emigration [7,8]. In addition, the early response cytokines TNF-α and IL-1β are synthesized in response to LPS or gram-

negative bacteria in the lungs, and receptors for these cytokines are essential to neutrophil emigration [9].

The coordinated expression of diverse genes may be mediated in part by common transcription factors. All of the above genes contain κB sites in their 5'-untranslated promoter regions, and mutation of these sites inhibits the inducible expression of reporter genes driven by these promoter regions (see reference [10] and references therein). Thus, NF-κB proteins may mediate the LPS-induced expression of genes controlling neutrophil emigration in the lungs.

LPS in the lungs induces the nuclear translocation of the NF-κB proteins RelA (also known as p65) and p50 [11,12]. RelA contains a transactivation domain which recruits coactivator complexes, increasing gene expression [13,14]. The genetic deficiency of RelA inhibits the LPS-induced pulmonary expression of the chemokines KC and MIP-2 and the adhesion molecule ICAM-1, resulting in decreased neutrophil emigration [15]. Thus, RelA is essential to the gene expression mediating neutrophil emigration induced by LPS in the lungs. p50 is more complex, capable of either increasing or decreasing gene expression *in vitro* [16-24]. During *E. coli* pneumonia, the deficiency of p50 increases the expression of multiple κB-regulated genes, resulting in increased neutrophil recruitment and excessive inflammatory injury [25].

In the present manuscript, we report that p50 deficiency had a very different effect on neutrophil recruitment elicited by *E. coli* LPS in the lungs. In marked contrast to *E. coli*, *E. coli* LPS elicited neutrophil accumulation which was significantly decreased by p50 deficiency. p50 deficiency did not diminish neutrophil accumulation elicited by heat-killed *E. coli*, indicating that p50 dependency could be overcome by bacterial products other than LPS. Peripheral blood neutrophils remained responsive to the chemokine KC in p50-deficient mice, arguing against a functional desensitization of chemokine receptors. IL-6 was overexpressed in p50-deficient lungs, and IL-6 can decrease neutrophil recruitment to intrapulmonary LPS [26,27], but excessive IL-6 was not responsible for the decreased neutrophil recruitment of p50-deficient mice. Although the mechanism remains unclear, these studies demonstrate for the first time that p50 facilitates neutrophil accumulation in response to some stimuli in the lungs, including *E. coli* LPS.

Results

Effect of p50 deficiency on LPS-induced neutrophil recruitment

Neither WT nor p50-deficient mice have neutrophils in their air spaces in the absence of experimental pulmonary inflammation [25]. The intratracheal instillation of *E. coli* LPS elicited neutrophil emigration in both genotypes, but there were significantly fewer emigrated neutrophils in the alveolar air spaces of p50-deficient mice compared to WT (Figures 1A,1B,1C). There was no difference between genotypes in neutrophils within the alveolar septae of p50-deficient mice (Figure 1D), and there were significantly more neutrophils in the peripheral blood of p50-deficient mice compared to WT during LPS-induced pulmonary inflammation (Figure 1E). Therefore, the decreased neutrophils in alveolar air spaces did not result from inadequate delivery of neutrophils to pulmonary capillaries. These data suggest that p50 deficiency may compromise transit from the pulmonary capillaries to the alveolar air spaces.

Effect of p50 deficiency on neutrophil recruitment elicited by heat-killed E. coli

The observation that p50 deficiency decreased neutrophil recruitment elicited by *E. coli* LPS in the lungs was surprising in light of previous studies indicating no such effect (or in fact the opposite effect) of p50 deficiency on neutrophil recruitment elicited by *E. coli* in the lungs [25]. Differing responses to LPS and living bacteria could be due to bacterial products other than LPS, or to the growth and metabolism of the bacteria. To determine whether bacterial products other than LPS were capable of overcoming p50 dependency in the absence of bacterial growth and metabolism, WT and p50-deficient mice received instillations of heat-killed *E. coli*. Neutrophils were observed in the alveolar air spaces after the instillation of heat-killed *E. coli*, and there were no significant differences between genotypes (Figure 2). Therefore, in contrast to *E. coli* LPS (Figure 1B), heat-killed *E. coli* induced neutrophil recruitment which does not require p50. These data indicate that other *E. coli* molecules are sufficient to bypass or overcome the p50-dependent pathway of neutrophil recruitment elicited by LPS in the lungs.

Increased LPS-induced gene expression due to p50 deficiency

p50 could be essential to neutrophil recruitment if it induces the expression of κB-regulated genes which mediate LPS-induced neutrophil recruitment, such as ICAM-1 [8], KC [2], or MIP-2 [4]. There were no significant differences in ICAM-1 RNA prior to LPS instillation (Figure 3). LPS increased ICAM-1 RNA in both WT and p50-deficient mice (Figure 3), and there was significantly more ICAM-1 in LPS-inflamed p50-deficient lungs compared to WT (Figure 3). Thus, LPS-induced ICAM-1 expression was increased by p50 deficiency, arguing against an essential role for p50 in inducing this gene.

Previous data indicated no detectable KC or MIP-2, measured at the protein level by ELISA, in bronchoalveolar lavage fluids (BALF) from WT or p50-deficient mice without

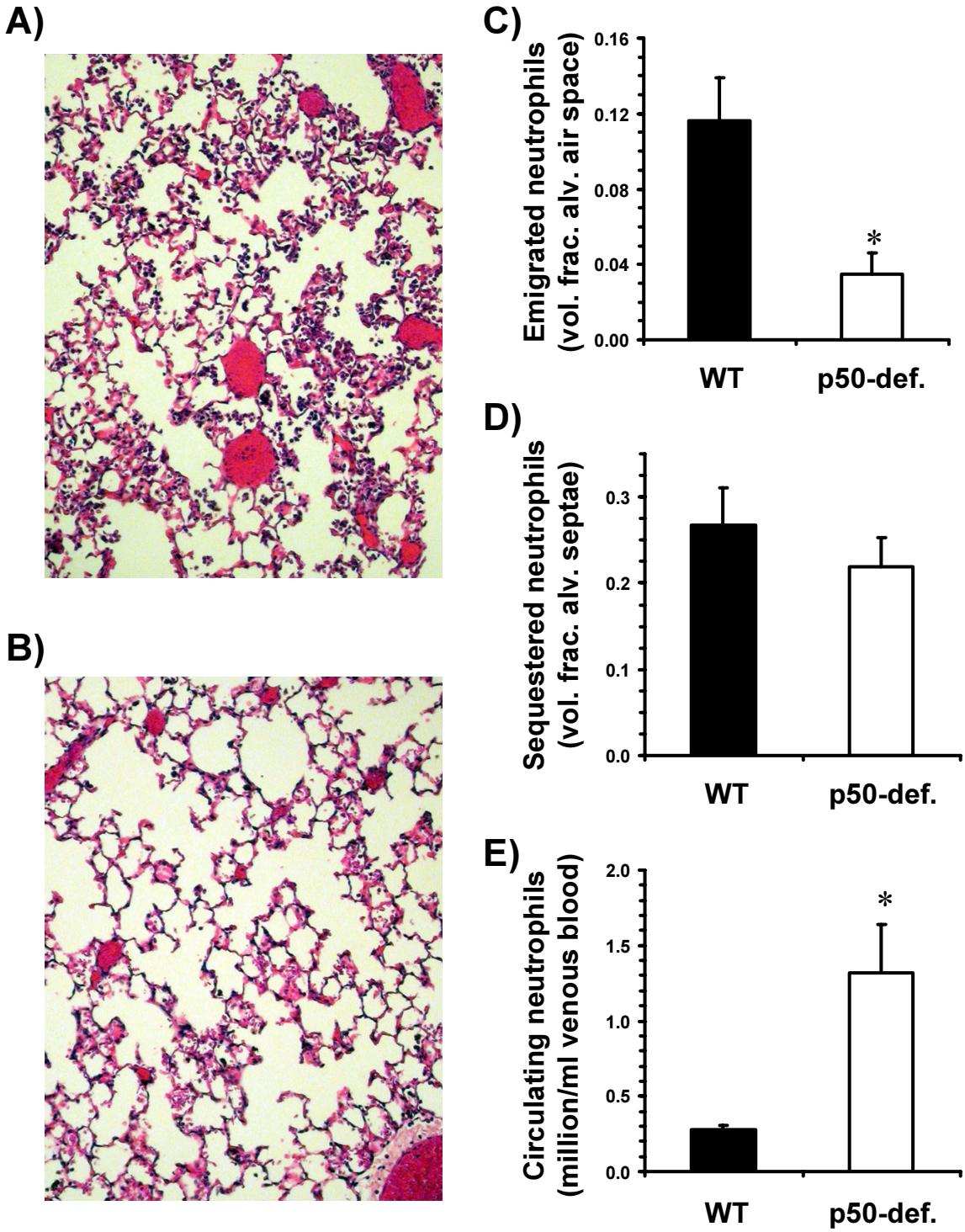

Figure 1

Effect of p50 deficiency on neutrophil recruitment elicited by LPS in the lungs. *E. coli* LPS was instilled intratracheally to WT and p50-deficient mice, and lungs and blood were collected after 24 hours. Representative histologic images from lungs of WT and p50-deficient mice are shown (A and B, respectively). Emigrated neutrophils (C) in the alveolar air spaces, and sequestered neutrophils (D) in the alveolar septae, were quantified using morphometric analyses of histologic sections. Circulating neutrophils (E) were quantified in blood samples collected from the inferior vena cava. Asterisks (*) denote significant differences from WT mice.

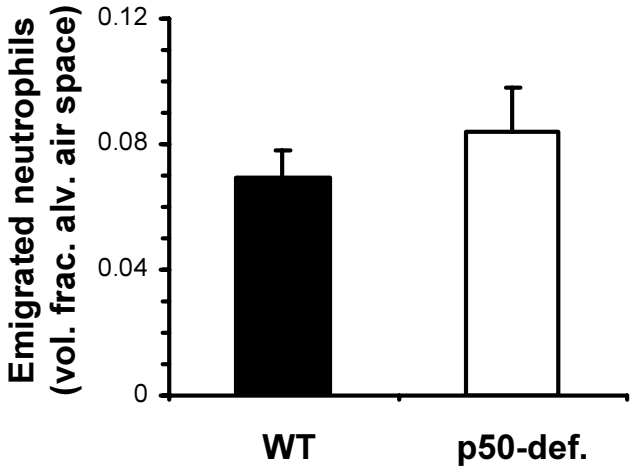

Figure 2
Effect of p50 deficiency on neutrophil recruitment elicited by heat-killed *E. coli* in the lungs. *E. coli* that had been killed by autoclaving (as indicated by an inability to grow in culture) were instilled intratracheally to WT and p50-deficient mice, and lungs were collected after 24 hours. Emigrated neutrophils in the alveolar air spaces were quantified using morphometric analyses of histologic sections. There were no significant effects of p50 deficiency.

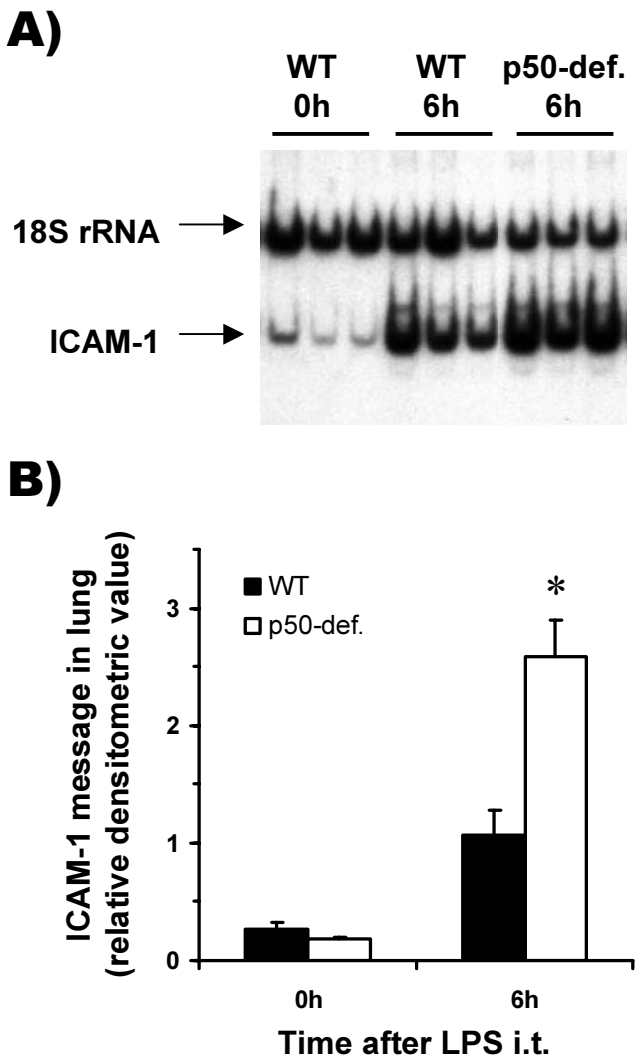

Figure 3
Effect of p50 deficiency on ICAM-1 expression in the lungs. cDNA was prepared from RNA in lungs collected from WT and p50-deficient mice. ICAM-1 expression was measured by semi-quantitative multiplex RT-PCR. The autoradiograph (A) shows representative samples, with each lane containing amplicons of ICAM-1 message and 18S rRNA from cDNA reverse transcribed from lung RNA of an independent mouse. The graph (B) depicts ICAM-1 message for each sample expressed relative to the densitometric value of 18S rRNA from the same PCR reaction tube. Bars depict mean and SEM from 4–7 mice per group, and asterisk (*) denotes significant difference from WT mice.

experimental inflammation [25]. Thus, p50 deficiency does not affect basal expression of these chemokines in the lungs. Both chemokines were detectable in all mice 6 and 24 hours after LPS instillation (Figure 4A,4B). There were significantly more of these neutrophil chemokines in BALF from p50-deficient mice compared to WT at the 6 hour time point (Figure 4A,4B). These data suggest that, as during *E. coli* pneumonia, p50 serves to limit rather than augment the expression of these chemokines. Therefore, insufficient expression of ICAM-1, KC, and MIP-2 was not responsible for the effect of p50 deficiency on neutrophil recruitment elicited by LPS in the lungs.

TNF-α and IL-1β contribute to neutrophil recruitment elicited by *E. coli* in the lungs [9]. There is little TNF-α (<10 pg/ml) or IL-1β (<4 pg/ml) in BALF from WT or p50-deficient mice without experimental inflammation, and no significant differences between genotypes [25]. These cytokines were elaborated in response to *E. coli* LPS (Figure 4C,4D). The concentrations of TNF-α were elevated in the BALF from p50-deficient mice compared to WT (Figure 4C). For IL-1β concentrations in the BALF, there was no significant effect of p50 deficiency (Figure 4D). Thus, decreased neutrophil recruitment did not result from insufficient elaboration of these early response cytokines.

Some cytokines, such as MIP-2 and TNF-α, are expressed very rapidly in response to LPS in the lungs [28,29]. Therefore, we also examined the concentrations of these cytokines at an earlier time point, 3 hours after LPS instillation. Both cytokines were detectable in all mice

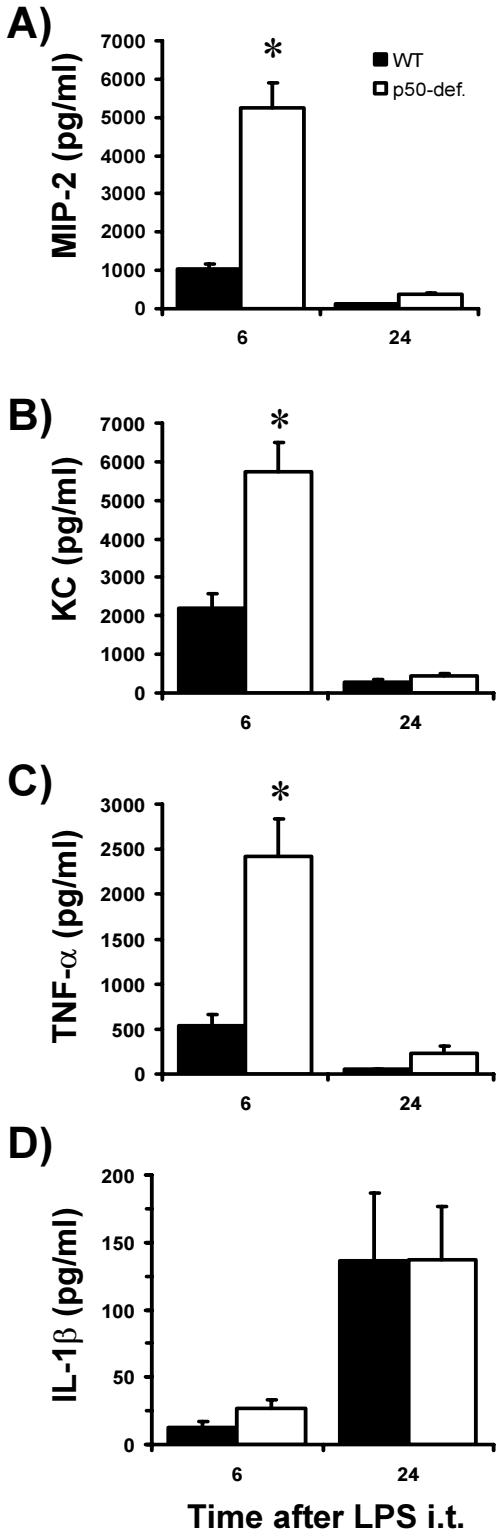

Figure 4

Effect of p50 deficiency on cytokine concentrations in the air spaces of the lungs during LPS-induced pulmonary inflammation. Bronchoalveolar lavage fluids were collected from WT and p50-deficient mice after *E. coli* LPS was instilled intratracheally. Concentrations of MIP-2 (A), KC (B), TNF-α (C), and IL-1β (D) in the lavage fluids were quantified by ELISA. Asterisks (*) denote significant differences from WT mice.

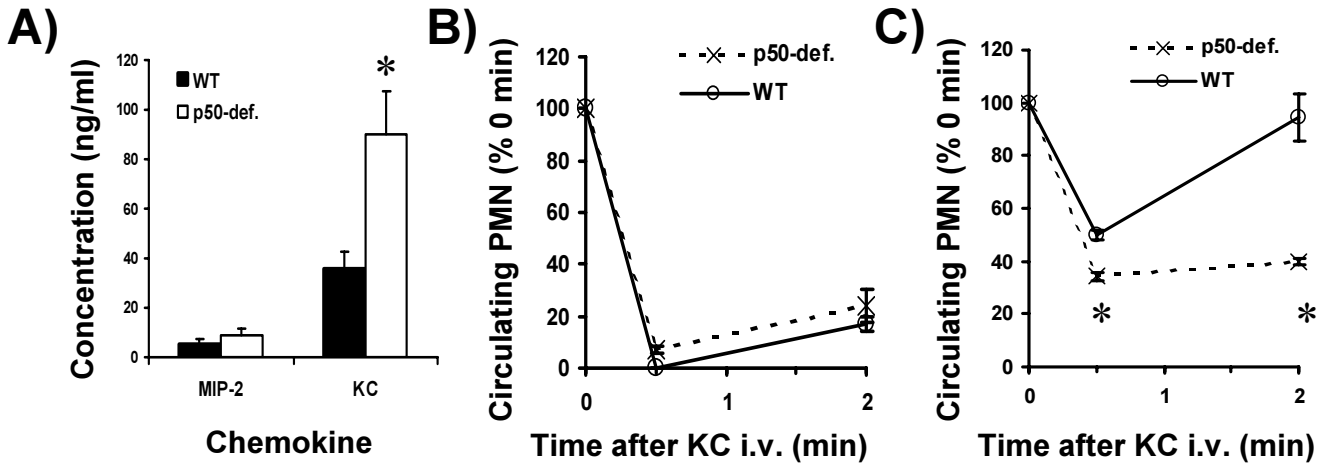

Figure 5

Effect of p50 deficiency on chemokine responsiveness of blood neutrophils. (A) Effect of p50 deficiency on concentrations of neutrophil chemokines in the blood. *E. coli* LPS was instilled intratracheally to WT and p50-deficient mice, blood was collected after 6 hours, and the concentrations of MIP-2 and KC were quantified by ELISA. Asterisks (*) denote significant differences from WT mice. (B) Effect of p50 deficiency on the ability of blood neutrophils to respond to the chemokine KC. Since chemoattractants induce cell stiffening which results in neutropenia, circulating neutrophils (PMN) were counted before and after the intravenous injection of recombinant murine KC. Circulating neutrophils were quantified by differential counting of blood samples collected from the inferior vena cava. There were no significant effects of p50 deficiency. (C) Effect of p50 deficiency on the ability of blood neutrophils to respond to the chemokine KC during LPS-induced pulmonary inflammation. *E. coli* LPS was instilled intratracheally to WT and p50-deficient mice, and 6 hours later neutrophil responsiveness to recombinant murine KC was assessed as described above. Asterisks (*) denote significant differences from WT mice.

examined (MIP-2 at concentrations of 367 ± 120 and 194 ± 70 pg/ml, and TNF-α at concentrations of 1268 ± 238 and 655 ± 70 pg/ml, in WT and p50-deficient mice, respectively). Concentrations did not significantly differ between WT and p50-deficient mice at this time point. Furthermore, concentrations of these cytokines at 3 hours failed to reach the values attained by p50-deficient mice at 6 hours, supporting the concept that p50 deficiency resulted in an overall increase in these cytokines in the air spaces after LPS exposure.

Capacity of p50-deficient neutrophils to respond to chemokine

Neutrophils can be desensitized to chemoattractants by exposure to excessive concentrations of them [30-32]. During LPS-induced pulmonary inflammation, circulating neutrophils in p50-deficient mice get exposed to increased levels of KC in the plasma (Figure 5A). Thus, p50-deficient neutrophils may get desensitized to the effects of KC. To determine whether neutrophils in the circulating blood of WT and p50-deficient mice could respond to this chemokine, neutropenia induced by intravenous KC was compared in WT and p50-deficient mice with and without LPS-induced pulmonary inflammation. In the absence of pulmonary inflammation, intravenous KC rapidly induced a near complete neutropenia in both WT and p50-deficient mice (Figure 5B). At least 93% of circulating neutrophils were responsive to KC in both genotypes (Figure 5B). Neutropenia induced by KC did not significantly differ between genotypes (Figure 5B). After 6 hours of LPS-induced pulmonary inflammation, intravenous injection of KC induced a more moderate neutropenia compared to in the absence of pulmonary inflammation (Figure 5C). Intravenous KC induced greater neutropenia in p50-deficient mice than in WT mice (Figure 5C). Thus, during LPS-induced pulmonary inflammation, a greater fraction of circulating neutrophils were responsive to KC in p50-deficient mice compared to WT mice. These data suggest that the decreased emigration of p50-deficient neutrophils did not result from an inability of blood neutrophils to respond to chemokines.

Effect of p50 deficiency on LPS-induced neutrophil recruitment in the absence of IL-6

IL-6 is a κB-regulated factor which decreases LPS-induced neutrophil recruitment in the lungs [26,27]. In the absence of experimental inflammation, IL-6 concentrations in BALF of WT and p50-deficient mice are low (<60 pg/ml) and not different between genotypes [25]. IL-6

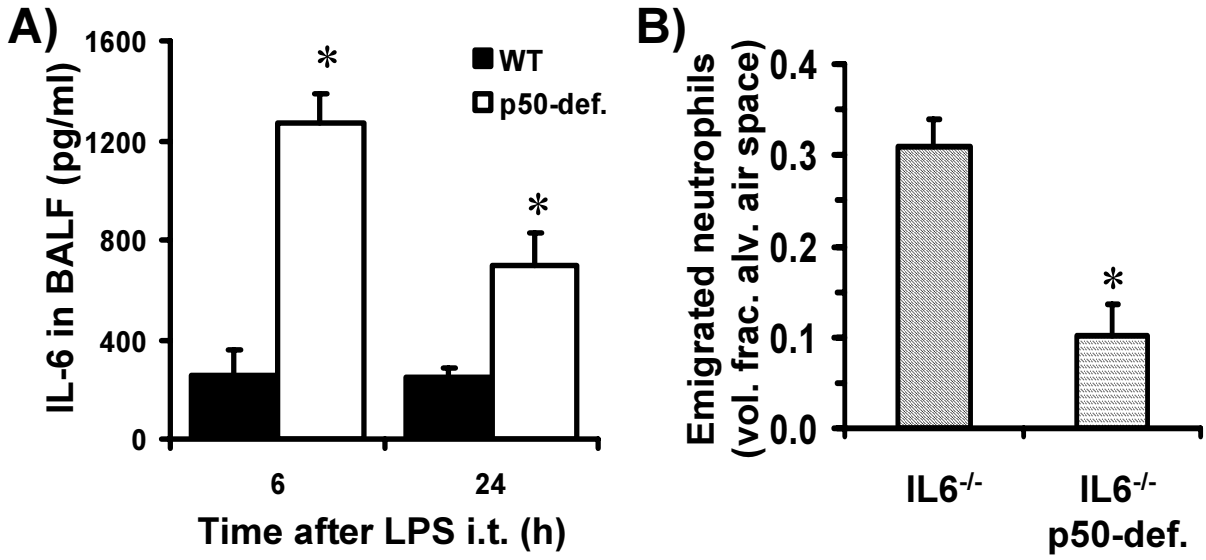

Figure 6

Role of IL-6 in the decreased neutrophil recruitment of p50-deficient mice during LPS-induced pulmonary inflammation. (A) Increased IL-6 in the lungs of p50-deficient mice. *E. coli* LPS was instilled intratracheally to WT and p50-deficient mice, bronchoalveolar lavage fluids were collected, and IL-6 concentrations were measured by ELISA. Asterisk (*) denotes significant difference from WT mice. (B) Effect of p50 deficiency on LPS-induced neutrophil recruitment in the absence of IL-6. IL-6-deficient and p50-deficient mice were crossed to generate double mutant mice deficient in both IL-6 and p50. *E. coli* LPS was instilled intratracheally to IL-6-deficient mice and double mutant mice deficient in both IL-6 and p50, and lungs were collected after 24 hours. Emigrated neutrophils in the alveolar air spaces were quantified using morphometric analyses of histologic sections. Asterisk (*) denotes significant differences from IL-6 deficient mice without mutation in the gene for p50.

concentrations were elevated in BALF from p50-deficient mice compared to WT mice during LPS-induced pulmonary inflammation (Figure 6A), suggesting that excessive IL-6 could be responsible for decreasing neutrophil recruitment in p50-deficient lungs. To test this hypothesis, we crossed p50 deficiency into an IL-6-deficient background and compared neutrophil recruitment in IL-6-deficient mice and double mutant mice deficient in both IL-6 and p50. IL-6-deficient mice had many emigrated neutrophils in their lungs after LPS instillation, and this neutrophil emigration was decreased by the absence of p50 (Figure 6B). Similar to in mice with WT IL-6 alleles (Figure 1A), p50 deficiency reduced emigrated neutrophils to approximately 30% of control values in the absence of IL-6 (Figure 6B). Therefore, the decreased neutrophil recruitment due to p50 deficiency during LPS-induced pulmonary inflammation is independent of any requirement for IL-6.

Discussion

The deficiency of NF-κB p50 decreased neutrophil recruitment elicited by *E. coli* LPS in the lungs to 30% of WT levels, indicating that maximal neutrophil recruitment to this stimulus requires this transcription factor. In contrast, neutrophil recruitment elicited by either living [25] or heat-killed *E. coli* was not decreased in p50-deficient mice compared to WT, but was 130% or 120% of WT levels, respectively. Thus, select pathways for neutrophil emigration depend on NF-κB p50, and these pathways are essential in response to LPS but not more complex gram-negative bacterial stimuli.

The LPS preparations used in these studies likely contained both LPS, which activates TLR4, and bacterial lipoproteins that signal through TLR2 [33]. Because this semi-purified LPS preparation induced neutrophil recruitment that was decreased by p50 deficiency, TLR2 and TLR4 together induce p50-dependent pathways. Based on an approximation of several ng LPS per million gram-negative bacteria [34], the instillate of heat-killed *E. coli* used here contained 4-5 orders of magnitude less LPS than the instillate of semi-purified LPS used here. Such an amount of semi-purified LPS is insufficient to induce neutrophil recruitment in the lungs (data not shown), indicating that factors other than LPS or in combination with the low levels of LPS are responsible for inducing neutrophil recruitment to heat-killed *E. coli*. The present results suggest that these bacterial products, in contrast to LPS, induce

neutrophil recruitment that does not require p50. Candidate bacterial products include unmethylated CpG DNA and n-formyl-methionine peptides; both are likely included with killed bacteria, and both are capable of binding host cell receptors and inducing neutrophil recruitment. In addition, killed bacteria may differ from semi-purified LPS in forming complexes with soluble host proteins, such as complement, natural antibody, and surfactant proteins, which may alter host signaling and molecular pathways used for neutrophil emigration.

p50 deficiency increased the expression of κB-regulated genes induced by LPS in the lungs. Similarly, p50 deficiency increases the expression of κB-regulated genes induced by living *E. coli* in the lungs [25]. These data together suggest that p50 usually functions as a gene repressor rather than activator during innate immune responses to bacterial stimuli in the lungs. The data interestingly contrast with the observation that p50 serves as a gene activator during acquired immune responses to antigen in the lungs, elicited by ovalbumin sensitization and challenge [35,36]. The molecular mechanisms responsible for determining whether p50 functions as a repressor or an activator remain to be determined, but may involve intracellular signaling pathways which alter the nuclear proteins associating with p50. For example, p50 homodimers usually recruit a repressor complex containing histone deacetylase 3 to the *KAI1* promoter, but MEKK1-mediated phosphorylation of a component of this complex causes it to dissociate from the p50 homodimer and be replaced by a histone acetyl transferase-containing complex which activates gene transcription [37]. p50 may recruit different complexes to different promoter regions for different purposes during innate and acquired immune responses in the lungs.

Excessive gene expression due to p50 deficiency may be responsible for the decreased neutrophil recruitment during LPS-induced pulmonary inflammation. Excess levels of chemokines can desensitize neutrophils *in vitro* [30,31], and can inhibit neutrophil recruitment *in vivo* [32]. Although KC and MIP-2 concentrations were increased in the BALF and KC concentrations were increased in the blood of p50-deficient mice, circulating neutrophils in p50-deficient mice remained responsive to the chemokine KC. Desensitization of chemokine receptors therefore seems unlikely to mediate the decreased neutrophil recruitment. However, excessive chemokine elaboration could still contribute to the decreased neutrophil recruitment. The dose-response curves for neutrophil chemoattractants is typically bell-shaped, with *in vitro* chemotaxis peaking at an optimum concentration and then decreasing at higher concentrations [38]. Net chemokine concentrations in p50-deficient lungs may have been beyond the optimal concentrations. Furthermore, increasing chemokine concentrations in the blood may collapse gradients of soluble and/or matrix-bound chemokine, preventing the directed migration of neutrophils [39]. The increased KC concentration in the blood of p50-deficient mice could have contributed to such a collapse of chemokine gradients.

Another strong candidate for decreasing neutrophil recruitment when overexpressed is IL-6. The effect of IL-6 on neutrophil emigration in the lungs is complex and stimulus-specific [40,41]. During LPS-induced pulmonary inflammation, adding IL-6 decreases neutrophil emigration [26] and interrupting IL-6 increases neutrophil emigration [27], suggesting that this cytokine limits LPS-induced neutrophil recruitment in the lungs. LPS-induced IL-6 was increased in the BALF of p50-deficient mice compared to WT. Therefore, we hypothesized that excessive IL-6 expression decreased LPS-induced neutrophil emigration in p50-deficient mice. We tested this hypothesis by generating double mutant mice deficient in both p50 and IL-6, and comparing neutrophil recruitment in double mutants and mice deficient in IL-6 alone. p50 deficiency decreased neutrophil recruitment in the absence of IL-6. These data demonstrate conclusively that p50 facilitates neutrophil recruitment independent of any requirement for IL-6.

The precise mechanism by which p50 facilitates neutrophil recruitment to *E. coli* LPS in the lungs remains to be elucidated. We have measured over 30 genes at the RNA or protein level (including the present data and data not shown), and all of them have been either increased or unchanged by p50 deficiency during LPS-induced pulmonary inflammation. We have observed no significant decreases in gene expression during innate immune responses in p50-deficient mice. Since p50 clearly limits the expression of many κB-regulated genes during innate immune responses to bacterial stimuli in the lungs, we favor the interpretation that p50 facilitates neutrophil recruitment in the lungs by decreasing gene expression which brakes neutrophil recruitment to LPS but not *E. coli* in the lungs. However, even within an extremely simplified system, such as a single cell type (fibroblasts) treated *in vitro* with a single stimulus (TNF-α), each NF-κB subunit has distinct roles that differ among different genes [42]. Although not yet observed, p50 may increase the expression of some genes during LPS-induced pulmonary inflammation. Thus, an alternative mechanistic hypothesis is that NF-κB p50 is essential for the production of genes yet to be identified which are essential for maximal neutrophil recruitment to LPS in the lungs.

Conclusions

In response to *E. coli* or *E. coli* LPS, p50 deficiency increases the expression of multiple κB-regulated genes,

suggesting that p50 usually limits the expression of these genes. LPS induces a p50-dependent pathway for neutrophil recruitment in the lungs, which can be overcome or bypassed with bacterial products other than LPS. The mechanism by which p50 facilitates neutrophil accumulation during LPS-induced pulmonary inflammation remains to be determined, but we hypothesize that p50 may limit gene expression that interferes with neutrophil emigration. Deficiency of p50 increases concentrations of chemokines and of IL-6, each of which can suppress neutrophil emigration. However, p50-deficient neutrophils remain responsive to chemokines, and p50 deficiency compromises neutrophil recruitment even when IL-6 is abrogated by gene targeting. Identifying the genes regulated by p50 to facilitate neutrophil recruitment to LPS in the lungs is an important goal for future studies.

Methods

Mice

Mice with targeted deletion of the *Nfkb1* gene [43], deficient in NF-κB p50, and WT mice of similar random hybrid genetic background (C57BL/6 × 129/Sv) were maintained under specific pathogen free conditions in a full barrier facility. In addition, IL-6-deficient (*Il6*$^{-/-}$) mice on a C57BL/6 background (from Jackson Laboratories; Bar Harbor, ME) were crossed with *Nfkb1*$^{-/-}$ mice, and selective breeding resulted in colonies of *Nfkb1*$^{+/+}$*Il6*$^{-/-}$ and *Nfkb1*$^{-/-}$*Il6*$^{-/-}$ mice on a similarly random hybrid background. Mice were 6–10 weeks of age at the time of experiments. All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals.

Intratracheal instillations

Mice were anesthetized by i.m. injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg). The trachea was surgically exposed, and 50 μl of sterile saline containing 100 μg *E. coli* LPS serotype O55:B5 (Sigma; St. Louis, MO) and colloidal carbon at 1%, marking the site of instillation, was instilled through an angiocatheter inserted via the trachea into the left bronchus. This dose of LPS was chosen to be consistent with other studies of molecules mediating neutrophil emigration in the lungs [12,15,44-46]. In a subset of experiments, the instillate contained heat-killed *E. coli* (strain 19138 from American Type Culture Collection; Manassas, VA) at a dose equivalent to 10^6 CFU. After enumeration by spectrophotometry and colony-counting, *E. coli* were autoclaved and stored in aliquots at -80°C. No living bacteria could be cultured from these aliquots.

Neutrophil recruitment

Mice were killed by inhalation of a lethal overdose of halothane anesthesia 24 h after LPS instillation. The hearts were tied off to maintain pulmonary blood, and peripheral blood samples were collected from the inferior vena cava. Lungs were excised and fixed by intratracheal instillation of 6% glutaraldehyde at a pressure of 23 cm H_2O. Emigrated and sequestered neutrophils were quantified by morphometric analyses of histologic lung sections, as previously described [9]. Investigators were blinded to the genotypes of the mice during morphometric examination. Circulating neutrophils were quantified in peripheral blood samples. After RBC lysis, leukocytes were counted using a hemacytometer, and differential distributions were assessed in blood smears stained with LeukoStat (Fisher Scientific; Pittsburgh, PA).

ICAM-1 expression

Pulmonary expression of ICAM-1 was measured by multiplex RT-PCR using sets of primers specific for ICAM-1 and 18S rRNA, as previously described [45]. Mice were killed by overdose of halothane at the indicated times after LPS instillation, and left lung lobes were snap-frozen and stored at -80°C. RNA was isolated from lungs using RNeasy kits (Qiagen; Valencia, CA), and cDNA was prepared using Advantage RT-for-PCR (Clontech; Palo Alto, CA). Preliminary experiments identified the PCR conditions that produced a linear relationship between input RNA and signal from amplified product. ICAM-1 RNA was amplified through 19 cycles of 25 s at 95 C, 35 s at 55 C, and 50 s at 72 C. PCR reactions contained α[^{32}P]ATP to label products for autoradiography and densitometry after separation by electrophoresis. Every PCR tube contained 50 pmol each of the following primers: GGGTCCAGGCAGGAGTCTCATCCAGCAGGC plus GGTGAAGTCTGTCAAACAGGA (for ICAM-1) and CTTAAAGGAATTGACGGAAG plus TCCGCAGGTTCACCTACGGA (for 18S rRNA). The ICAM-1 primers were specific for sequences in exons 6 and 7 that are contained in all full-length and alternatively spliced forms of murine ICAM-1 that have been described [45,47,48]. Sequencing of the amplified product demonstrated 100% homology to the expected ICAM-1 sequence. ICAM-1 expression for each lung sample was expressed relative to the densitometric value of 18S rRNA from the same PCR reaction tube.

Cytokine concentrations

Cytokine concentrations were measured in the BALF and blood plasma collected from WT and p50-deficient mice at indicated times after LPS instillation. Mice were killed by lethal overdose of halothane. Blood was collected by venipuncture of the inferior vena cava. Plasma was separated from blood cells by centrifugation. The trachea was cannulated, and lungs were lavaged 12 times with 0.8–1.0 ml volumes of PBS. The 12 lavageates were pooled, and cells and debris were removed by centrifugation. Aliquots of BALF and of plasma were snap-frozen and stored at -

80°C until analyzed by ELISA (R&D Systems; Minneapolis, MN).

Chemokine responsiveness

The intravascular administration of chemoattractants causes responsive leukocytes to stiffen and become transiently sequestered in the pulmonary capillaries, resulting in a rapid removal of responsive cells from the circulation [49]. This system was used as an *in vivo* assay for the abilities of neutrophils to respond to chemokines within the relevant environment (i.e., the blood of that genotype, with or without LPS-induced pulmonary inflammation). Mice were anesthetized by i.m. injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg). The peritoneal cavity was opened to expose the inferior vena cava, and 100 μl of sterile, pyrogen-free saline containing recombinant murine KC (0.01 mg/kg body weight; R&D Systems) was injected into a tail vein. A 50 μl blood sample was collected from the inferior vena cava before and both 30 and 120 seconds after KC injection. Mice were killed by halothane overdose after the last blood draw. Circulating neutrophils were quantified in peripheral blood samples, as above.

Statistics

When WT and p50-deficient mice were examined at a single time point, data were compared using a t-test for independent samples. When WT and p50-deficient mice were examined at multiple time points, data were compared using factorial ANOVA and individual groups were compared *post hoc* using the Tukey Honest Significant Difference test. Graphs show the mean and SE from 4–10 mice per group. Groups were considered significantly different when $p < 0.05$.

Authors' contributions

JM conceived of the study, participated in its design and coordination, performed the statistical analyses, and drafted the manuscript. ML carried out *in vivo* studies of neutrophil function, and participated in their design and interpretation. MS carried out molecular studies including immunoassays, RNA analyses, and genotyping, and participated in their design and interpretation. All authors read and approved the final manuscript.

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