Nuclear Factor- κ B Activation in Neonatal Mouse Lung Protects against Lipopolysaccharide-induced Inflammation

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Rationale: Injurious agents often cause less severe injury in neonates as compared with adults.

Objective: We hypothesized that maturational differences in lung inflammation induced by lipopolysaccharide (LPS) may be related to the nature of the nuclear factor (NF)- κ B complex activated, and the profile of target genes expressed.

Methods: Neonatal and adult mice were injected with intraperitoneal LPS. Lung inflammation was assessed by histology, and apoptosis was determined by TUNEL (terminal deoxynucleotidyl transferase UTP nick-end labeling). The expression of candidate inflammatory and apoptotic mediators was evaluated by quantitative real-time polymerase chain reaction and Western immunoblot.

Results: Neonates demonstrated reduced inflammation and apoptosis, 24 hours after LPS exposure, as compared with adults. This difference was associated with persistent activation of NF- κ B p65p50 heterodimers in the neonates in contrast to early, transient activation of p65p50 followed by sustained activation of p50p50 in the adults. Adults had increased expression of a panel of inflammatory and proapoptotic genes, and repression of antiapoptotic targets, whereas no significant changes in these mediators were observed in the neonates. Inhibition of NF- κ B activity in the neonates decreased apoptosis, but heightened inflammation, with increased expression of the same inflammatory genes elevated in the adults. In contrast, inhibition of NF- κ B in the adults resulted in partial suppression of the inflammatory response.

Conclusions: NF- κ B activation in the neonatal lung is antiinflammatory, protecting against LPS-mediated lung inflammation by repressing similar inflammatory genes induced in the adult.

Keywords: acute lung injury; apoptosis; gene expression regulation

Acute respiratory distress syndrome (ARDS) remains one of the leading causes of death in pediatric and adult intensive care units (1–3). Activation of endothelial and infiltrating inflammatory cells results in the production of injurious cytokines and chemokines, and disruption of the alveolar epithelial and capillary barriers contributes to the accumulation of protein-rich fluid in the alveoli, decreasing lung compliance (4–6).

Increased nuclear translocation of the transcription factor nuclear factor (NF)- κ B contributes to the pathogenesis of acute lung injury. Transgenic mice expressing luciferase under the

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pediatric patients demonstrate decreased lung injury in response to injurious agents as compared with adults. However, the molecular mechanisms responsible for this protection are not well defined.

What This Study Adds to the Field

We demonstrate developmental differences in nuclear factor- κ B activation in a murine model of LPS-induced acute lung injury, producing antiinflammatory effects in neonatal mice and proinflammatory effects in adult mice.

control of an NF- κ B promoter demonstrate increased reporter activity in the lung after systemic LPS exposure, with increased expression of NF- κ B-regulated proinflammatory genes, including tumor necrosis factor (TNF)- α and monocyte chemoattractant protein (MCP)-1 (7). Inhibition of NF- κ B in adult mice after cecal ligation and puncture results in decreased inflammatory gene expression in the lungs and improvement in pulmonary mechanics (8). In addition, alveolar macrophages from patients with ARDS have increased NF- κ B activity as compared with critically ill patients without evidence of lung injury (9).

Although multiple factors can precipitate ARDS, sepsis and the systemic inflammatory response remain the most common cause of ARDS in adults (1). In contrast, ARDS in children occurs more commonly as a result of direct lung injuries, such as pneumonia and aspiration (10). Pediatric patients also have decreased ARDS-related mortality rates (10). This protection against severe lung injury has been replicated in animal models of zymosan-induced sepsis (11), lung injury induced by hyperoxia (12), and ventilator-induced lung injury (13, 14). In response to hyperoxia, this protective effect observed in the neonates was related to increased activation of NF- κ B. It remains, however, to be defined how activation of NF- κ B could be antiinflammatory in this neonatal model and proinflammatory in adult models of lung injury, and whether this difference underlies the protection against ARDS-related mortality observed in the pediatric population.

We hypothesized that the nature of NF- κ B activation in response to LPS is different in the neonatal and the adult lung, and that this difference would confer protection to the neonate based on the expression of unique target genes. We found that whereas both adult and neonatal mice activate NF- κ B in the lung in response to LPS, the temporal activation of the complexes activated was distinct in the two groups. The neonatal animals have sustained activation of p65p50 heterodimers, whereas the adults only transiently activate p65p50, followed by persistent

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activation of p50p50 homodimers. Furthermore, the neonates had reduced lung inflammation and apoptosis after LPS administration as compared with the adults. The adult animals demonstrated a heightened expression of proinflammatory cytokines, repression of antiapoptotic mediators, and expression of p53-mediated proapoptotic genes, as compared with the neonates. Although inhibition of NF- κ B activity in the adult resulted in a partial suppression of inflammatory gene induction, inhibition in the neonatal lung resulted in increased neutrophil and macrophage infiltration and increased expression of proinflammatory cytokines. Therefore, our data suggest that the neonatal protection against LPS-induced lung inflammation was in part related to NF- κ B-mediated repression of a number of proinflammatory genes that are increased in the adult.

Some of the results of these studies have been previously reported in the form of abstracts (15, 16).

METHODS

Animal Model

C57BL/6 neonatal (5 d old), and adult (4 mo old) mice were maintained in the appropriate facility and fed food and water *ad libitum*. Experimental sepsis was induced by intraperitoneal administration of 20 mg/kg *Escherichia coli* 0127:B8 LPS (Sigma-Aldrich, St. Louis, MO). Control animals were injected with an equal volume of sterile saline. Dose-response curves were first obtained using doses of LPS ranging from 1 to 50 mg/kg. Additional neonatal and adult animals were treated intraperitoneally with either 10 mg/kg of the NF- κ B inhibitor BAY 11-7082 (Calbiochem, San Diego, CA) or vehicle, either alone or 1 hour before LPS. At various time points, mice were killed using sodium pentobarbital, and the lungs were harvested, perfused through the right ventricle with phosphate-buffered saline, and inflation fixed with formalin for morphometric and histologic analyses. Additional lungs were frozen for use in electrophoretic mobility shift assays, Western immunoblots, and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Immunohistochemistry and TUNEL Assay

Immunohistochemistry was performed on formalin-fixed lung sections using techniques previously described (17) with primary antibodies against p65 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), Mac-3 (1:100; BD Pharmingen, San Jose, CA), and Ly7/4 (1:200; Serotec, Raleigh, NC). Apoptotic cells were detected using the ApopTag In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA). Positive cells were counted in a blinded fashion in five representative sections from each animal, and expressed as the average number of positive cells per field at $\times 40$ magnification.

Electrophoretic Mobility Shift Assay

Nuclear extracts obtained from whole mouse lung were used for DNA binding assays using γ - 32 P-labeled double-stranded oligonucleotides containing the NF- κ B consensus sequence (Promega, Madison, WI) as previously described (12). Signal specificity was ensured by competition reactions using a 100-fold excess of nonradiolabeled NF- κ B oligonucleotide (Promega), or 100-fold excess of nonradiolabeled mutant oligonucleotide (Santa Cruz Biotechnology). Supershift experiments were conducted with the addition of 5 μ l of anti-p50, p65, cRel, or RelB antibodies (Santa Cruz Biotechnology) to the nuclear extracts before incubation with the labeled NF- κ B probe.

Western Immunoblots

Proteins were resolved as previously described (17). Membranes were incubated with primary antibodies against cellular FLICE inhibitory protein (cFLIP) (1:500; R&D Systems, Minneapolis, MN), cellular inhibitor of apoptosis-1 (cIAP-1) (1:500; R&D Systems), bcl-2 (1:500; Abcam, Cambridge, MA), and bcl-xL (1:500; Abcam), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Membranes were reprobed with primary antibody against β -actin (Abcam) to ensure equal loading of samples.

qRT-PCR

RNA was extracted from whole lung tissue using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was reverse transcribed with SuperScript III RT (Invitrogen). Real-time absolute quantification of gene expression levels was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan gene expression primers. The expression level of each gene was normalized to the expression of β -actin using a standard curve method of quantification.

Statistical Analysis

All data are presented as mean $\pm$ SEM. The number of animals used for each experimental group is indicated in the figure legends. Statistical significance between multiple groups was evaluated by one-way analysis of variance, followed by the Newman-Keuls multiple comparison post-test. Statistical significance between two groups was evaluated by Student's *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

LPS Induces Increased Lung Inflammation in Adult versus Neonatal Mice

Dose-response data using LPS doses ranging from 1 to 50 mg/kg demonstrated equivalent survival rates in the neonatal and adult animals in response to this range of doses (Figure E1 of the online supplement). Neonatal and adult mice were administered 20 mg/kg of *E. coli* LPS, based on early data demonstrating that this was the lowest dose of LPS that consistently produced a similar degree of NF- κ B binding in both neonatal and adult lungs. We evaluated the degree of lung inflammation and apoptosis in the neonatal versus adult mice at 6, 24, and 48 hours after LPS injection. Using qualitative analysis of histology at all time points, we observed that the neonatal animals treated with LPS had few inflammatory cells infiltrating the lung parenchyma. In contrast, lung sections obtained from LPS-treated adult mice demonstrated alveolar wall thickening and inflammatory cell infiltration, which was most severe 24 hours after LPS exposure (Figure 1A).

We then quantified the degree of lung inflammation by immunostaining with antibodies against Mac-3 (Figure 1B), a cell marker for macrophages, and Ly7/4 (Figure 1C), a cell marker for neutrophils, cells that accumulate during the acute phase of ARDS. Systemic LPS resulted in a mild increase in macrophages and neutrophils in the neonatal lung that was not statistically significant (Figure 1D). In contrast, adult mice treated with LPS demonstrated a fourfold increase in macrophages ($p < 0.001$), and an almost eightfold increase in neutrophils ($p < 0.01$), as compared with control animals.

LPS Induces Increased Apoptosis in Adult versus Neonatal Mice

Patients with ARDS have increased apoptosis of alveolar epithelial cells, in part secondary to an up-regulation of the Fas/FasL system (18). Increased epithelial cell apoptosis is also found in animal models of acute lung injury as evidenced by TUNEL (terminal deoxynucleotidyl transferase UTP nick-end labeling) staining, caspase activity assays, and electron microscopic features (19, 20). We compared the degree of apoptosis by TUNEL staining in the neonatal and adult mouse lungs at 6 and 24 hours after LPS exposure. As has been previously described, there was a greater amount of apoptotic cells present in the neonatal lungs under control conditions, representing one mechanism allowing for alveolar formation and septation (21). Systemic LPS induced an apoptotic response in both the neonatal and adult lungs that was maximal at 24 hours (Figures 2A and 2B). However, adult mice had a greater (eightfold) increase in the number of apoptotic cells, compared with a more modest (twofold) increase

Figure 1. LPS induces increased lung inflammation in adult versus neonatal mice. (A) Representative formalin-fixed lung sections stained by hematoxylin and eosin demonstrate increased inflammatory cell infiltration, alveolar thickening, and edema in the adult lung in contrast to little gross histologic change observed in the neonatal lung (Neo) 24 hours after LPS exposure. Immunostaining of representative sections demonstrates increased (B) Mac-3- and (C) Ly 7/4-positive cells in LPS-treated adult lungs as compared with neonatal lungs at 24 hours. (D) Quantification of Mac-3- and Ly 7/4-positive cells per field in control (Con) and LPS-treated neonatal and adult mice. Bars represent mean $\pm$ SEM for control (n = 3) and LPS-treated adult and neonatal mice (n = 4 for both). **p < 0.01 and ***p < 0.001 versus control adults. Scale bar represents 50 μ m.

observed in the LPS-treated neonatal mice (p = 0.01) (Figure 2C).

LPS Induces Distinct NF- κ B Complexes in Adult versus Neonatal Mice

To determine whether there were differences in the nature of the NF- κ B complex activated in the neonatal and adult mouse lung, we performed electrophoretic mobility shift assays (EM-SAs) using nuclear extracts obtained from whole lung homogenates at various time points after LPS administration. NF- κ B complex activation occurred in both neonatal and adult lungs as early as 1 hour after systemic LPS exposure, peaked in both groups at 2 hours, and returned toward basal levels by 4 hours (Figure 3A). The complexes present in both the neonatal and adult animals at 1 hour appeared to have a similar composition. However, by the peak time point of activation, the NF- κ B complex activated in the adult lung was a more rapidly migrating complex compared with the one activated in the neonate lung. We performed supershift analysis using specific antibodies against the various NF- κ B/Rel family members (p65, p50, cRel, and RelB) to further define the subunits of the complexes present at the peak time point of activation (2 h). The addition of antibodies against the p50 protein supershifted the complex present in both the neonatal and adult lung extracts at 2 hours (Figures 3B and 3C). In contrast, the addition of an antibody against p65 decreased the intensity of binding of the complex present at 2 hours in the neonate (Figure 3B) but not in the adult lung (Figure

3C). This decrease in band intensity rather than an upward shift is in agreement with studies by others suggesting that the p65 antibody may interfere with the oligonucleotide binding site (22–25). Administration of antibodies against cRel and RelB were also unable to affect the binding of the adult complex. This indicated that the neonatal NF- κ B complex activated in the lung at 2 hours consisted of a p65 and p50 heterodimer, whereas the faster migrating complex in the adult lung present at the same time point consisted of a p50 homodimer.

To further demonstrate that the p65 protein was a significant component of the neonatal, but not the adult, NF- κ B complex at the peak time point of activation, we immunostained formalin-fixed lung sections obtained from neonatal and adult mice 2 hours after LPS exposure with antibodies against the NF- κ B subunit p65. Control animals in both age groups had diffuse staining for p65 protein in the cytoplasm of lung epithelial cells, endothelial cells, and alveolar macrophages (Figure 4). However, exposure to systemic LPS resulted in the widespread translocation of p65 from the cytoplasm into the nucleus of multiple cell types in the neonatal lung, including alveolar epithelial cells, alveolar macrophages, and pulmonary endothelial cells, in contrast to minimal p65 nuclear translocation evident in the adult lung.

Decreased Antiapoptotic and Increased Proapoptotic Factors in LPS-treated Adult versus Neonatal Mice

We then investigated whether differences in pro- and antiapoptotic proteins known to be under transcriptional control of

Figure 2. LPS induces increased apoptosis in adult versus neonatal mice. (A) Representative TUNEL staining of formalin-fixed lung sections demonstrates increased positive cells in adult mice in response to LPS exposure. (B) Quantification of TUNEL-positive cells from five representative lung sections in a blinded fashion demonstrates increased apoptotic cells in neonatal lungs under control conditions. Both LPS-treated adults and neonates had increased TUNEL-positive cells as compared with control animals. Bars represent mean $\pm$ SEM for control ($n = 3$) and LPS-treated adult and neonatal mice ($n = 4$ for both). * $p < 0.05$ versus control neonates and ** $p < 0.01$ versus control adults. (C) A greater fold increase was seen in the number of apoptotic cells in the LPS-treated adults as compared with the LPS-treated neonates. * $p = 0.01$ versus LPS treated neonates. Scale bar represents 50 μm .

NF- κ B could account for the increased degree of LPS-induced apoptosis in the adult lungs. Activation of NF- κ B can increase the expression of genes that either promote or prevent apoptosis. Most commonly, NF- κ B activation results in decreased apoptosis secondary to an increase in the transcription of gene products that inhibit apoptosis, including cIAP-1, cFLIP, bcl-xL, and bcl-2 (26). We found that systemic LPS resulted in significantly decreased levels of antiapoptotic NF- κ B targets cIAP-1 ($p < 0.001$) and cFLIP ($p < 0.01$) in the adult animals at 24 hours (Figures 5A and 5B), but no significant difference was found in the LPS-treated neonates. The levels of antiapoptotic factors bcl-2 and bcl-xL were not significantly different in any of the four groups (data not shown).

In contrast to these antiapoptotic effects, NF- κ B can also promote apoptosis through the repression of the p53 inhibitor, the murine double minute-2 protein (Mdm2), allowing for stabilization of p53, and increased transcription of p53-mediated proapoptotic genes (27). We therefore determined the expression of Mdm2, p53, and the p53 downstream targets, Noxa, Fas, and death receptor-5 (DR5), by qRT-PCR. Neonatal animals had increased levels of Mdm2 at baseline as compared with adults ($p < 0.05$) (Figure 5C). Exposure to LPS further exaggerated this difference, with expression of Mdm2 in the adults less than half that of the neonates 6 hours after LPS exposure ($p < 0.01$),

although by 24 hours, expression of Mdm2 in LPS-treated adults and neonates was similar (data not shown). The expression of p53 was not different in the four groups at either time point (data not shown). This early repression of Mdm2 in the adults was followed by an increase in p53-mediated proapoptotic factors, with increased expression of Fas and Noxa at 6 hours ($p < 0.01$ for both) (data not shown), and persistent up-regulation of Noxa, DR5, and Fas at 24 hours post-LPS exposure ($p < 0.05$ for all) (Figures 5D–5F). In contrast, the LPS-treated neonates demonstrated only a transient increase in Fas expression at 6 hours ($p < 0.001$), but no significant elevation of these three proapoptotic factors at 24 hours.

Increased Expression of Inflammatory Mediators in LPS-treated Adult versus Neonatal Mice

We also evaluated the mediators that might account for the difference in inflammation observed in the adult and neonatal mice, by determining the expression of proinflammatory mediators associated with NF- κ B activation by qRT-PCR at 6 and 24 hours. Although there was a trend in the adult animals toward increasing inflammatory mediators by 6 hours, this was not statistically significant (data not shown). However, by 24 hours, the LPS-treated adults had a more than tenfold increase in expression of the cytokine TNF- α ($p < 0.05$) (Figure 6A). This was

Figure 3. Systemic LPS activates distinct nuclear factor (NF)- κ B complexes in adult and neonatal mouse lungs. (A) Nuclear extracts from lung homogenates were incubated with radiolabeled oligonucleotides containing the NF- κ B consensus sequence. Activated NF- κ B complexes were present in the nuclear extracts of both LPS-treated neonatal and adult mice, beginning at 1 hour (lanes 3 and 11), peaking at 2 hours (lanes 4, 5, 12, 13) and decreasing by 4 hours (lanes 6 and 14). However, the primary complex in the neonates (lanes 4 and 5) was noted to have a slower speed of migration as compared with the primary complex found in the adults (lanes 12 and 13). Specificity of the bands was confirmed by the disappearance of the bands with the addition of 100-fold excess of cold oligonucleotide (Cold oligo) (lanes 7 and 15), and the reappearance of the bands with the addition of 100-fold excess of cold, mutated oligonucleotide (Mutant oligo) (lanes 8 and 16). Supershift analysis of the neonatal (B) and adult (C) NF- κ B complexes present at 2 hours was performed by the addition of antibodies against the Rel family proteins: p65, p50, Rel B, and cRel. There is an upward shift of the neonatal NF- κ B complex with the addition of antibodies against p50, and decreased intensity of the band with the addition of antibodies against p65. Supershift analysis of the adult complex demonstrates a shift in the position or intensity of the band only by the addition of p50 antibodies. Images are representative examples of $n \geq 3$ individual experiments.

also true for the expression of MCP-1, a chemokine important in the recruitment and adherence of monocytes to the endothelium, and for the macrophage inflammatory protein (MIP)-2, the murine analog of IL-8, a potent neutrophil chemokine ($p < 0.001$ for both) (Figures 6B and 6C). In addition, the expression of inducible nitric oxide synthase (iNOS) ($p < 0.001$) and interferon- γ -inducible protein-10 (IP-10) ($p < 0.05$) was similarly up-regulated in the LPS-treated adults, but there was no induction of IFN- γ (Figures 6D–6F). In contrast, the LPS-treated neonates demonstrated only an isolated and transient increase in the level of IP-10, 6 hours post-LPS exposure (data not shown). Although there were trends toward mild increases in some of these mediators at 24 hours, the levels were not significantly different from control levels (Figures 6A–6F).

Administration of BAY 11-7082 Decreases NF- κ B Binding in Neonatal Mice

Because NF- κ B activation in neonate mice was associated with decreased LPS-mediated inflammation and apoptosis as compared with adult mice, we sought to determine whether NF- κ B mediated this protection. The selective NF- κ B inhibitor BAY 11-7082 prevents the phosphorylation and subsequent degradation of the inhibitory protein I κ B α , thereby preventing nuclear translocation and transcriptional activity of p65-containing complexes (28). Effects of BAY 11-7082 appear to be specific for NF- κ B as this compound does not globally inhibit kinase activity (28), nor does it affect the DNA binding of other stress-activated transcription factors, such as activator protein-1 (AP-1) (29). We administered 10 mg/kg of BAY 11-7082 by intraperitoneal

Figure 4. Increased nuclear translocation of p65 NF- κ B in LPS-treated neonates compared with adults. Representative immunostaining for the p65 subunit in formalin-fixed lung sections demonstrates abundant cytoplasmic staining of p65 in both adult and neonatal mice under control conditions. Increased nuclear translocation of p65 was found in the neonatal lung 2 hours after LPS exposure as compared with minimal p65 nuclear translocation in the adult lung. Scale bar represents 50 μ m. Images are representative examples from groups of adult and neonatal control ($n = 3$) and LPS-treated ($n = 4$) mice.

Figure 5. Decreased antiapoptotic and increased proapoptotic factors in LPS-treated adult mice. (A, B) Western immunoblot analysis demonstrated decreased optical density (OD) cellular inhibitor of apoptosis-1 (cIAP-1) (A) and cellular FLICE inhibitory protein (cFLIP) (B) levels in LPS-treated adults compared with LPS-treated neonates at 24 hours. Bars represent mean $\pm$ SEM for control and LPS-treated adult and neonatal mice ($n = 3$ for each group). ** $p < 0.01$ and *** $p < 0.001$ versus control adults. (C) At 6 hours, higher basal expression of murine double minute-2 protein (Mdm2) in the neonates compared with adults under control conditions, and decreased expression of Mdm2 in the LPS-treated adults versus LPS-treated neonates. Bars represent mean $\pm$ SEM for control and LPS-treated adult and neonatal mice ($n = 3$ for each group). # $p < 0.05$ versus control neonates and ** $p < 0.01$ versus LPS-treated neonates. (D–F) LPS mediated up-regulation of the p53-dependent genes Noxa (D), DR5 (E), and Fas (F) in adult animals at 24 hours, in contrast to no significant elevation of these mediators in the neonates at this time point. Bars represent mean $\pm$ SEM for control and LPS-treated adult and neonatal mice ($n = 3$ for each group). * $p < 0.05$ versus control adults.

injection to neonatal and adult mice, 1 hour before the administration of 20 mg/kg of LPS. Nuclear extracts obtained from neonatal and adult lungs after treatment with vehicle, BAY 11-7082 alone, or BAY 11-7082 1 hour before LPS exposure were used for EMSA. Treatment with vehicle or BAY 11-7082 alone had no effect on NF- κ B binding in the neonatal lungs (Figure E3). However, BAY 11-7082 administration 1 hour before LPS effectively reduced the amount of NF- κ B binding present in the neonatal lungs at 2 hours by 42% ($p = 0.0013$) (Figure 7A). In contrast, treatment of the adult animals with BAY 11-7082 did not decrease DNA binding of the p50 homodimers (Figure 7B). This result is consistent with studies demonstrating that the affinity of I κ B α for the p50p50 homodimer is 50-fold weaker than

Figure 6. Increased expression of proinflammatory cytokines and chemokines in LPS-treated adult mice. Increased expression of tumor necrosis factor (TNF)- α (A), monocyte chemoattractant protein (MCP)-1 (B), macrophage inflammatory protein (MIP)-2 (C), inducible nitric oxide synthase (iNOS) (D), and interferon- γ -inducible protein-10 (IP-10) (E) observed in adult lungs, 24 hours after LPS exposure, in contrast to no significant increase in these mediators in LPS neonatal lungs. Neither adult nor neonatal lungs demonstrated statistically significant increases in the expression of IFN- γ (F) in response to LPS exposure. Bars represent mean $\pm$ SEM for control and LPS-treated adult and neonatal mice ($n = 3$ for each group). * $p < 0.05$ and *** $p < 0.001$ versus control adults.

that for the p65p50 heterodimers, and agents such as BAY 11-7082 that prevent the phosphorylation of I κ B α do not interfere with p50p50 nuclear translocation (30, 31).

Inhibition of NF- κ B Activity Suppresses LPS-mediated Apoptosis in the Neonatal Lung

To determine whether the neonatal NF- κ B complex was responsible for the less severe LPS-mediated lung apoptosis observed in the neonates as compared with the adults, we performed TUNEL staining of lung sections obtained from those neonates treated with the NF- κ B antagonist BAY 11-7082. Neonates treated with BAY 11-7082 before LPS exposure were found to have a marked reduction in the degree of apoptosis as compared with neonates receiving LPS alone ($p < 0.05$) (Figure 8). This suggested that the p65p50 complex was mediating the modest increase in apoptosis observed in the neonates treated with LPS.

Figure 7. Administration of the selective NF- κ B inhibitor BAY 11-7082 decreases NF- κ B binding *in vivo*. Nuclear extracts obtained from neonates treated with LPS alone, or BAY 11-7082 before LPS, were used to evaluate the amount of NF- κ B–DNA binding by electrophoretic mobility shift assay (EMSA). (A) Increased binding of NF- κ B complexes in the LPS-treated neonates (lanes 4–7) 2 hours after exposure, as compared with controls (lanes 1–3). Administration of BAY 11-7082 1 hour before LPS exposure (lanes 9–12) decreased the NF- κ B band intensity. Specificity of the bands was demonstrated by the disappearance of the band with the addition of 100-fold excess of cold oligonucleotide (lanes 8 and 13). Quantification of the bands shown in (A) by densitometry revealed a 42% reduction in the degree of NF- κ B–DNA binding by the administration of BAY 11-7082 before LPS exposure. Bars represent mean $\pm$ SEM for control ($n = 3$), LPS-treated ($n = 4$), or BAY 11-7082 + LPS-treated neonates ($n = 4$), with $*p = 0.008$ versus LPS treated neonates by Student's t test. (B) EMSA using nuclear extracts obtained from adults treated with LPS alone (lanes 1–3), or with BAY 11-7082 before LPS (lanes 5–7), demonstrates that BAY 11-7082 does not decrease the DNA binding of the p50p50 complex.

However, we were unable to determine the mechanism accounting for this effect, as BAY 11-7082 treatment increased the expression of proapoptotic DR5 and Fas ($p < 0.01$), reduced the level of antiapoptotic cFLIP ($p < 0.05$), and had no effect on cIAP-1 (data not shown). Therefore, additional NF- κ B–dependent proapoptotic effects must exist in the neonatal lung, and remain to be defined. In addition, because NF- κ B activity in both the neonatal and adult is associated with increased apoptosis, the more severe apoptosis in the adults is likely due to the widespread reduction of antiapoptotic factors, and increased proapoptotic factors, observed in association with the p50p50 complex.

Inhibition of NF- κ B Activity Heightens LPS-induced Inflammation in the Neonatal Lung

In contrast to the suppression of mild apoptosis in the neonates, NF- κ B inhibition using BAY 11-7082 resulted in increased in-

flammatory cells adhering to the pulmonary vascular endothelium and infiltrating the lung parenchyma, evident on hematoxylin-and-eosin-stained lung sections 24 hours after LPS exposure. Immunostaining of these same lung sections with antibodies against Mac-3 and Ly7/4 demonstrated almost twice the number of macrophages ($p < 0.01$) and neutrophils ($p < 0.05$) in the lungs of those neonates treated with the NF- κ B inhibitor as compared with neonates treated with LPS alone (Figure 9).

Inhibition of NF- κ B Induces Proinflammatory Gene Expression in the Neonatal Lung

This increase in inflammation in the neonatal lung was associated with widespread increases in inflammatory mediators, consistent with de-repression of these agents by the NF- κ B inhibitor BAY 11-7082. The expression of TNF- α , a chemokine mildly up-regulated in the LPS-treated neonates, was significantly increased in the neonates treated with BAY 11-7082 before LPS as compared with control neonates and neonates treated with LPS alone ($p < 0.05$) (Figure 10A). Similarly, the expression of MCP-1 and MIP-2 was de-repressed by the p65p50 NF- κ B inhibition, with greater than 10-fold increases in both after administration of BAY 11-7082 ($p < 0.001$) (Figures 10B and 10C). Likewise, the expression of iNOS and IP-10 (molecules up-regulated in LPS-treated adults but not in neonates) was markedly increased in those neonates receiving BAY 11-7082 in addition to LPS, as compared with neonates receiving LPS alone ($p < 0.001$) (Figures 10D and 10E). There was also a significant increase in the expression of IFN- γ in the neonates treated with BAY 11-7082, a cytokine not significantly elevated in the LPS-treated adults (Figure 10F). In contrast, administration of BAY 11-7082 to the adult animals before LPS resulted in a significant decrease in the expression of iNOS and IP-10 (Figure 11), providing further evidence for NF- κ B–mediated proinflammatory effects in adult mice, yet antiinflammatory effects in neonates. There was a trend toward decreased expression of TNF- α , MCP-1, MIP-2, and IFN- γ ; however, this did not reach statistical significance.

DISCUSSION

In this study, we demonstrated distinct, temporal patterns of NF- κ B activation in the lungs of neonatal and adult mice after systemic LPS exposure, with neonatal mice activating solely p65p50 heterodimers, and adult mice transiently activating p65p50 heterodimers, followed by persistent activation of p50 homodimers. This difference in NF- κ B complex activation was associated with increased lung inflammation in the adult mice as compared with the neonates and induction of the inflammatory mediators TNF- α , MCP-1, MIP-2, IP-10, and iNOS. By administering an NF- κ B antagonist to the neonatal animals before LPS exposure, we were able to confirm a protective role of the neonatal p65p50 complex in repressing the heightened expression of the inflammatory mediators observed in the adult lungs.

Our data demonstrating that NF- κ B functions to repress LPS-induced inflammation in the neonatal mouse lung are particularly intriguing as they are in contrast to the well-described function of NF- κ B in the propagation of the inflammatory response (8, 32). However, despite much evidence supporting the role of NF- κ B as a proinflammatory regulator, recent studies have demonstrated that NF- κ B activation may have an additional role in the resolution of inflammation (33). For example, transgenic mice that lack p50, and are haploinsufficient for p65, develop more profound colonic inflammation than control mice in response to the microflora *Helicobacter hepaticus*, and are also more sensitive to lethal LPS-mediated shock (34, 35). In both conditions, the transgenic mice lacking intact NF- κ B signaling had increased expression of a number of proinflammatory

Figure 8. Inhibition of NF- κ B suppresses LPS-induced apoptosis in the neonatal lung. TUNEL staining of formalin-fixed lung sections 24 hours after LPS exposure demonstrates decreased TUNEL-positive cells in neonatal mice treated with the NF- κ B inhibitor BAY 11-7082 as compared with neonates receiving LPS alone. Bars represent mean $\pm$ SEM for control (n = 3), LPS-treated (n = 4), or BAY 11-7082 + LPS-treated (n = 4) neonates. *p < 0.05 versus LPS treated neonates. Scale bar represents 50 μ m.

mediators, including TNF- α , MCP-1, IP-10, and MIP-2. Although it has been demonstrated previously that the degree of NF- κ B activation can vary with development (36, 37), we have shown here that, in response to the same stimulus, adult and neonatal mice temporally activate distinct profiles of NF- κ B complexes. This is associated with increased expression of inflammatory mediators in the adult, and repression of these same mediators in the neonate.

Although we demonstrate that the p65p50 complex represses inflammatory mediator expression in neonate mice, this heterodimer has been associated with increased inflammatory gene

regulation in adult animals, suggesting that the differences we observed in gene expression cannot be due to complex composition alone (7). NF- κ B transcriptional activity can be modified by interaction with various coactivator and corepressor proteins. Some cofactors function to increase NF- κ B recruitment to the chromatin and to enhance NF- κ B-mediated transcription (38, 39), whereas others allow NF- κ B-DNA binding, but directly interfere with NF- κ B-dependent transcription, repressing gene expression (40). It may be that the neonatal p65p50 complex can bind the promoters of inflammatory genes but, in association with a repressive cofactor, is unable to activate transcription. In

Figure 9. Inhibition of NF- κ B increases LPS-induced inflammation in the neonatal lung. (A) Hematoxylin and eosin staining of formalin-fixed lung sections demonstrates increased inflammatory cell infiltration and alveolar thickening in neonates treated with BAY 11-7082 as compared with neonates treated with LPS alone. (B, C) Quantification of (B) Mac-3- and (C) Ly 7/4-positive cells by immunostaining of representative sections demonstrates increased macrophages and neutrophils in the lungs of neonates treated with NF- κ B inhibition as compared with neonates given LPS alone. Bars represent mean $\pm$ SEM for control (n = 3), LPS-treated (n = 4), or BAY 11-7082 + LPS-treated (n = 4) animals, with *p < 0.05 and **p < 0.01 versus LPS-treated neonates. Scale bar represents 50 μ m.

Figure 10. Inhibition of NF- κ B increases inflammatory gene expression in LPS-treated neonates. Quantitative reverse transcriptase-polymerase chain reaction at 24 hours demonstrated increased expression of TNF- α (A), MCP-1 (B), MIP-2 (C), iNOS (D), interferon- γ -inducible protein-10 (IP-10) (E), and IFN- γ (F) in neonates treated with BAY 11-7082 before LPS as compared with LPS alone. The administration of BAY 11-7082 in the absence of LPS did not induce inflammatory gene expression. Bars represent mean $\pm$ SEM for control (n = 3), LPS-treated (n = 3), or BAY 11-7082 + LPS-treated (n = 3) animals. *p < 0.05 versus control neonates, and *p < 0.05, **p < 0.01, and ***p < 0.001 versus control, BAY alone, and LPS-treated neonates.

this way, the inactive p65p50 complex may prevent the binding of transcriptionally active factors to the same promoters. When the nuclear translocation of the p65p50 complex is suppressed with BAY 11-7082, these additional transcription factors could gain access and induce the expression of target inflammatory mediators. Future studies to identify developmentally regulated cofactors will be necessary to investigate if this is one potential mechanism allowing for the varied effects of p65p50 observed in neonates and adults.

The p50p50 complex is also capable of either activating (41) or repressing transcription (42, 43), depending on its association with other factors, such as Bcl-3 (28). In our system, we identified activation of NF- κ B inflammatory gene products in association with persistent binding of the p50p50 complex. However, we were not able to decrease p50p50-DNA binding in the adults with the administration of BAY 11-7082. Additional strategies to specifically inhibit p50p50 nuclear translocation would be necessary to demonstrate that the induction of inflammatory genes we observed in the adult mice was directly dependent on p50p50 complex activity. Interestingly, despite the inability of BAY 11-7082 to decrease p50p50 binding, it did result in decreased expression of iNOS and IP-10 in the adult animals. It is possible that BAY 11-7082 inhibits the early, transient activation of p65p50 in the adult mice, and that the effect of this

Figure 11. Effect of BAY 11-7082 on inflammatory gene expression in the LPS-treated adults. Quantitative reverse transcriptase-polymerase chain reaction at 24 hours in the adult animals treated with BAY before LPS as compared with LPS alone demonstrates no significant effect on the expression of TNF- α (A), MCP-1 (B), MIP-2 (C), and IFN- γ (F). However, BAY 11-7082 administration did reduce the expression of iNOS (D) and interferon- γ -inducible protein-10 (IP-10) (E) in the LPS-treated adults, providing further evidence of the proinflammatory role of NF- κ B in the adult mice. Bars represent mean $\pm$ SEM for control (n = 3), LPS-treated (n = 3), or BAY 11-7082 + LPS-treated (n = 3) animals. *p < 0.05 and **p < 0.01 versus control adults, and *p < 0.05 and ##p < 0.01 versus LPS-treated adults.

complex is proinflammatory as opposed to the antiinflammatory effects of the sustained activation of this complex in the neonate mice. Another possibility is that BAY 11-7082 targets a later source of p65p50 complex activation in the adult beyond 4 hours that is also proinflammatory. Nonetheless, it strengthens our finding that there appear to be age-dependent, contrasting effects of NF- κ B activation, with proinflammatory effects predominating in the adult, and antiinflammatory effects in the neonate.

In addition to maturational differences in lung inflammation, we also observed differences in LPS-mediated apoptosis in the neonatal and adult mouse lung. Initiation of apoptosis can occur via the intrinsic pathway by disruption of the mitochondrial membrane, or via the extrinsic pathway by signaling through death receptors such as Fas and DR5 (44). It appears that the heightened apoptotic response in the adult mice is a consequence of activation of both pathways in response to LPS. The decrease in the expression of the antiapoptotic factors cIAP-1, cFLIP, and Mdm2 in the adult mice is in keeping with the repressive role of the p50p50 homodimer. Furthermore, repression of Mdm2 by the p50p50 complex would also account for the up-regulation of the proapoptotic genes Fas, DR5, and Noxa, by allowing for stabilization and increased transcriptional activity of p53.

In contrast to hyperoxia-induced lung injury in the neonate, where it appeared that NF- κ B activity was antiapoptotic (12),

we have shown that LPS-mediated lung injury is associated with proapoptotic NF- κ B activity in the neonate, because inhibition of the neonatal p65p50 complex prevented LPS-induced apoptosis. We were, however, unable to account for the p65p50-dependent apoptotic response on the basis of changes in the pro- and anti-apoptotic mediators evaluated in this study, because only modest trends in the appropriate direction were observed. A more comprehensive evaluation of pro- and antiapoptotic factors with a genomic or proteomic approach would be necessary to identify novel targets to account for the p65p50-mediated apoptotic response observed in the neonatal lung in response to LPS.

The functional consequence of the differing rates of LPS-induced apoptosis in the adult and neonatal mice was not evaluated in this study. Physiologic apoptosis is a mechanism important in pre- and postnatal lung development. However, it is possible that pathologic apoptosis during the critical window of postnatal lung development could result in the loss of cells critical for normal alveolar growth (45). Alternatively, apoptosis may be important in the resolution of lung inflammation, allowing for the removal of infiltrating leukocytes (6, 46). It could be that the suppression of apoptosis in our neonates treated with the NF- κ B inhibitor allowed for the persistence of neutrophils and macrophages in the lung parenchyma, and subsequent propagation of inflammatory gene expression.

In summary, our experimental study is the first to define maturational differences in the pattern of NF- κ B complex activation resulting in the regulation of downstream target genes that mediate the severity of lung inflammation and apoptosis in response to LPS. Of great interest is the fact that NF- κ B activation serves to protect the immature lung against endotoxin-mediated inflammation in contrast to the proinflammatory effect of NF- κ B activation in the mature mouse. This effect may represent an evolutionary mechanism to prevent concurrent proinflammatory effects accompanying the NF- κ B activity occurring in the setting of normal central nervous system, skin, and lymphoid development (47–50). Further studies in a clinical population would be important to determine whether NF- κ B activation in association with ARDS may be protective in young patients and injurious in older individuals with the same lung insult.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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