p53 Attenuates Lipopolysaccharide-Induced NF-κB Activation and Acute Lung Injury[1]

Gang Liu, Young-Jun Park, Yuko Tsuruta, Emmanuel Lorne, and Edward Abraham[2]

The transcriptional factor p53 has primarily been characterized for its central role in the regulation of oncogenesis. A reciprocal relationship between the activities of p53 and NF-κB has been demonstrated in cancer cells, but there is little information concerning interactions between p53 and NF-κB in inflammatory processes. In this study, we found that neutrophils and macrophages lacking p53, i.e., $p53^{-/-}$, have elevated responses to LPS stimulation compared with $p53^{+/+}$ cells, producing greater amounts of proinflammatory cytokines, including TNF-α, IL-6, and MIP-2, and demonstrating enhanced NF-κB DNA-binding activity. $p53^{-/-}$ mice are more susceptible than are $p53^{+/+}$ mice to LPS-induced acute lung injury (ALI). The enhanced response of $p53^{-/-}$ cells to LPS does not involve alterations in intracellular signaling events associated with TLR4 engagement, such as activation of MAPKs, phosphorylation of IκB-α or the p65 subunit of NF-κB, or IκB-α degradation. Culture of LPS-stimulated neutrophils and macrophages with nutlin-3a, a specific inducer of p53 stabilization, attenuated NF-κB DNA-binding activity and production of proinflammatory cytokines. Treatment of mice with nutlin-3a reduced the severity of LPS-induced ALI. These data demonstrate that p53 regulates NF-κB activity in inflammatory cells and suggest that modulation of p53 may have potential therapeutic benefits in acute inflammatory conditions, such as ALI. *The Journal of Immunology,* **2009, 182: 5063–5071.**

Macrophages and neutrophils are among the first cells that interact with invading microbial pathogens and respond by producing proinflammatory mediators, including cytokines and chemokines, reactive oxygen species, and antimicrobial peptides, which participate in host defense mechanisms aimed at eradicating bacterial or viral infection (1–4). However, although inflammatory responses are crucial in mounting effective antimicrobial defense, overly exuberant inflammation can be deleterious, resulting in organ dysfunction, including acute lung injury (ALI)[3] (5, 6).

Inflammatory cells recognize distinct microbial products, which exist as pathogen-associated molecular patterns (PAMPs), through TLRs (7–10). Association of TLRs with PAMPs leads to recruitment of adaptor proteins and kinases, such as MyD88, IL-1R-associated kinase 1, IIL-1R-associated kinase 4, and TNF receptor-associated factor 6, to the TLR intracellular domain (7–10). The TLR-associated complex then activates downstream signaling cascades resulting in activation of MAPKs and kinases, such as the IκB kinase (IKK), which are involved in activating NF-κB (7–10). IKK phosphorylates IκB-α and leads to its degradation, allowing NF-κB to be translocated to the nucleus, where it binds to the promoters of target genes, including *TNF-α*, *MIP-2*, *IκB-α*, and cellular inhibitor of apoptosis, activating their transcription (7–10).

Increased activation of NF-κB is found in PBMC, neutrophils, and alveolar macrophages after exposure to the TLR4 ligand, LPS, and in patients with sepsis (11–14). In addition, greater or more persistent nuclear accumulation of NF-κB is associated with higher mortality and more severe organ dysfunction in such patients, probably due to excessive induction of proinflammatory cytokines and delayed apoptosis of immune cells such as neutrophils (11, 12, 15, 16).

p53 is a transcriptional factor that induces the expression of a number of downstream target genes involved in apoptosis, cell cycle arrest, and DNA repair (17–19). In resting cells, p53 is maintained at low levels, primarily through the actions of its negative regulator, murine double minute (Mdm2), an E3 ubiquitin ligase (18). In response to DNA damage and other cellular stresses, p53 is phosphorylated by ATM (ataxia-telangiectasia, mutated), ATR (ATM and Rad3 related), and/or ChK1/2 (checkpoint kinase 1/2) (18). p53 phosphorylation disrupts interactions between p53 and Mdm2, thus leading to p53 stabilization and increased transcriptional activity (18, 20). Recently, a compound (nutlin-3a) that specifically blocks the interaction between p53 and Mdm2 has been developed. Nutlin-3a stabilizes p53 without inducing DNA damage and has been shown to enhance p53 activity, such as induction of apoptosis and cell cycle arrest of cancer cells (21–23).

Previous studies have demonstrated mutual regulation between NF-κB and p53 in cancer cells (24–26). For example, NF-κB was shown to inhibit p53 transcriptional activity (25). This was suggested to be a major mechanism by which NF-κB promotes oncogenesis (25). In addition, several p53 mutants were found to induce the expression of NF-κB subunits, including p100 (27). However, it is less clear how p53 itself regulates NF-κB activity, especially in inflammatory cells after TLR engagement by PAMPs or other ligands.

In this study, we investigated the involvement of p53 in the responses of inflammatory cells to LPS. We found that p53 not

Department of Medicine, University of Alabama at Birmingham, AL 35294

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[2] Address correspondence and reprint requests to Dr. Edward Abraham, Department of Medicine, University of Alabama at Birmingham, School of Medicine, 420 Boshell Building, 1808 7th Avenue South, Birmingham, AL 35294. E-mail address: eabraham@uab.edu

[3] Abbreviations used in this paper: ALI, acute lung injury; PAMP, pathogenesis-associated molecular patterns; IKK, IκB kinase; BAL, bronchoalveolar lavage; PI, propidium iodide; MPO, myeloperoxidase; ChIP, chromatin immunoprecipitation.

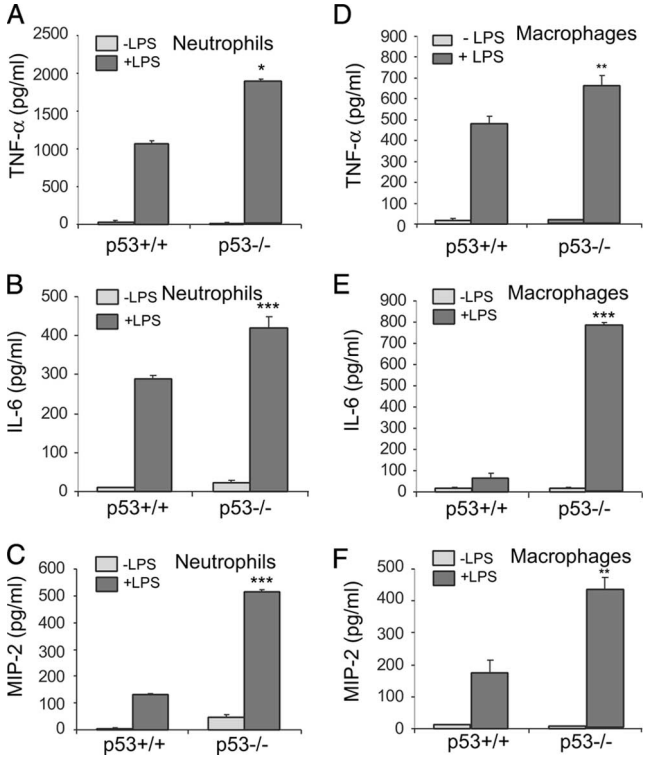

FIGURE 1. $p53^{-/-}$ inflammatory cells demonstrate enhanced activation by LPS. *A–C,* $p53^{-/-}$ neutrophils produced significantly greater amounts of proinflammatory cytokines than did $p53^{+/+}$ cells after LPS stimulation. Two $\times$ 10^6 $p53^{+/+}$ or $p53^{-/-}$ neutrophils were treated with 10 ng/ml LPS for 6 h. Levels of TNF-α (*A*), IL-6 (*B*), and MIP-2 (*C*) in culture supernatants were measured by ELISA. *, $p < 0.05$ and ***, $p < 0.001$ compared with $p53^{+/+}$ cells. *D–F,* $p53^{-/-}$ macrophages produced significantly greater amounts of proinflammatory cytokines than $p53^{+/+}$ cells after LPS stimulation. In brief, 0.5 $\times$ 10^6 $p53^{+/+}$ or $p53^{-/-}$ peritoneal macrophages were treated with 5 ng/ml LPS for 6 h. Levels of TNF-α (*D*), IL-6 (*E*), and MIP-2 (*F*) in culture supernatants were determined by ELISA. **, $p < 0.01$ and ***, $p < 0.001$ compared with $p53^{+/+}$ cells. Values are the mean ± SD of triplicate experiments.

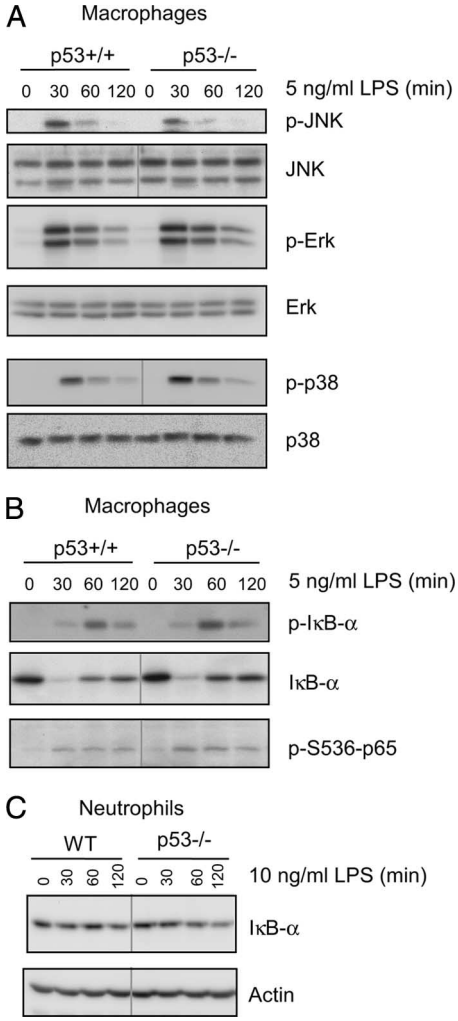

FIGURE 2. Signaling events after TLR4 engagement are not altered in $p53^{-/-}$ inflammatory cells. *A,* $p53^{+/+}$ or $p53^{-/-}$ peritoneal macrophages were treated with 5 ng/ml LPS for the indicated times. The levels of total and phosphorylated JNK, ERK1/2, and p38 were determined by Western blotting. *B,* IκB-α degradation, IκB-α phosphorylation, and p65 phosphorylation were comparable in $p53^{+/+}$ and $p53^{-/-}$ macrophages after LPS stimulation. $p53^{+/+}$ or $p53^{-/-}$ peritoneal macrophages were treated as in *A. C,* IκB-α degradation was comparable in $p53^{+/+}$ and $p53^{-/-}$ neutrophils after LPS stimulation. $p53^{+/+}$ or $p53^{-/-}$ neutrophils were treated with 10 ng/ml LPS for the indicated times and then cell extracts were obtained for Western blotting. Data are representative of at least three repeated experiments. WT, wild type.

only negatively regulates activation of inflammatory cells, including neutrophils and macrophages, by LPS, but also diminishes the severity of LPS-induced ALI.

Materials and Methods

Mice

p53-deficient ($p53^{-/-}$) mice on the C57BL/6 background were gifts from Dr. K. Roth (University of Alabama at Birmingham). Age- and sex-matched wild-type C57BL/6 ($p53^{+/+}$) mice were purchased from the National Cancer Institute-Frederick. Eight- to 10-wk-old male animals were used for experiments. The mice were kept on a 12-h light/dark cycle with free access to food and water. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Materials

Nutlin-3a was purchased from Cayman Chemical. LPS from *Escherichia coli* 0111:B4 and rabbit anti-actin Abs were from Sigma-Aldrich. PAM3CSK4 was from InvivoGen. Abs specific for phosphorylated JNK, total JNK, phosphorylated ERK, total ERK, phosphorylated p38, total p38, phosphorylated IκB-α (S32/S36), IκB-α, and phosphorylated p65 (S635) were from Cell Signaling. Rabbit anti-p65, anti-GAPDH, and anti-p53 Abs were from Santa Cruz Biotechnology. Custom mixture Abs and negative selection columns for neutrophil isolation were purchased from StemCell Technologies. Protein G-agarose beads were from Pierce.

In vivo ALI model

The murine ALI model was used as previously described (28, 29). Briefly, mice were anesthetized with isoflurane. The tongue was then gently extended and the LPS solution (1 mg/kg LPS in 50 μl) deposited into the oropharyx. With this model, ALI, as characterized by neutrophil infiltration into the pulmonary interstitium, development of interstitial edema, and increased proinflammatory cytokine production, occurs after injection of LPS, with the greatest accumulation of neutrophils into airways and histologic injury being present 24 h after LPS exposure.

Harvest of bronchoalveolar lavage (BAL) fluid

BAL fluid samples were obtained from LPS-treated or control mice by lavaging the lungs three times with 1 ml of iced PBS. Total cell counts were measured in the BAL fluid with a hemocytometer and protein concentrations were measured with a Bio-Rad protein assay kit.

Isolation of neutrophils

Mouse neutrophils were purified from bone marrow cell suspensions as described previously (28, 29). Briefly, the femur and tibia of a mouse were

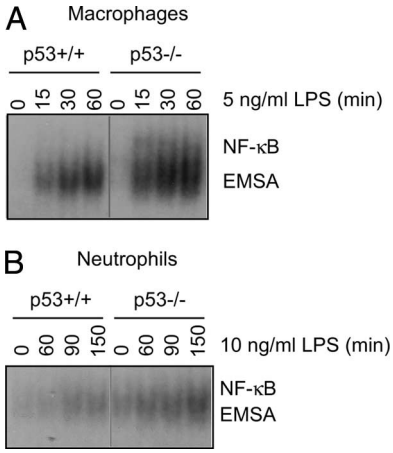

FIGURE 3. LPS-induced NF-κB DNA-binding activities are increased in p53$^{-/-}$ cells. *A* and *B,* p53$^{+/+}$ and p53$^{-/-}$ macrophages (*A*) or neutrophils (*B*) were treated with 5 or 10 ng/ml LPS for the indicated periods of time. The cells were collected and nuclear extracts were prepared. EMSA was performed as described in *Materials and Methods*. Data are from three independent experiments.

flushed with RPMI 1640 and the cells were passed through a 40-μm cell strainer (BD Biosciences). The cell pellets were resuspended in PBS and then incubated for 15 min, rotating at 4°C, with 20 μl of primary Abs specific for the cell surface markers F4/80, CD4, CD45R, CD5, and TER119. This custom mixture is specific for T and B cells, RBC, monocytes, and macrophages. Anti-biotin tetrameric Ab complexes (100 μl) were then added, and the cells were incubated for an additional 15 min at 4°C. Following this, 60 μl of colloidal magnetic dextran iron particles were added to the suspension and incubated for 15 min, rotating at 4°C. The cell suspension was then placed into a column surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection.

Isolation of peritoneal macrophages

Mice were injected i.p. with 1.5 ml of 4% thioglycolate solution. 4 days after injection, mice were sacrificed and the peritoneal cavities were flushed with 10 ml of DMEM. The peritoneal lavage fluids were centrifuged and the cells were resuspended with DMEM plus 10% FBS and plated. After incubation for 1 h at 37°C, the cells were washed three times and nonadherent cells were removed by aspiration. The attached cells were peritoneal macrophages.

Flow cytometry assays

Neutrophils or macrophages were treated with 0, 10, 20, or 40 μM nutlin-3a for 6 h. The cells were then collected, washed once with cold PBS, resuspended with binding buffer containing FITC-annexin V (Calbiochem) and incubated at room temperature for 15 min. Propidium iodide (PI)

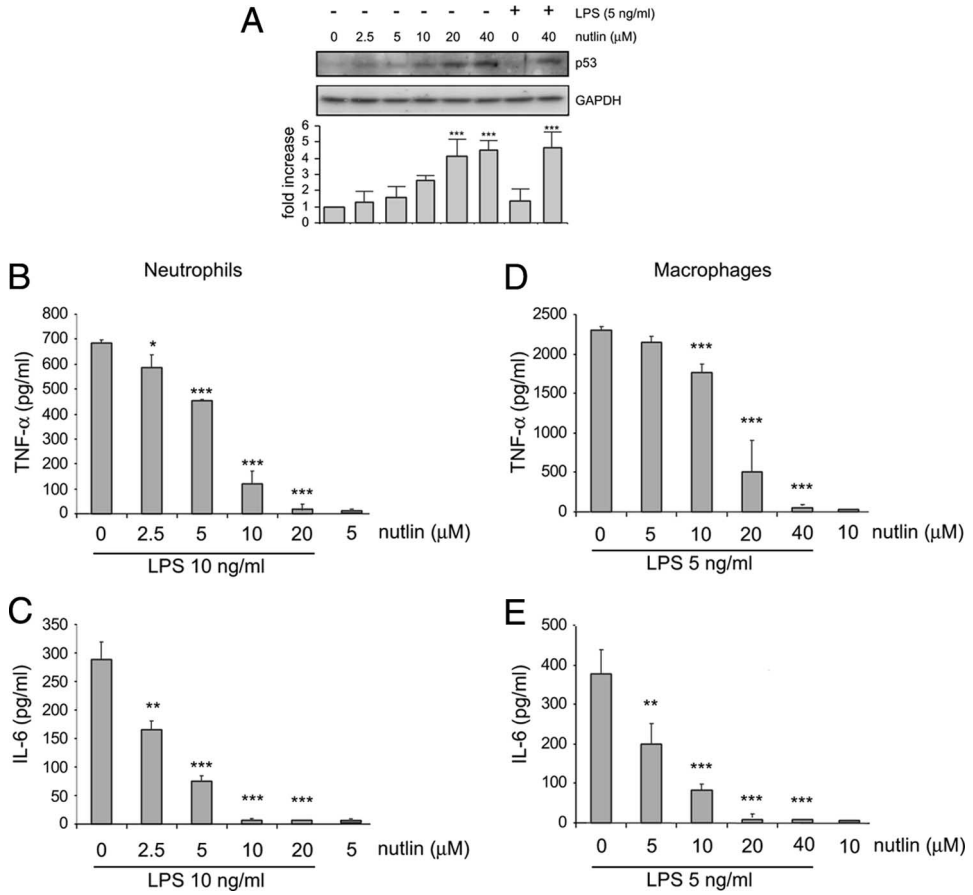

FIGURE 4. Nutlin-3a up-regulates p53 levels, but down-regulates the response of inflammatory cells to LPS. *A*, Nutlin-3a induced p53 in macrophages. p53$^{+/+}$ peritoneal macrophages were treated with the indicated concentrations of nutlin-3a for 6 h. Western blots were analyzed with densitometry. The ratio of the levels of p53 to GAPDH in the cells treated without LPS or nutlin-3a was regarded as 1. The fold increase shown is the relative increase for each indicated treatment compared with no treatment. Values are presented as mean ± SD of four independent experiments. ***, $p < 0.001$ compared with no treatment. *B–E*, Nutlin-3a attenuates the activation of macrophages and neutrophils by LPS. Two × 10^6 p53$^{+/+}$ neutrophils (*B* and *C*) or 2 × 10^6 peritoneal macrophages (*D* and *E*) were pretreated with nutlin-3a at the indicated concentrations for 1 h. The neutrophils (*B* and *C*) or macrophages (*D* and *E*) were then treated without or with 5 (macrophages) or 10 (neutrophils) ng/ml LPS for 6 h. TNF-α and IL-6 concentrations in the culture supernatants were measured by ELISA. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the control group without nutlin-3a treatment. Values are presented as mean ± SD of quadruplicate experiments. Data are representative of three independent experiments.

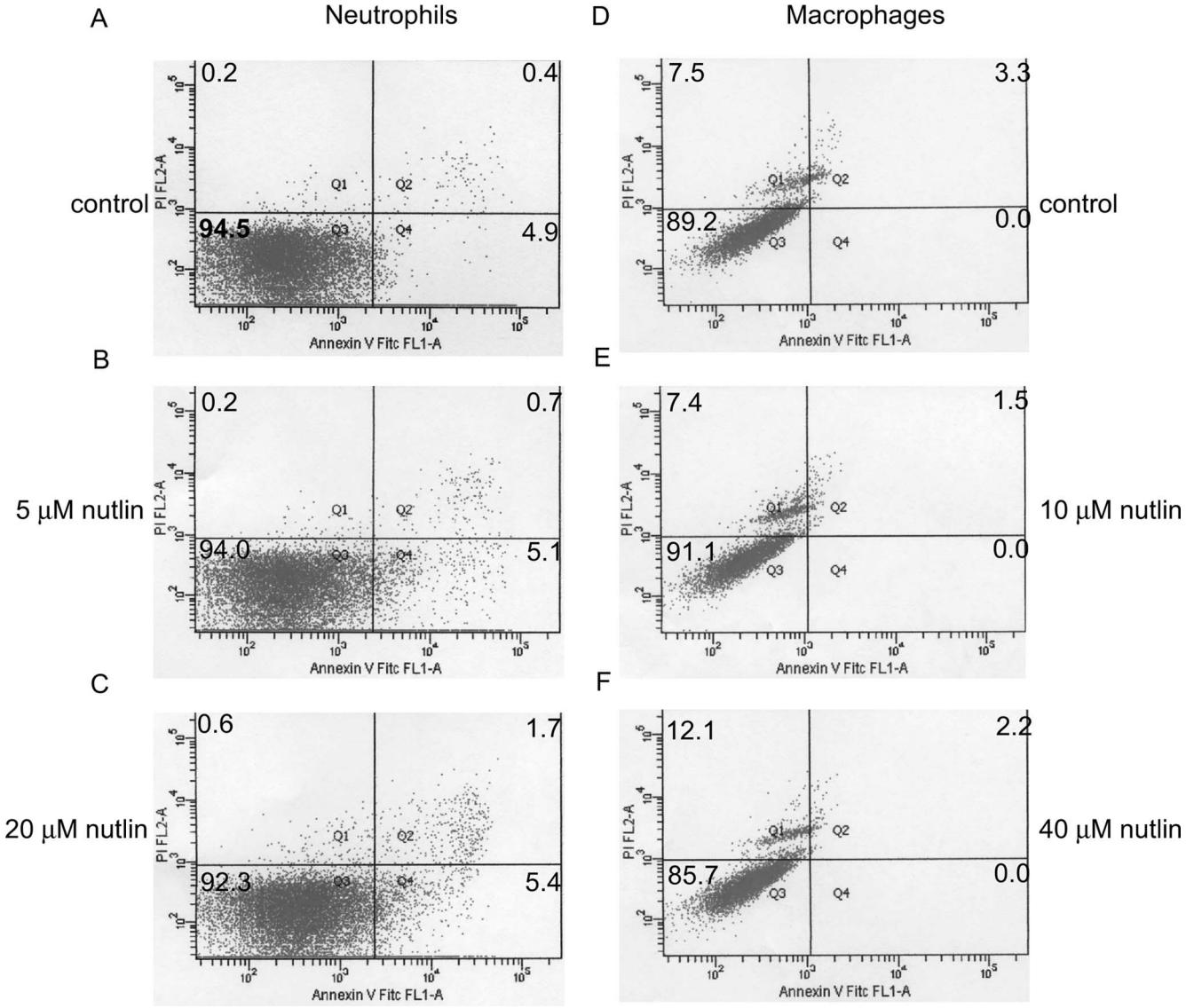

FIGURE 5. Nutlin-3a does not induce apoptosis of macrophages or neutrophils. *A–C*, $p53^{+/+}$ neutrophils were treated with 0, 5, or 20 μM nutlin-3a for 6 h. Cells were then collected and flow cytometry was performed after staining with annexin V and PI to determine the levels of apoptosis. *D–F*, $p53^{+/+}$ macrophages were treated with 0, 10, or 40 μM nutlin-3a for 6 h. The cells were then collected and stained with annexin V and PI, as above, to determine the levels of apoptosis. Data are representative of three independent experiments.

(Calbiochem) was then added into the buffers. The cells were then analyzed by a BD Biosciences LSR II system. The *x*-axis is FITC and *y*-axis is PI fluorescence. Cells with FITC-positive staining were regarded as apoptotic.

Western blotting assay

Western blotting assays were performed essentially as previously described (30).

Cell stimulation

Neutrophils or macrophages were pretreated without or with nutlin-3a at various concentrations for 1 h. The cells were then stimulated with LPS or PAM3CSK4 for various lengths of time. The supernatants or cells were collected for the following analysis.

Cytokine and chemokine ELISA and protein assays

Immunoreactive TNF-α, MIP-2, and IL-6 were quantified using DuoSet ELISA Development kits (R&D Systems) according to the manufacturer's instructions.

Myeloperoxidase (MPO) assay

MPO was measured using a modification of a previously described method (28, 29). In brief, lung tissue was homogenized using a Glas-Col homogenizer in 0.5 ml of 0.5% hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer (pH 6.0). The homogenate was centrifuged at 14,000 × *g* for 30 min at 4°C and the supernatant was collected for assay of MPO activity as determined by measuring the H_2O_2-dependent oxidation of *o*-dianisidine solution (3,3′-dimethoxybenzidine dihydrochloride in potassium phosphate buffer, pH 6.0) at 450 nm.

Wet:dry lung weight ratios

All mice used for lung wet:weight ratios were of identical ages. Lungs were excised, rinsed briefly in PBS, blotted, and then weighed to obtain the "wet" weight. Lungs were then dried in an oven at 80°C for 7 days to obtain the "dry" weight.

EMSA

Nuclear extracts were prepared and assayed by EMSA as previously described (28). For analysis of NF-κB, the κB DNA sequence of the Ig gene was used. Synthetic double-stranded sequences (with enhancer motifs underlined) were filled in and labeled with [γ-^{32}P]dATP (PerkinElmer) using T_4 polynucleotide kinase as follows: κB sequence, 5′-GCCATGGGGG GATCCCCGAAGTCC-3′ (Promega).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was essentially performed as previously described (30). Briefly, cells were fixed with 1% of formaldehyde for 10 min. Genomic DNA was then sheared by sonication to lengths ranging from 200 to 1000 bp. For

input determination, 5% of cell extract was taken and the rest of the extract was incubated with rabbit anti-p65 polyclonal Abs overnight, followed by precipitation with protein G-agarose beads. Genomic DNA in the immunocomplexes was purified by a Qiagen miniprep column, and the NF-κB-responsive elements in the promoter of NF-κB target genes were amplified by PCR. The primer sequence for amplification of NF-κB-responsive elements in the promoter of mouse *IκB-α* gene was sense 5′-TGGCGAGGTCT GACTGTTGTGG-3′ and antisense 5′-GCTCATCAAAAAGTTCCCTGTGC-3′. The primer sequence for amplification of the mouse *MIP2* promoter was sense 5′-CAGGAAAGGCAATCCCAGAAAGG-3′ and antisense 5′-GGAGGGT GCTGAACACTTGTAAGG-3′.

Statistical analysis

For each experimental condition, the entire group of animals was prepared and studied at the same time. Data are presented as mean ± SD (in vitro experiments) or ± SEM (in vivo experiments) for each experimental group. ANOVA was used for comparisons between multiple groups. Student's *t* test was used for comparisons between two groups. A value of $p < 0.05$ was considered significant.

Results

$p53^{-/-}$ inflammatory cells demonstrate enhanced activation by LPS

To examine the participation of p53 in the response of inflammatory cells to LPS, bone marrow neutrophils were isolated from $p53^{+/+}$ and $p53^{-/-}$ mice and treated with 10 ng/ml LPS. As shown in Fig. 1, *A–C*, $p53^{-/-}$ neutrophils produced significantly more proinflammatory cytokines, including TNF-α, IL-6, and MIP-2, than did $p53^{+/+}$ neutrophils. To determine whether this is a cell type-specific effect, peritoneal macrophages were harvested from $p53^{+/+}$ and $p53^{-/-}$ mice and treated with 5 ng/ml LPS. As was the case with $p53^{-/-}$ neutrophils, $p53^{-/-}$ macrophages produced more TNF-α, IL-6, and MIP-2 after LPS stimulation than did $p53^{+/+}$ macrophages (Fig. 1, *D* and *E*). These data suggest that p53 negatively regulates the responses of inflammatory cells, including neutrophils and macrophages, to LPS stimulation.

TLR4-associated signaling events are not affected in $p53^{-/-}$ inflammatory cells

To determine why $p53^{-/-}$ inflammatory cells have enhanced responses to LPS, we examined cytoplasmic signaling events that occur after TLR4 engagement. As shown in Fig. 2*A*, LPS induced rapid phosphorylation of JNK, ERK, and p38 in macrophages. However, there was no difference in MAPK activation between $p53^{+/+}$ and $p53^{-/-}$ macrophages. Furthermore, IκB-α degradation, IκB-α phosphorylation, and p65 phosphorylation were similar between $p53^{+/+}$ and $p53^{-/-}$ macrophages after LPS stimulation (Fig. 2*B*). Like macrophages, $p53^{+/+}$ and $p53^{-/-}$ neutrophils demonstrated no difference in LPS-induced IκB-α degradation (Fig. 2*C*). These data suggest that the enhanced responses of $p53^{-/-}$ inflammatory cells are not the result of increased TLR4-associated signaling events in response to LPS stimulation.

LPS-induced NF-κB DNA-binding activity is elevated in $p53^{-/-}$ cells

After TLR4 engagement, NF-κB translocates to the nucleus where it binds to the promoters of target genes and activates their transcription (7–10). We thus investigated the DNA-binding activity of NF-κB in nuclear extracts obtained from $p53^{+/+}$ and $p53^{-/-}$ inflammatory cells following LPS stimulation. As shown in Fig. 3*A*, DNA-binding activity of NF-κB in nuclear extracts from LPS-activated $p53^{-/-}$ macrophages was increased compared with that found in $p53^{+/+}$ cells. Similarly, DNA-binding activity of NF-κB from $p53^{-/-}$ neutrophils cultured with LPS were also higher than that present in $p53^{+/+}$ macrophages (Fig. 3*B*).

Nutlin-3a up-regulates p53 levels, but down-regulates the response of inflammatory cells to LPS

Our initial experiments demonstrated that $p53^{-/-}$ macrophages and neutrophils have enhanced responses to LPS stimulation. We next asked whether a further increase in p53 levels in $p53^{+/+}$ cells can attenuate their responses to LPS. To examine this issue, $p53^{+/+}$ neutrophils or macrophages were treated with nutlin-3a, a specific inhibitor of p53 and Mdm2 interaction (21). As shown in Fig. 4*A*, nutlin-3a increased p53 levels in a dose-dependent manner in macrophages. Next, neutrophils or macrophages were pretreated with nutlin-3a for 1 h and then subjected to LPS stimulation for 4 h. We found that nutlin-3a treatment resulted in diminished LPS-induced TNF-α and IL-6 production in both macrophages and neutrophils (Fig. 4, *B–E*). Of note, nutlin-3a alone did not activate either neutrophils or macrophages to produce proinflammatory cytokines (Fig. 4, *B–E*).

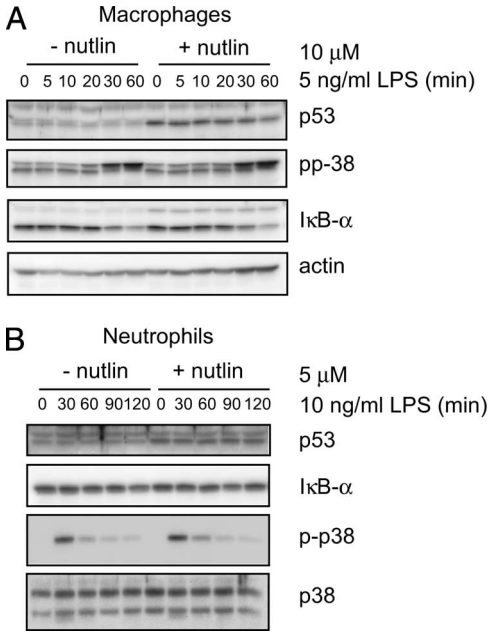

FIGURE 6. Nutlin-3a does not affect the TLR4-associated signaling events in inflammatory cells. *A* and *B*, Nutlin-3a does not affect the degree of IκB-α degradation or p38 phosphorylation in inflammatory cells after LPS stimulation. $p53^{+/+}$ macrophages (*A*) or neutrophils (*B*) were pretreated with nutlin-3a for 60 min. The cells were then stimulated with LPS for the indicated times. Cell extracts were prepared and the levels of p53, IκB-α, phosphorylated p38, total p38, and actin were determined by Western blotting. Data are from three independent experiments.

Since p53 appears to regulate NF-κB DNA-binding activity in TLR4-stimulated inflammatory cells, we asked whether this was a TLR4-specific response or might also occur after engagement of TLR. To address this question, we examined the effects of nutlin-3a on neutrophils and macrophages treated with PAM3CSK4, a TLR2 ligand. As shown in supplemental Fig. 1,[4] nutlin-3a significantly decreased the activation of PAM3CSK4-treated neutrophils and macrophages. These data indicate that an increase in p53 levels from baseline in control $p53^{+/+}$ inflammatory cells attenuates their responses not only to TLR4 ligands, such as LPS, but also to activation through TLR2.

Exposure to nutlin-3a does not produce apoptosis of macrophages or neutrophils

p53 induces cell cycle arrest and/or apoptosis, depending on the nature of stimuli and the cell population (26). Because macrophages and neutrophils are terminally differentiated cells, the effects of p53 on these cells cannot involve its role in regulating cell cycle arrest. However, accelerated entry into apoptosis may result in an attenuated response to LPS. To investigate whether the dampened responses of

[4] The online version of this article contains supplemental material.

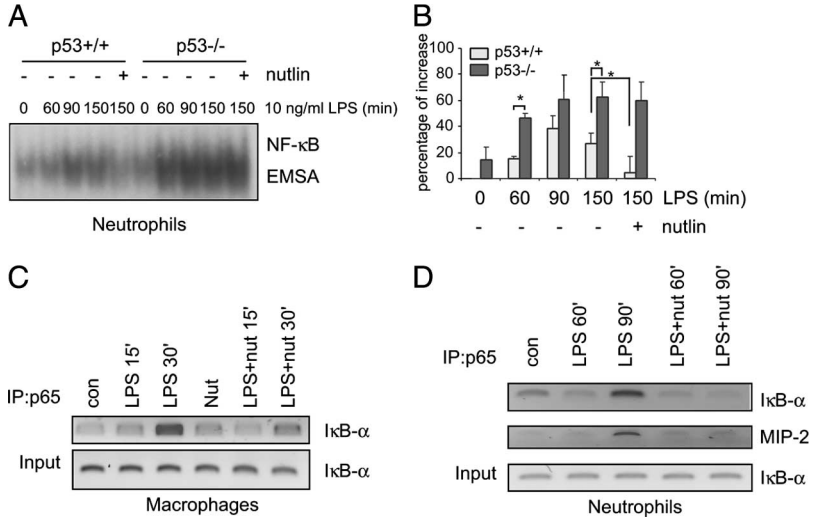

FIGURE 7. Nutlin-3a decreases LPS-induced NF-κB binding to DNA. *A*, Nutlin-3a diminishes NF-κB nuclear translocation in LPS-treated $p53^{+/+}$, but not $p53^{-/-}$ neutrophils. $p53^{+/+}$ or $p53^{-/-}$ neutrophils were pretreated with vehicle alone (DMSO) or 5 μM nutlin-3a in DMSO for 1 h. The cells were then left untreated or were cultured with 10 ng/ml LPS for the indicated times. Nuclear extracts were prepared and EMSAs were performed as described in *Materials and Methods*. *B*, Densimetric quantitation of *A*. The densimetric values from $p53^{+/+}$ cells without treatment were used as controls. The percentage increase for each treatment was calculated by determining the ratio of the indicated treatment—control values to control. Data are presented as mean ± SD from triplicate experiments. ∗, $p < 0.05$. *C*, Nutlin-3a diminishes NF-κB binding to the *IκB-α* promoter. $p53^{+/+}$ macrophages were pretreated with vehicle alone (DMSO) or 10 μM nutlin-3a for 1 h. The cells were then left untreated or were cultured with 5 ng/ml LPS for the indicated times. The ChIP assay was performed as described in *Materials and Methods*. *D*, Nutlin-3a diminishes NF-κB binding to the *IκB-α* and *MIP-2* promoters in LPS-stimulated neutrophils. $p53^{+/+}$ neutrophils were pretreated with vehicle alone (DMSO) or with 5 μM nutlin-3a in DMSO for 1 h. The cells were then left untreated or were cultured with 10 ng/ml LPS for the indicated times. ChIP assays were then performed. Data are from three independent experiments.

nutlin-3a-treated inflammatory cells to LPS are results of enhanced apoptosis, we performed annexin V-PI staining and found low levels of apoptosis in nutlin-3a-treated macrophages and neutrophils, even when the concentration of nutlin-3a was four times higher than that needed to significantly decrease the responses of the cells to LPS (Fig. 5, *A–F*).

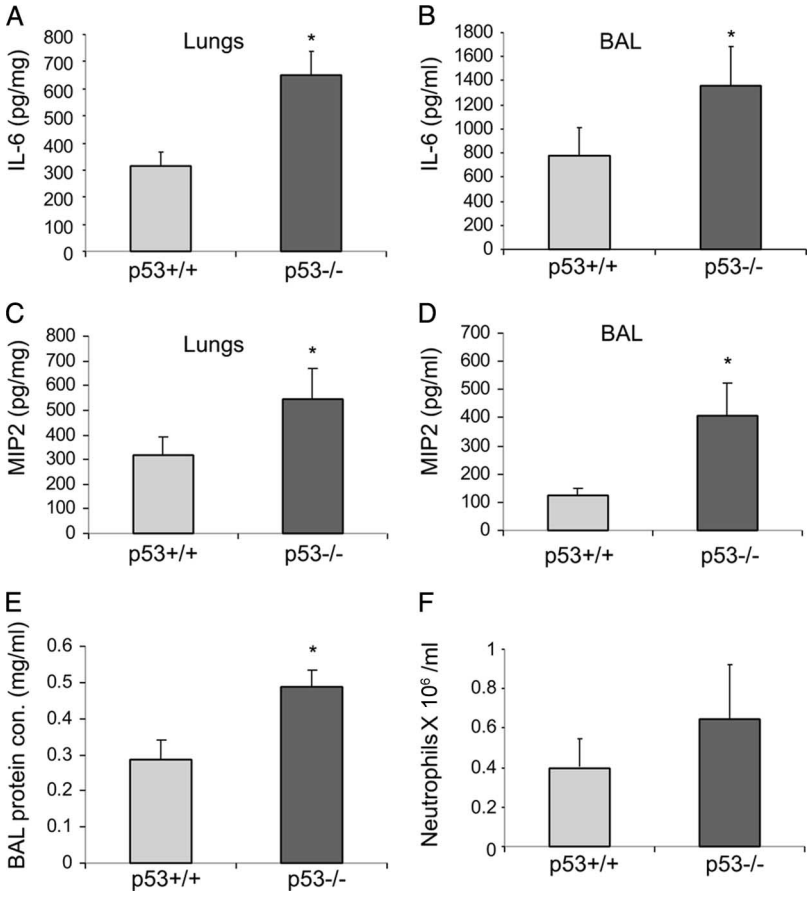

FIGURE 8. Increased severity of LPS-induced ALI in $p53^{-/-}$ mice. *A–D*, Concentrations of proinflammatory cytokines in lung homogenates and BAL fluid of $p53^{-/-}$ mice were significantly higher than those in $p53^{+/+}$ mice. $p53^{+/+}$ or $p53^{-/-}$ mice ($n = 5$ in each group) were intratracheally injected with 1 mg/kg LPS dissolved in 50 μl of PBS. At 24 h after LPS instillation, the mice were sacrificed and BAL fluid and lung homogenates were collected as described in *Materials and Methods*. Levels of IL-6 and MIP-2 in lung homogenates (*A* and *B*) and BAL fluid (*C* and *D*) were determined by ELISA. ∗, $p < 0.05$ compared with $p53^{+/+}$ mice. *E*, Total protein levels in the BAL fluid of $p53^{-/-}$ mice were significantly higher than those present in $p53^{+/+}$ mice. ∗, $p < 0.05$ compared with $p53^{+/+}$ mice. *F*, Neutrophil counts in the BAL fluid of $p53^{-/-}$ mice were significantly higher than those in $p53^{+/+}$ mice. Values are presented as mean ± SEM. Data are representative of two independent experiments.

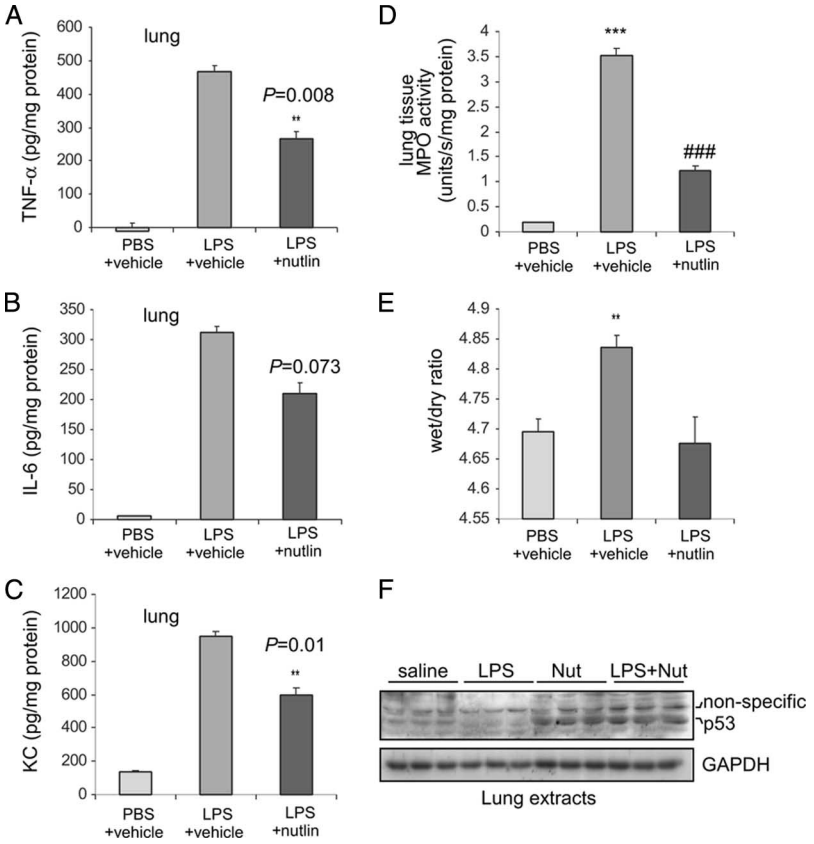

FIGURE 9. Nutlin-3a treatment attenuates LPS-induced ALI. *A–C*, Nutlin-3a decreases cytokine levels in the lungs after LPS instillation. $p53^{+/+}$ mice were injected i.p. with 25 mg/kg nutlin-3a every 24 h for 2 days in 50 μl of DMSO, while control mice were injected i.p. with 50 μl of vehicle (DMSO; $n = 5$ mice in each group). Intratracheal LPS at 1 mg/kg dissolved in 50 μl of PBS or PBS alone was administered 4 h after the second injection of nutlin-3a or vehicle. At 24 h after LPS administration, the mice were sacrificed and lung homogenates were prepared. TNF-α (*A*), IL-6 (*B*), and KC (*C*) levels were measured by ELISA. *D*, Nutlin-3a treatment diminished LPS-induced lung MPO activity. $p53^{+/+}$ mice were treated as in *A–C*. MPO activity was determined as described in *Materials and Methods*. ***, $p < 0.001$ when compared with the PBS + vehicle group; ###, $p < 0.001$ when compared with the LPS + vehicle group. *E*, Nutlin-3a treatment diminished LPS-induced increases in lung wet:dry ratios. $p53^{+/+}$ mice were treated as in *A–C*. Lung wet:dry ratios were determined as described in *Materials and Methods*. **, $p < 0.01$ when compared with the PBS + vehicle or LPS + nutlin group. *F*, Nutlin-3a increased p53 levels in lung tissues. $p53^{+/+}$ mice ($n = 3$) were pretreated every 24 h for 2 days with 25 mg/kg nutlin-3a i.p. in 50 μl of DMSO or vehicle (DMSO) alone. At 4 h after the second injection of nutlin-3a or vehicle, LPS (1 mg/kg) dissolved in 50 μl of saline or saline alone was administered intratracheally. Lung homogenates were collected 24 h after LPS injection and p53 levels were determined by Western blotting. GAPDH was used as a loading control. Values are presented as mean ± SEM. Data are representative of two independent experiments.

Nutlin-3a does not affect TLR4-associated signaling pathways in inflammatory cells

To determine whether nutlin-3a affects the inflammatory responses of macrophages and neutrophils through modulation of signaling cascades induced by TLR4 engagement, $p53^{+/+}$ macrophages were pretreated with 10 μM nutlin-3a for 1 h and then stimulated with LPS for differing lengths of time. As shown in Fig. 6*A*, the kinetics of p38 phosphorylation and IκB-α degradation were comparable in macrophages pretreated with or without nutlin-3a. Similarly, LPS-induced p38 phosphorylation and IκB-α degradation were not affected in neutrophils pretreated with nutlin-3a (Fig. 6*B*). These data suggest that p53 induction does not affect signaling events, including MAPK and IKK activation, induced by TLR4 engagement in inflammatory cells.

Nutlin-3a decreases LPS-induced NF-κB DNA-binding activity

Since nutlin-3a does not affect TLR4-associated signaling events, we next determined whether nutlin-3a regulates NF-κB activity in the nucleus. As shown in Fig. 7, *A* and *B*, NF-κB DNA-binding activity in neutrophils was increased by LPS stimulation. LPS-treated $p53^{-/-}$ neutrophils demonstrated increased NF-κB- binding activity compared with $p53^{+/+}$ cells (Fig. 7, *A* and *B*). Culture with nutlin-3a decreased LPS-induced NF-κB binding to DNA in $p53^{+/+}$ neutrophils (Fig. 7, *A* and *B*). Furthermore, nutlin-3a treatment did not affect NF-κB DNA-binding activity in LPS-stimulated $p53^{-/-}$ neutrophils, indicating that the effects of nutlin-3a on the NF-κB DNA-binding activities are specifically dependent on p53 induction (Fig. 7, *A* and *B*).

To examine whether nutlin-3a regulates NF-κB binding to the promoters of target genes, we performed ChIP assays in LPS-stimulated cells. As shown in Fig. 7*C*, LPS stimulation increased p65 binding to the *IκB-α* promoter in macrophages. However, nutlin-3a pretreatment decreased LPS-induced p65 binding to the *IκB-α* promoter (Fig. 7*C*). Similarly, nutlin-3a pretreatment also down-regulated p65 binding to the promoters of *IκB-α* and *MIP2* in LPS-activated neutrophils (Fig. 7*D*).

$p53^{-/-}$ mice demonstrate increased severity of LPS-induced ALI

Neutrophils and macrophages play a central role in the pathogenesis of ALI (1, 12, 31, 32). Because our in vitro experiments demonstrated enhanced NF-κB DNA- binding activity and increased production of proinflammatory cytokines in LPS-activated $p53^{-/-}$ neutrophils and macrophages, we hypothesized that LPS-induced ALI would be more severe in $p53^{-/-}$ mice. As shown in Fig. 8, *A–D*, there were significantly higher

levels of the proinflammatory cytokines IL-6 and MIP-2 in lung tissue and BAL fluid from $p53^{-/-}$ mice compared with $p53^{+/+}$ mice. Furthermore, protein concentrations in BAL fluid, an indicator of lung leak, were significantly higher in LPS-treated $p53^{-/-}$ mice than those found in $p53^{+/+}$ mice (Fig. 8*E*). In addition, neutrophil counts in BAL fluids from $p53^{-/-}$ mice were increased (Fig. 8*F*). These data suggest that the enhanced responses of $p53^{-/-}$ macrophages and neutrophils to LPS, and perhaps of other pulmonary cell populations as well, contribute to more severe LPS-induced lung injury in $p53^{-/-}$ mice.

Nutlin-3a attenuates LPS-induced ALI

The ability of nutlin-3a to attenuate inflammatory responses of neutrophils and macrophages prompted us to ask whether nutlin-3a can also reduce the severity of LPS-induced ALI. As shown in Fig. 9, *A–C*, treatment with nutlin-3a before LPS administration significantly decreased levels of proinflammatory cytokines, including TNF-α, IL-6, and KC, in the lungs. Pulmonary MPO activity, a marker of neutrophil infiltration into the lungs (28), was also reduced in nutlin-3a-treated mice (Fig. 9*D*). Furthermore, lung wet:dry ratios, a measure of interstitial pulmonary edema and severity of ALI, was diminished in nutlin-3a-treated mice compared with those found in mice treated with vehicle (Fig. 9*E*). As expected, p53 expression was increased in the lung tissues of nutlin-3a-treated mice (Fig. 9*F*).

Discussion

The reciprocal regulation of the activities of NF-κB and p53 has been the focus of numerous studies (33, 34). In cancer cells, these transcriptional factors appear to have opposite roles in modulating cellular functions (35). For example, NF-κB promotes inflammation and inhibits apoptosis, while p53 induces cell cycle arrest and promotes apoptosis (36, 37). The evidence that NF-κB signaling regulates p53 activity is ample. For example, one of the NF-κB family molecules, Bcl-3, up-regulates Mdm2 expression, thereby inhibiting p53 transcriptional activity (38). Another NF-κB subunit, p52, can specifically bind to the promoter of the p53 target gene, *p21*, and inhibits p53-dependent p21 basal expression (39). How p53 modulates NF-κB activity remains less clear. p53 mediates increased IκB-α expression and resultant decreases in NF-κB activation (35). It has also been shown that p53 suppresses IKK activity and subsequent activation of NF-κB (40). In addition, reciprocal sequestration of coactivators, including CBP and p300, has been thought to be a major mechanism underlying the mutual suppression that exists between p53 and NF-κB (26, 41).

In this study, we found that p53, even at basal levels, is involved in regulation of NF-κB activity since $p53^{-/-}$ inflammatory cells demonstrated enhanced responses to LPS. These findings, as well as studies from other groups demonstrating that significantly more proinflammatory cytokines are produced in the thymus of LPS-treated $p53^{-/-}$ mice than in wild-type mice (42), suggest that overtly exuberate responses of $p53^{-/-}$ inflammatory cells to LPS stimulation could be one of the mechanisms explaining the enhanced LPS-induced lung injury and increased susceptibility to LPS-associated mortality in $p53^{-/-}$ mice. However, elevation of NF-κB-dependent inflammatory cytokines in $p53^{-/-}$ mice may not be the sole explanation for their sensitivity to endotoxemia. Komarova et al. (42) also found that $p53^{-/-}$ macrophages had decreased phagocytic activity. Defects in the ingestion of apoptotic cells, and specifically of apoptotic neutrophils, are associated with unfavorable outcomes from inflammatory diseases (43–45). p53 has become a novel therapeutic target for treatment of inflammatory diseases and modulation of p53 by a natural product, genistein, was shown to be able to attenuate TLR4-induced activation of monocytes (46).

Intracellular events induced by TLR4 engagement, such as activation of MAPK and IKK, were comparable in p53 wild-type and knockout cells. These findings indicate that p53 negatively regulates NF-κB activity downstream of IKK activation and IκB-α degradation. Indeed, we observed increases in NF-κB DNA-binding activity in LPS-treated $p53^{-/-}$ macrophages and neutrophils compared with that found in $p53^{+/+}$ cells. Nevertheless, we were unable to detect p53 binding to the IκB-α promoter in LPS-stimulated $p53^{+/+}$ macrophages (data not shown), suggesting that there is no direct and physical involvement of p53 in modulating binding of NF-κB to the promoters of its target genes. Since enhanced interaction with the coactivator p300/CBP increases NF-κB DNA-binding activity and subsequent transcriptional activity, it may be a mechanism by which $p53^{-/-}$ cells demonstrate increased responses to LPS stimulation.

Exposure of LPS-stimulated macrophages and neutrophils to nutlin-3a reduced their production of proinflammatory cytokines. Treatment with nutlin-3a also decreased the severity of LPS-induced ALI. Nutlin-3a is a specific inhibitor of the interaction between p53 and Mdm2 and thereby enhances the activity of p53 (47). Compared with other physiological and nonphysiological inducers of p53, such as chemotherapeutic drugs and ion irradiation, nutlin-3a creates no DNA damage, but does specifically stabilize and activate p53. Nutlin-3a induces cell cycle arrest and apoptosis of a variety of cancer cells both in vitro and in vivo (48). The decrease in the proinflammatory responses of nutlin-3a-treated cells to LPS was not caused by cell death since nutlin-3a did not induce any apparent alteration in apoptosis among either neutrophils or macrophages after 4 h of treatment. Furthermore, nutlin-3a exposure did not alter proximal signaling events, such as activation of MAPK and IKK, after TLR4 engagement, which is consistent with the findings by Dey et al. (49) that nutlin-3a does not affect TNF-α- or IL-1-induced IκB-α phosphorylation and degradation or p65 phosphorylation in cancer cells. However, nutlin-3a treatment did inhibit the binding of NF-κB to promoters of target genes in LPS-treated macrophages and neutrophils. These inhibitory effects of nutlin-3a on NF-κB activation appear to be specific for nutlin-3a-induced alterations in p53 as nutlin-3a failed to attenuate NF-κB binding to DNA in $p53^{-/-}$ cells.

One potential mechanism by which nutlin-3a-induced p53 activation negatively regulates NF-κB activity is through sequestration of NF-κB-binding coactivators such as p300/CBP (26, 41). Although a recent study found that p53 is involved in the regulation of IKK kinase activity (40), we found no alterations in IκB-α phosphorylation or degradation in LPS-treated $p53^{-/-}$ neutrophils or macrophages or in $p53^{+/+}$ cells treated with nutlin-3a. Although these results indicate that p53 does not participate in regulating the activation of IKKβ, which is the primary IKK isoform responsible for IκB-α phosphorylation after TLR4 engagement (50), they do not rule out a role for interactions between p53 and other IKK isoforms, such as IKKα, in modulating NF-κB activity. IKKα does not participate in IκB-α degradation (50). However, it is translocated to the nucleus where it phosphorylates histone H3, among other activities. Phosphorylation of histone H3 promotes the accessibility of NF-κB as well as cofactors to promoter regions, thereby facilitating NF-κB-dependent transcription (51, 52). Whether inhibition of IKKα-induced histone H3 phosphorylation is one of the mechanisms by which p53 modulates NF-κB binding to promoters and transcription of NF-κB-regulated genes needs further exploration.

Although we only found minimal apoptosis of neutrophils and macrophages 4 h after nutlin-3a treatment that was not different from the degree of apoptosis present among untreated cells, there is still a possibility that nutlin-3a enhances apoptosis of these inflammatory cell populations over more extended periods of time. Neutrophils are short-lived cells and play a central role in development and perpetuation of LPS-induced lung injury by accumulating in the lungs and producing high levels of proinflammatory mediators, including cytokines, chemokines, and reactive oxygen species (53). Increased neutrophil apoptosis and resultant clearance from the lungs during sepsis

has been shown to be beneficial (53, 54). Thus, some of the beneficial effects of nutlin-3a and p53 activation in ALI could come from enhanced neutrophil apoptosis induced by p53.

In conclusion, we found that p53 negatively regulates NF-κB activity by decreasing binding of NF-κB to the promoters of genes for proinflammatory cytokines, thereby contributing to the increased response of $p53^{-/-}$ inflammatory cells to LPS stimulation and enhancing lung injury in LPS-treated $p53^{-/-}$ mice. Modulation of p53 by nutlin-3a diminished the response of neutrophils and macrophages to stimulation through TLR2 or TLR4 and also attenuated LPS-induced ALI.

Disclosures

The authors have no financial conflict of interest.

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