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NADPH Oxidase Limits Lipopolysaccharide-Induced Lung Inflammation and Injury in Mice through Reduction-Oxidation Regulation of NF-κB Activity

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Although reactive oxygen species (ROS) produced by NADPH oxidase are known to regulate inflammatory responses, the impact of ROS on intracellular signaling pathways is incompletely understood. In these studies, we treated wild-type (WT) and p47phox−/− deficient mice with LPS to investigate mechanisms by which NADPH oxidase regulates signaling through the NF-κB pathway. After intratracheal instillation of LPS, ROS generation was impaired in p47phox−/− mice, whereas these mice had increased neutrophilic alveolitis and greater lung injury compared with WT controls. In mice interbred with transgenic NF-κB reporters (HIV-long terminal repeat/luciferase [HLL]), we found exaggerated LPS-induced NF-κB activation and increased expression of proinflammatory cytokines in lungs of p47phox−/−/HLL mice compared with controls. Both lung macrophages and bone marrow–derived macrophages (BMDMs) isolated from p47phox−/−/HLL mice showed enhanced LPS-stimulated NF-κB activity compared with controls. Although nuclear translocation of NF-κB proteins was similar between genotypes, EMSAs under nonreducing conditions showed increased DNA binding in p47phox−/−/HLL mice compared with controls. Both lung macrophages and bone marrow–derived macrophages (BMDMs) isolated from p47phox−/−/HLL mice showed enhanced LPS-stimulated NF-κB activity compared with controls. Although nuclear translocation of NF-κB proteins was similar between genotypes, EMSAs under nonreducing conditions showed increased DNA binding in p47phox−/−/HLL mice compared with WT BMDMs, pointing to NADPH oxidase modulating intracellular redox status in macrophages. Treatment with the Ref-1–specific inhibitor E3330 or hydrogen peroxide inhibited LPS-induced NF-κB activation in p47phox−/−/HLL BMDMs but not in WT/HLL cells. Consistent with these findings, small interfering RNA against Ref-1 selectively reduced NF-κB activity in LPS-treated p47phox−/−/HLL BMDMs. Together, these results indicate that NADPH oxidase limits LPS-induced NF-κB transcriptional activity through regulation of intracellular redox state. The Journal of Immunology, 2013, 190: 000–000.

A cute and chronic lung inflammation is associated with increased production of reactive oxygen species (ROS) (1, 2). NADPH oxidase, which is the major source of ROS in activated phagocytes, is activated by recruiting its cytoplastic subunits p47phox, p67phox, and p40phox and rac to the plasma membrane or neutrophil secondary granules and forming a complex with membrane-bound flavocytochrome gp91phox and p22phox (3). Activated NADPH oxidase converts molecular oxygen to superoxide anion that can in turn be converted to antimicrobial ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid. Chronic granulomatous disease (CGD) results from disabling mutations of ROS metabolites, such as H2O2 and hypohalous acid.
activity (21). We previously showed that NADPH oxidase–deficient (p47\(^{phox}−/−\)) mice develop increased lung inflammation and pro-inflammatory cytokine production compared with wild-type (WT) mice in response to intratracheal (TT) zymosan, a fungal cell wall–derived product that ligates TLR2 and dendrin-1 (22). Increased inflammation in this model, as well as increased inflammation following TT injection of the TLR4 ligand lipopolysaccharide (LPS), is associated with impaired activation of the transcription factor Nrf2 and increased activity of NF-\(\kappa\)B in the lungs of p47\(^{phox}−/−\) mice (23).

The current study evaluates mechanisms by which NADPH oxidase regulates NF-\(\kappa\)B activation in the lungs. In this study, we show that ROS production after IT injection of Escherichia coli LPS is NADPH oxidase dependent and that impaired ROS production results in increased NF-\(\kappa\)B activation as well as excessive lung inflammation and injury. After LPS treatment, p47\(^{phox}−/−\) macrophages have increased NF-\(\kappa\)B transcriptional activity, as opposed to differences in nuclear translocation of NF-\(\kappa\)B proteins. Further, we found that NADPH oxidase limits NF-\(\kappa\)B activity after LPS treatment through modulation of the intracellular redox state. Together, these findings point to modulation of redox status of NF-\(\kappa\)B proteins as a major mechanism by which NADPH oxidase regulates LPS-induced inflammatory responses and subsequent lung injury.

## Materials and Methods

### Animals

Mice with a targeted disruption of the p47\(^{phox}\) gene were used in these studies (21). NF-\(\kappa\)B reporter mice (HIV-long terminal repeat/luciferase [HLL]; Invivogen, McGraw Park, IL) and counting 400–600 cells in complete cross sections. Animals were anesthetized with isoflurane, and the trachea was exposed by surgical dissection. LPS (serotype 055:B5; Sigma-Aldrich, St. Louis, MO) was diluted in sterile PBS and injected (3 \(\mu\)g/\(g\) body weight) into the trachea via a 27-gauge needle. The neck wound was closed with sterile sutures under aseptic conditions.

### Histology and immunohistochemistry

After euthanasia, lungs were inflated with 1 ml 10% neutral buffered formalin, removed en bloc after tracheal ligation, preserved in 10% neutral buffered formalin, removed en bloc after tracheal ligation, preserved in 10% neutral buffered formalin, and subsequently embedded in paraffin. Lung-tissue sections (5 \(\mu\)m) were stained with H&E. The degree of lung inflammation/injury in the alveolar tissue was assessed on 20 sequential sections. Intranuclear NF-\(\kappa\)B activity (21). We previously showed that NADPH oxidase–deficient (p47\(^{phox}−/−\)) mice develop increased lung inflammation and pro-inflammatory cytokine production compared with wild-type (WT) mice in response to intratracheal (TT) zymosan, a fungal cell wall–derived product that ligates TLR2 and dendrin-1 (22). Increased inflammation in this model, as well as increased inflammation following TT injection of the TLR4 ligand lipopolysaccharide (LPS), is associated with impaired activation of the transcription factor Nrf2 and increased activity of NF-\(\kappa\)B in the lungs of p47\(^{phox}−/−\) mice (23).

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### IT LPS administration

Each mouse was anesthetized with isoflurane, and the trachea was exposed by surgical dissection. LPS (serotype 055:B5; Sigma-Aldrich, St. Louis, MO) was diluted in sterile PBS and injected (3 \(\mu\)g/\(g\) body weight) into the trachea via a 27-gauge needle. The neck wound was closed with sterile sutures under aseptic conditions.

### Luciferase activity

To measure NF-\(\kappa\)B transcriptional activity, lung homogenates from NF-\(\kappa\)B reporter mice or macrophages were prepared, and luciferase activity was measured using a standard assay (Promega, Madison, WI) (24). Luciferase activity was expressed as relative light units normalized to lung protein content as measured by the Bradford assay (23).

### Bioluminescence imaging of ROS production

An ROS-sensing probe, L-012 (Wako Chemicals, Richmond, VA), was used to detect ROS production in living mice. L-012 is an analog of luminol and reacts with superoxide anion to produce luminescence (26). The probe was injected retro-orbitally (20 \(\mu\)g), and bioluminescence was detected by an IVIS 200 system device (Xenogen, Alameda, CA). Data were collected and analyzed using Living Image software v.4.1 (Xenogen). The exposure time was 40 s.

### BAL, cell counts, and cytokine measurements

After euthanasia, bronchoalveolar lavage (BAL) was performed with three 700-\(\mu\)l aliquots of ice-cold PBS. Lung lavage fluid was centrifuged at 400 \(\times\) g for 10 min to separate cells from supernatant. Supernatant was saved separately and frozen at \(-70^\circ\)C. The cell pellet was suspended in serum-free RPMI 1640 culture medium, and total cell counts were determined on a granulocytometer. Differential cell counts were determined by staining cytospin slides with a modified Wright stain (Diff-Quik; Baxter, McGraw Park, IL) and counting 400–600 cells in complete cross sections. Levels of cytokines in the cell-free BAL supernatant were measured using a Mouse 20-Plex Cytokine Bead Array (Invitrogen).

### Wet-to-dry ratio

Lungs were removed and the wet weight recorded. Lungs were then placed in an incubator at 65\(^\circ\)C for 48 h, and dry weight was determined.

### Isolation and immortalization of bone marrow–derived macrophages

Primary mouse bone marrow–derived macrophages (BMDMs) were isolated using a previously described method with modifications (27). Femurs were removed postmortem, bone marrow was flushed out, and cells were collected by centrifugation at 400 \(\times\) g for 5 min at 4\(^\circ\)C. Cell pellets were resuspended in 1 ml RBC lysis buffer (BioLegend), kept on ice for 5 min, and centrifuged again at 400 \(\times\) g for 5 min. The remaining cells were resuspended in DMEM infected with a retrovirus carrying v-raf and v-myc (9, 27) (gift from Dr. Jeffrey Hasday, University of Maryland, Baltimore, MD). 1.75 \(\times\) 10\(^6\) U/ml GM-CSF (R&D Systems, Minneapolis, MN) and 10 mg/ml polyethylene (Sigma-Aldrich). After 24 h, supernatant was removed, and cells were cultured and expanded in fresh DMEM supplemented with 10% FBS and GM-CSF (1.75 \(\times\) 10\(^3\) U/ml) for 7 d.

### Isolation of lung macrophages

Perfused lungs were digested in RPMI 1640 medium containing collage-nase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 \(\mu\)g/ml; Sigma-Aldrich) to obtain single-cell suspensions. After treatment with RBC Lysis Buffer (BioLegend), single-cell suspensions were cultured in RPMI 1640 medium for 2 h, and adherent cells were collected.

### Superoxide measurement

Superoxide production was measured from BMDMs after treatment with LPS using a commercially available Lumimax superoxide kit from Strat-agen (La Jolla, CA).

### Real-time quantitative PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA), digested with DNase (Ambion, Austin, TX), and reverse transcribed into cDNA by iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad). The relative amount of each mRNA species was calculated based on its threshold cycle (C\(T\)) and normalized to GAPDH expression. The primer sequences were: GAPDH (5'–GGCATGCACTGTGGTC-3' and 5'–GGCATGCACTGTGGTC-3'); CXCL1 (5'–CCGAAATGATCCGCA CACTCAA-3' and 5'–GCACGTCTGTCTTCTTCCGTAC-3'); and TNF-\(\alpha\) (5'–AACGTTCTG AGGCCCCAGTGGT-3' and 5'–GGACACCACTCAGTCTGTG-3').

### Extraction of nuclear proteins from BMDMs and EMSA

Nuclear extracts were prepared from BMDMs by hypotonic cell lysis followed by high-speed extraction. Briefly, BMDMs were harvested, and buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM PMSF, 0.6% [v/v] Nonidet P-40, 0.5 mM DTT, 0.5 mM 2-ME, and protease inhibitor mixture [Roche]) was added and then incubated on ice for 10 min followed by a 6-min microcentrifugation at 6500 rpm. The supernatant was collected as the cytoplasmic extract. The pellet was resuspended in buffer B (20 mM HEPES, 25% [v/v] glycerol, 1.5 mM MgCl\(_2\), 250 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM 2-ME, 1 mM PMSF, and protease in-hibitor mixture [Roche]) and incubated on ice for 30 min with intermittent vortexing. Following a 6-min microcentrifugation at 14,000 rpm, the supernatant was collected as the nuclear extract. Samples were stored at
NF-κB DNA-binding assay

Nuclear protein (5 μg) was analyzed for NF-κB binding activity using the TransAm NF-κB family kit (Active Motif) according to the manufacturer’s instructions.

Western blot analysis

Fifteen micrograms nuclear protein from BMDMs were separated on a 10% acrylamide gel. Western blot analysis were performed with Abs against thioredoxin 1 (Trx-1; Millipore), NF-κB p65, redox factor 1 (Ref-1), actin, and TATA box binding protein (TBP; Santa Cruz Biotechnology), with the Odyssey infrared system (LI-COR).

Transfection with small interfering RNA for knockdown of Ref-1

Ref-1 knockdown was performed using siGENOME SMARTpool from Dharmacon (Thermo Scientific). The nontargeting small interfering RNA (siRNA) was used as a control. siRNA transfection was performed using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s protocol. After 48 h of transfection, cells were treated with LPS and harvested for measurement.

Reduced glutathione and glutathione disulfide measurement by HPLC

Intracellular glutathione concentrations were measured by HPLC as previously described (28). After LPS treatment, acid soluble thiols were extracted with 5% perchloric acid and 0.2 M boric acid and were further derivatized with iodooacetic acid and dansyl chloride. Reduced glutathione (GSH) and glutathione disulfide (GSSG) were analyzed by HPLC using a propylamine column (YMC Pack, NH2; Waters) and an automated HPLC system (Alliance 2695; Waters). The measured concentrations were normalized to protein content (Bio-Rad).

Statistical analysis

Results from at least three separate experiments were averaged and expressed as means ± SEM. Statistical analyses were performed with GraphPad Prism software version 5.04 for Windows (GraphPad, La Jolla, CA) using unpaired t test for comparisons between two groups and two-way ANOVA with Bonferroni posttest for comparisons that assess the interaction between genotype and treatment variables.

Results

IT LPS causes exaggerated lung inflammation, injury, and cytokine production in p47phox−/− mice

To study the role of NADPH oxidase in mediating LPS-induced lung inflammation and injury, we challenged WT and p47phox−/− mice with IT LPS (3 μg/g of body weight) and harvested lungs 4 and 24 h later. Compared to WT mice, p47phox−/− mice had increased numbers of total cells and neutrophils recovered from BAL fluid (Fig. 1A, 1B). In addition to increased numbers of inflammatory cells, p47phox−/− mice also showed evidence of greater lung injury compared with WT mice. Protein concentration in BAL fluid, a marker of lung injury, was increased at 24 h after LPS in p47phox−/− compared with WT mice (Fig. 1C). At this dose of IT LPS, BAL protein content was only minimally increased in WT mice. Consistent with these findings, lung wet/dry ratio (a measurement of lung edema) was increased.

FIGURE 1. IT LPS administration causes increased lung inflammation, injury, and cytokine levels in p47phox−/− mice compared with WT mice. BAL inflammatory cells (A), BAL neutrophils (B), BAL protein concentration (C), and lung wet-to-dry ratio (D) were measured at baseline, 4 h, and 24 h following IT LPS (3 μg/g body weight). (E and F) H&E-stained lung sections (original magnification ×200) and lung injury score harvested 24 h after LPS treatment. (G) Representative immunohistochemistry (original magnification ×200) for myeloperoxidase-positive cells in lung sections 24 h after LPS treatment. (H) BAL cytokines were measured at 4 h following LPS treatment. Results are presented as means ± SEM; n = 4–7 per group. *p < 0.05. KC, Keratinocyte chemoattractant.
in p47phox−/− mice compared with WT mice at 24 h after LPS treatment (Fig. 1D). By histological evaluation of H&E-stained lung-tissue sections, edema, interstitial thickening, and inflammatory cell influx were more prominent in p47phox−/− mice compared with WT mice (Fig. 1E, 1F). Additionally, staining for myeloperoxidase, a marker for neutrophils, revealed a marked increase in neutrophils in lung tissue of LPS-treated p47phox−/− mice compared with controls (Fig. 1G). In BAL fluid, the levels of a variety of proinflammatory cytokines, including IL-6, GM-CSF, MCP-1, IL-2, IP-10, MIG, keratinocyte chemotactrant, and IL-12, were significantly increased in p47phox−/− mice compared with WT mice at 4 h after LPS administration (Fig. 1H). Together, these results demonstrate that intact NADPH oxidase limits LPS-induced lung inflammation, injury, and proinflammatory cytokine production. These results run contrary to the notion that ROS potentiate inflammation and injury and indicate that NADPH oxidase can induce signaling pathways that restrain excessive inflammation.

**Inverse correlation between ROS production and NF-κB activation in lungs following IT LPS**

LPS primes NADPH oxidase activation in neutrophils in vitro (29), but the requirement of NADPH oxidase for ROS generation after IT LPS injection is not clear. Therefore, we used a bioluminescence imaging method to detect ROS production in vivo. WT and p47phox−/− mice were treated with LPS followed by retro-orbital administration of L-012 (20 μg/g), a luminol analog that reacts with ROS and emits light. At 4 h after LPS administration, a significant increase in photon emission was observed over the chest in WT mice, followed by a return to baseline at 24 h. In contrast, ROS generation in p47phox−/− mice was unchanged after LPS (Fig. 2A, 2B). Interestingly, the basal level of ROS production tended to be lower in p47phox−/− mice following LPS treatment. These results show that phagocyte NADPH oxidase is the major source of LPS-induced ROS generation in lungs.

We next assessed LPS-stimulated NF-κB activation in WT and p47phox−/− mice that harbor a reporter construct containing NF-κB–driven luciferase (identified as WT/HLL and p47phox−/−/HLL mice, respectively). By measurement of luciferase activity in lung tissue, p47phox−/−/HLL mice showed significantly increased NF-κB activity in lungs at 4 h after LPS treatment compared with WT/HLL mice (Fig. 2C). Consistent with this finding, NF-κB activity was increased in total BAL cells and in lung macrophages isolated from lung tissue in p47phox−/−/HLL mice compared with LPS-treated WT/HLL mice at 4 h after LPS administration (Fig. 2D, 2E). These results show that NADPH oxidase limits LPS-induced NF-κB activity in vivo and that induction of ROS correlates inversely with NF-κB activity in lung cells in this model.

**NADPH oxidase regulates NF-κB activity and proinflammatory cytokine expression in macrophages**

Macrophages are critical for initiating the innate immune response, resolving acute inflammation, and promoting repair following injury. We and others have shown that NF-κB plays an important role in these processes (30). To gain mechanistic knowledge about how NADPH oxidase modulates NF-κB function in macrophages, we immortalized BMDMs from WT/HLL and p47phox−/−/HLL mice and treated the cells with LPS. Superoxide production was significantly increased at 1 and 4 h after LPS (0.2 μg/ml) treatment in WT/HLL cells, but remained at basal levels in p47phox−/−/HLL cells (Fig. 3A), indicating that ROS generation was NADPH oxidase dependent. We then evaluated the effect of NADPH oxidase on LPS-stimulated NF-κB transcriptional activity using the luciferase reporter system. Luciferase activity was significantly increased in NF-κB–dependent luciferase activity in lung homogenates of p47phox−/−/HLL and controls (WT/HLL) at baseline and at 4 and 24 h after IT LPS. (C) NF-κB–dependent luciferase activity in lung homogenates of p47phox−/−/HLL mice crossed with an NF-κB reporter line (HLL) and controls (WT/HLL) at baseline and at 4 and 24 h after IT LPS. (D) Luciferase activity in cells from NF-κB–luciferase reporter mice obtained from BAL. (E) Luciferase activity in lung macrophages isolated by adherence from NF-κB–luciferase reporter mice at 4 h after LPS treatment. Results are presented as means ± SEM; n = 3–6 per group. *p < 0.05, **p < 0.001, respectively.
increased in p47^phox^−/−/HLL BMDMs from 2–8 h after LPS treatment (Fig. 3B). Consistent with this finding, mRNA levels of NF-κB-dependent proinflammatory cytokines, CXCL1 and TNF-α, were also increased in p47^phox^−/−/HLL BMDMs compared with WT/HLL BMDMs after LPS treatment (Fig. 3C, 3D). Collectively, our results show that NADPH oxidase deficiency results in impaired ROS production after LPS treatment, thereby enhancing NF-κB activity and mRNA expression of proinflammatory cytokines in BMDMs.

NF-κB DNA binding activity, but not nuclear translocation, is altered in p47^phox^−/−/macrophages

We evaluated whether NADPH oxidase in macrophages modulates NF-κB activation by regulating its nuclear translocation. Although nuclear p65 levels were increased after LPS treatment in both WT/HLL and p47^phox^−/−/HLL BMDMs, no differences were found between groups at any time point (Fig. 4A, 4B). To confirm these measurements, we quantified p65 levels in the nucleus using a TransAM ELISA (Active Motif). Findings were similar to those obtained by Western blot (Table I). In addition to measuring p65 levels, we also evaluated nuclear levels of additional NF-κB family components and found no differences in any of these proteins between genotypes (Table I).

Next, we measured NF-κB binding activity in the nucleus by EMSA. As with measurements of individual NF-κB components, no differences in DNA binding of NF-κB between WT/HLL and p47^phox^−/−/HLL BMDMs were identified at 1 h after LPS by standard EMSA (Fig. 4C). Typically, reducing agents are added to the DNA binding buffer in a standard EMSA to maximize binding of protein and DNA. Because we wondered whether redox status of NF-κB might be altered by NADPH oxidase, we performed EMSAs under nonreducing conditions and found enhanced DNA binding in p47^phox^−/−/HLL BMDMs compared with WT/HLL cells (Fig. 4D). Consistent with prior studies (31), p65 and p50 were the major components of NF-κB of the DNA-binding complex in the nucleus after LPS treatment based on supershift assays (data not shown). Together, these data suggest that redox status affects NF-κB binding activity in BMDMs and that NADPH oxidase-dependent alteration in redox status could explain the differences observed in NF-κB transcriptional activity in WT/HLL and p47^phox^−/−/HLL macrophages after LPS treatment.

NADPH oxidase modulates NF-κB activity through regulation of the intracellular redox environment in macrophages

NF-κB binding to DNA has been shown to be influenced by the redox status of Cys62 of p50 and possibly Cys38 of p65 (32). In the nucleus, active NF-κB is kept in a reduced state by Trx1 and Ref-1 (32, 33). Because we observed increased DNA binding of NF-κB to DNA without altering nuclear translocation, we hypothesized that redox status could be altered by NADPH oxidase. NF-κB activity is kept in a reduced state by Trx1 and Ref-1 (32, 33). Because we observed increased DNA binding of NF-κB to DNA without altering nuclear translocation, we hypothesized that redox status could be altered by NADPH oxidase.
NF-κB subunits in nuclear extracts of p47phox−/−/HLL BMDMs compared with WT/HLL BMDMs following LPS stimulation, we investigated whether NADPH oxidase affects the intracellular redox state. We measured the intracellular ratio of GSH/GSSG as well as nuclear Trx1 and Ref-1 levels as indicators of reducing capacity. The ratio of intracellular GSH/GSSG was significantly higher in p47phox−/−/HLL BMDMs compared with WT/HLL cells at baseline and after LPS treatment, indicating increased reducing capacity in p47phox−/−/HLL BMDMs (Fig. 5A). In the nucleus, we found increased levels of Ref-1 in p47phox−/−/HLL BMDMs but no differences in Trx1 between p47phox−/−/HLL and WT/HLL cells (Fig. 5B, 5C). In contrast, levels of cytoplasmic Ref-1 were unchanged (Figs. 5D).

We then evaluated the effect of a specific Ref-1 inhibitor, E3330 (33), on NF-κB reporter expression in WT/HLL and p47phox−/−/HLL macrophages after LPS treatment. E3330 (25 μM) suppressed NF-κB reporter expression at 4 h after LPS treatment in p47phox−/−/HLL BMDMs to levels similar to those found in LPS-stimulated WT/HLL cells (Fig. 6A). In contrast, E3330 treatment did not significantly affect LPS-induced NF-κB activation in WT/HLL cells. In separate experiments, BMDMs were treated with hydrogen peroxide (5 μM) just after addition of LPS. Consistent with the results of Ref-1 inhibition studies, hydrogen peroxide suppressed LPS-stimulated NF-κB reporter expression only in p47phox−/−/HLL BMDMs (Fig. 6B).

To further evaluate the role of Ref-1 in regulating NF-κB activation, we used siRNA against Ref-1 in BMDMs (Fig. 6C, 6D). Ref-1 expression was significantly reduced after treatment with Ref-1 siRNA. Following LPS treatment, knocking down Ref-1 suppressed NF-κB reporter expression in p47phox−/−/HLL BMDMs but did not affect NF-κB reporter expression in WT/HLL cells. Together, these results point to NADPH oxidase limiting LPS-induced NF-κB activation in macrophages by modulating the intracellular redox environment.

**Discussion**

We evaluated the role of NADPH oxidase in LPS-induced lung inflammation and injury, a process that is recognized to be regulated through the NF-κB pathway. Data presented in this study show that NADPH oxidase limits lung inflammation and injury as well as NF-κB activation and downstream cytokine responses following LPS challenge. Consistent with these in vivo findings, loss of NADPH oxidase resulted in increased LPS-induced NF-κB transcriptional activity in cultured BMDMs. Although nuclear translocation of NF-κB proteins was comparable between genotypes, we observed increased DNA binding of p65/p50 heterodimers in p47phox−/− compared with WT macrophages following LPS treatment. Additional studies showed that NADPH oxidase regulates intracellular redox status in macrophages, which in turn modulates LPS-induced NF-κB transcriptional activity. In the
nucleus, basal levels of Ref-1 were increased in p47\textsuperscript{phox}/−/− versus WT macrophages. Both chemical inhibition and siRNA-mediated knockdown of Ref-1 selectively inhibited NF-κB transcription activity in p47\textsuperscript{phox}/−/− cells. Together, these data elucidate an important mechanism by which NADPH oxidase limits inflammation through redox-dependent, Ref-1–mediated modulation of NF-κB transcriptional activation.

ROS regulate a broad array of physiological and pathological responses from cell proliferation to gene expression (34, 35). Evidence has accumulated that ROS generated by NADPH oxidase play roles in regulating host inflammatory responses via modulation of redox-sensitive pathways. In some settings, NADPH oxidase can protect against excessive neutrophilic inflammation and injury. For example, mice with NADPH oxidase deficiency have worse outcomes in an experimental model of acid aspiration-induced lung injury (7, 36). In addition, IT administration of zymosan or LPS results in increased and prolonged lung inflammation in NADPH oxidase–deficient mice (23, 37). This protection is likely due to a dual effect of NADPH oxidase in limiting inflammatory signaling and inducing activation of Nrf2, a redox-sensitive transcription factor that stimulates antioxidant and cytoprotective responses (23, 36). In contrast, hepatic injury is potentiated by NADPH oxidase after exposure to a peroxisomal proliferator or in alcohol-induced hepatitis (38, 39). Thus, NADPH oxidase can modulate inflammation in different ways depending on the experimental model, the inflammatory stimulus, and the organ involved.

Although NADPH oxidase can be induced to rapidly generate high levels of superoxide in phagocytes after an inflammatory stimulus, our studies show that the basal intracellular redox status in macrophages is also dependent on NADPH oxidase. Treatment of macrophages with a Ref-1 inhibitor or H\textsubscript{2}O\textsubscript{2} (at the concentration used in these studies) did not affect NF-κB transcriptional activity in WT macrophages, suggesting that intact NADPH oxidase limits the ability of Ref-1 to regulate NF-κB activity. However, redox-dependent increases in nuclear localization and/or activity of Ref-1 in the setting of deficient NADPH oxidase appear to maintain NF-κB components in a state with higher DNA binding affinity, resulting in greater levels of transcriptional activity. These findings may have implications for patients with CGD, in whom interventions to restore normal intracellular redox balance might protect from excessive responses to inflammatory stimuli.

NF-κB is recognized as a redox-sensitive transcription factor. We and others have shown that antioxidant therapy can inhibit NF-κB activity in some models (40–42). However, reduction of specific cysteine residues within the NF-κB DNA-binding domain has been shown to be important for DNA binding activity, maintaining Cys\textsuperscript{62} of the p50 subunit (or Cys\textsuperscript{59} in mouse) in a reduced state regulates DNA binding through a mechanism that depends on Ref-1 and Trx1 (32, 33). In addition, several reports have demonstrated that Ref-1 activates DNA-binding activity of many redox-sensitive transcription factors by directly reducing their cysteine residues (31–33, 43). Our findings extend these studies by showing that NADPH oxidase regulates Ref-1 expression and activity in macrophages in vitro and in vivo and that Ref-1–regulated NF-κB binding activity has important functional consequences in the lungs. In addition to redox regulation of NF-κB subunits, several other posttranslational modifications (e.g., phosphorylation and acetylation) modulate NF-κB binding and transcription activity. Although we did not find any differences in phosphorylation status of NF-κB p65 at Ser\textsuperscript{276}, Ser\textsuperscript{536}, or Ser\textsuperscript{468} between p47\textsuperscript{phox}/−/− and WT cells in these studies (data not shown), we have not excluded the possibility that other posttranslational protein modifications could be altered by NADPH oxidase.

**FIGURE 6.** Blockade of Ref-1 or addition of hydrogen peroxide selectively inhibits LPS-induced NF-κB activation in macrophages from p47\textsuperscript{phox}/−/− mice. (A) NF-κB–dependent luciferase activity in BMDMs from WT/HLL mice and p47\textsuperscript{phox}/−/−/HLL mice at baseline and 4 h after treatment with LPS (0.2 μg/ml) and Ref-1–specific inhibitor E3330 (25 μM). (B) NF-κB–dependent luciferase activity at baseline and 4 h after treatment with LPS ± H\textsubscript{2}O\textsubscript{2} (5 μM). (C) BMDMs were transfected with Ref-1 (or nontargeting control) siRNA, and the levels of Ref-1 in whole-cell lysate were determined by Western blot and quantified by densitometry. (D) NF-κB–dependent luciferase activity with siRNA-mediated knockdown of Ref-1. Results are mean ± SEM; n = 3–6/group. *p < 0.001.
Because macrophages are important for amplifying inflammatory signals and activating NF-κB activity in other cell types after IT LP (44), it is likely that increased inflammation in the lungs of p47phox−/− mice results from exaggerated activation of macrophages. Mice with a spontaneous mutation of the p47phox gene (Ncf1) that harbor a transgene with the WT Ncf1 gene under the human CD68 promoter gain functional NADPH oxidase activity in macrophages, as well as monocytes and dendritic cells. The hyperinflammatory phenotype observed in globally NADPH oxidase–deficient mice is abolished in transgenic mice with myeloid-specific NADPH oxidase reconstitution in a number of acute and chronic inflammatory models (45, 46). We observed that these transgenic mice phenocopied WT mice with regard to mild self-limited zymosan-induced lung inflammation, whereas globally, NADPH oxidase–deficient mice had a persistent neutrophilic alveolitis and consolidative lung lesions (W. Han, H. Li, J. Cai, L.A. Gleaves, V.V. Polosukhin, B.H. Segal, F.E. Yull, and T.S. Blackwell, manuscript in preparation). Together, these findings point to NADPH oxidase in the monocyte/macrophage lineage having a broad role in regulating inflammation by modulating NF-κB and other redox-sensitive pathways. However, direct effects of NADPH oxidase deficiency in other cell types, including neutrophils, may also contribute to the observed phenotype.

In conclusion, our studies demonstrate that NADPH oxidase–derived ROS regulate LPS-induced lung inflammation and injury through redox regulation of NF-κB activity. NADPH oxidase reduces NF-κB binding to DNA and transcriptional activity through regulation of intracellular redox state. This pathway for modulation of NF-κB is likely to be important in limiting the inflammatory response following infection and other proinflammatory and injurious stimuli. LPS and other microbial products lead to NF-κB activation, cytokine and chemokine production, neutrophil recruitment, and priming of NADPH oxidase. Although the immediate effects of ROS release can be injurious and proinflammatory, our results point to another role for NADPH oxidase in limiting NF-κB activity by modulating the intracellular redox state, thereby averting excessive inflammation and injury. Knowledge of the molecular mechanisms for redox regulation of NF-κB activity may lead to new therapeutic approaches for CGD and, more broadly, for diseases (e.g., sepsis, multiorgan failure) driven by inflammation in the lungs of p47phox−/− mice.

Disclosures

The authors have no financial conflicts of interest.

References

7. NADPH Oxidase Limits NF-κB Activation