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Protective Role of Reactive Oxygen Species in Endotoxin-Induced Lung Inflammation through Modulation of IL-10 Expression

Jing Deng,*† Xuerong Wang,* Feng Qian,‡ Stephen Vogel,§ Lei Xiao,* Ravi Ranjan,* Hyesuk Park,* Manjula Karpurapu,* Richard D. Ye,‡ Gye Young Park,* and John W. Christman*†‡§

Reactive oxygen species (ROS) generated by NADPH oxidase are generally known to be proinflammatory, and it seems to be counterintuitive that ROS play a critical role in regulating the resolution of the inflammatory response. However, we observed that deficiency of the p47phox component of NADPH oxidase in macrophages was associated with a paradoxical accentuation of inflammation in a whole animal model of noninfectious sepsis induced by endotoxin. We have confirmed this observation by interrogating four separate in vivo models that use complementary methodology including the use of p47phox−/− mice, p47phox−/− bone marrow chimera mice, adoptive transfer of macrophages from p47phox−/− mice, and an isolated perfused lung edema model that point to a relationship between excessive acute inflammation and p47phox deficiency in macrophages. Mechanistic data indicate that ROS deficiency in both cells and mice results in decreased production of IL-10 in response to treatment with LPS, at least in part, through attenuation of the Akt-GSK3β signal pathway and that it can be reversed by the administration of rIL-10. Our data support the innovative concept that generation of ROS is essential for counterregulation of acute lung inflammation. The Journal of Immunology, 2012, 188: 5734–5740.

The NADPH oxidase (NOX) is a family of proteins that transfer electrons to oxygen and generate superoxide, which is a precursor for reactive oxygen species (ROS) (1). In phagocytic cells, such as macrophages and neutrophils, NOX is a multiprotein complex that includes the transmembrane proteins gp91phox and gp22phox, the plasma proteins p47phox and p67phox, and the small GTPase-Rac2. Activation of NOX requires the transfer of cytosolic proteins to the membrane that bind to gp91phox and gp22phox complex (2, 3) resulting in the expression of a functional protein complex on the cell surface that generates ROS. It is well known that superoxide generation is important for phagocytic cells to clear tissue-invasive bacteria and to protect the host from pathogenic infection (4). Data are also reported that patients with chronic granulomatous disease, which is a genetic NOX deficiency, have difficulty in controlling bacterial infection because of loss of bactericidal activity in resident and recruited phagocytes (5, 6). However, it is also possible that NOX-dependent ROS production has an additional role in regulating signaling events related to immune regulation of noninfectious inflammation. Although ROS certainly contribute to a proinflammatory phenotype, paradoxically, leukocytes from granulomatous disease patients that lack NOX have a “hyperinflammatory phenotype” with increased production of proinflammatory cytokines in response to stimulation with TLR agonists (7, 8). Furthermore, studies using gp91phox−/− mice showed elevated concentrations of the proinflammatory chemokines and cytokines, including MCP-1, IL-6, IL-1β, and TNF-α, and an increased myeloperoxidase (MPO) activity in lungs in response to treatment with endotoxin (9). Taken together, these findings suggest the novel hypothesis that ROS have a critical role in maintaining homeostasis by protecting the host against inappropriate or excessive inflammatory responses.

IL-10 is one of the key anti-inflammatory cytokine that plays an important role in regulating an excessive inflammatory response (10). Mice that are genetically deficient in IL-10 spontaneously develop severe inflammation (11, 12). We investigated the possibility that ROS are involved in regulating macrophage phenotype, which is necessary for resolution of lung inflammation and restoration of lung health. Our data show that mice that lack the p47phox component of NOX have an exaggerated inflammatory response to endotoxemia, and this is associated with decreased activation of Akt/GSK3β pathway that results in deficiency of IL-10 production.

Materials and Methods

Mice

We have previously reported that the HLL transgenic and p47phox−/−/HLL mice express Photinus luciferase CDNA under the control of a proximal 5′ HIV-1 long terminal repeat promoter, which is sensitive to NF-κB-mediated signaling (13). Mice that were 8–12 wk of age were used in this study. All experiments involving mice were conducted with protocols approved by the Institutional Animal Care and Use Committee of the University of Illinois (Chicago, IL).
Preparation of bone marrow-derived macrophages
Femoral and tibia bone marrow cells were isolated from HLL and p47phox−/− HLL mice by methods described previously (14). Mouse bone marrow cells flushed from femurs and tibias were washed with Ca2+/Mg2+-free HBSS. Then, bone marrow progenitor cells were cultured in DMEM containing the 10% FBS and supplemented with 10% L929 cell culture media. After 7 d, bone marrow-derived macrophages (BMDMs) (∼99% macrophages based on flow cytometry using anti-F4/80) were collected for our experiments.

Measurement of cytokines
Mouse peritoneal macrophages were treated with or without LPS (100 ng/ml; Sigma-Aldrich, St. Louis, MO) and diphenylene iodonium (DPI) (5 µM; EMD Biosciences, Gibbstown, NJ) for 8 h in the presence or absence of Akt inhibitor X (Sigma; EMD Biosciences). The cells were then collected and lysed. The cell lysate was analyzed by immunoblot using Abs against phospho–GSK3-β, Akt, and total Akt (Cell Signaling Technology, Beverly, MA).

Western blot analysis
BMDMs were plated in 6-well plates at 2 × 105/well and starved overnight in serum-free DMEM. BMDMs were challenged with 100 ng/ml LPS for 8 h in the presence or absence of Akt inhibitor X (Sigma, St. Louis, MO). The cells were then collected and lysed. The cell lysate was analyzed by immunoblot using Abs against phospho–GSK3-β, Akt, and total Akt (Cell Signaling Technology, Beverly, MA).

Superoxide production assays
Superoxide production by BMDMs was determined in a luminol-ECL assay. BMDMs were seeded in a 96-well white plate with reaction buffer containing 40 U/ml HRP (Invitrogen, Grand Island, NY) and 100 µM luminol (Sigma-Aldrich) in HBSS containing 1% BSA. After stimulation with LPS, chemiluminescence was measured in a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA).

Mouse model of LPS-induced acute lung injury
Mice were injected i.p. with 8 mg/kg LPS. After 2 h, mice were euthanized with bottled CO2, followed with cervical dislocation. Lungs were flushed and excised; the same lobe of lung was ground in 1 ml reporter lysis buffer (Promega) to 20 µl of the homogenated lung tissue that was ground in reporter lysis buffer. Luciferase activity was measured in postmortem tissue samples by adding 100 µl freshly prepared luciferase assay buffer (Promega) to 20 µl of the homogenated lung tissue that was ground in reporter lysis buffer. Luciferase activity was measured as relative light units per milligram protein.

Luciferase activity in lung tissue
Luciferase activity was measured in postmortem tissue samples by adding 100 µl freshly prepared luciferase assay buffer (Promega) to 20 µl of the homogenated lung tissue that was ground in reporter lysis buffer. Luciferase activity was measured as relative light units per milligram protein. The total protein concentration in the cell lysates was determined by Bradford assay.

Fetal liver cell transplantation
Fetal liver cell (FLC) transplantation was performed as reported previously (15, 16). Briefly, FLCs were isolated from pregnant p47phox−/−/HLL and HLL mice. The recipient mice were irradiated by using 10-Gray radiations in two split doses of 5 Gray each in which the first dose of radiation was followed by a second dose after 2 h. FLCs were transplanted by tail vein injection into recipient p47phox−/−/HLL and HLL mice. Four weeks following the transplantation, a single dose of liposomal clodronate was administered intratracheal (i.t.) injection to eliminate any residual pulmonary macrophages that were of the recipient genotype and to facilitate reconstitution of the pulmonary macrophages of the donor genotype. Two weeks later, the p47phox−/−/HLL and HLL bone marrow chimera mice were injected i.p. with LPS (8 mg/kg) and were then sacrificed after 24 h. The bone marrow cells were genotyped, and the luciferase and MPO activity of the lung tissue were determined.

Measurement of MPO activity in lung tissue
Frozen lungs were homogenized in 50 mM phosphate buffer. The homogenate was centrifuged at 13,000 × g for 30 min, and then, the cell pellet was resuspended in 1 ml 0.5% hexadecyl trimethylammonium boronide (Sigma-Aldrich). After treatment with three cycles of freezing, thawing, and sonication, the homogenate was centrifuged at 13,000 × g for 20 min at 4˚C. The supernatant was incubated with 16 mM tetratetramethylene dibenzidine (Sigma-Aldrich) and 15 mM H2O2 (Sigma-Aldrich), and absorbance at 460 nm was determined. One unit of MPO activity was defined as the change in OD at 460 nm/mg protein/min.

Adaptive transfer of mouse macrophages to HLL mice
Adaptive transfer of mouse macrophages was performed as reported previously (17). Macrophages were obtained from peritoneal lavage after euthanizing the mice. Cells were washed twice with sterile 1× PBS before being resuspended in PBS and instilled into the trachea using aseptic techniques. After being anesthetized with ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.p.), HLL mice were treated with i.t. administration of peritoneal macrophages (3 × 106 cells/mouse) in 30 µl 1× PBS. Mouse tracheas were directly exposed by surgical resection, pierced with a 27G-inch needle, and injected with macrophages. The neck wound was closed with sterile sutures under aseptic conditions. After overnight, recipient HLL mice were injected with LPS (8 mg/kg) via i.p. route.

In vivo measurement of luciferase gene expression by bioluminescence imaging with IVIS
Mice were anesthetized, and the hair was removed from the chest and abdomen before imaging. At 0, 8, and 24 h after i.p. injection of LPS, mice were anesthetized with isoflurane inhalation and were subsequently i.p. injected with 100 µl 300 mg/ml luciferin solution (Caliper Life Sciences). Bioluminescence imaging with a charge-coupled device camera of IVIS was initiated 10–15 min after injection. Quantitative analysis was performed in a manually defined area from the region of interest (ROI) over the chest area, and the data were expressed as a photon value (photons per second). All bioluminescent data were collected and analyzed using IVIS.

Lung capillary leakage and lung edema measurement
Mice were injected (i.p.) with LPS and were administered Evans blue-labeled albumin (EBA) (20 mg/kg) via a tail vein after 24 h. After 60 min of EBA injection, the mice were euthanized, and their right lungs were excised. Lung homogenates were incubated with 2 volumes formalin for 18 h at 60˚C. The homogenate was centrifuged at 5000 × g for 30 min, and the OD of the supernatant was read at 620 nm. EBA concentration was calculated against a standard curve and presented as milligrams of EBA per gram of lung tissue. The left lungs were excised, weighed, and dried in the oven at 60˚C for 18 h. Quantitative analysis of lung wet-to-dry ratios was measured.

Liposomal clodronate preparation
Liposomal encapsulation of clodronate was performed as described previously (18). Briefly, a mixture of 8 mg cholesterol (Avanti, Alabaster, AL) and 86 mg egg-phosphatidylcholine (Avanti) was dissolved into chloroform solution and then evaporated under nitrogen. Chloroform was further removed under vacuum in a speedvac Savant concentrator. Dichloro- methylene diphosphonic acid (1.2 g; Sigma-Aldrich) was dissolved in 5 ml sterile 1× PBS, added to the liposome preparation, and mixed thoroughly. The solution was sonicated and ultracentrifuged at 10,000 × g for 1 h at 4˚C. The liposome pellet was removed, resuspended in 5 ml 1× PBS, and used within 48 h. The final concentration of the liposomal clodronate was 5 mg/ml.

Ex vivo lung studies
Mice anesthetized with ketamine/xylazine were administered with 100 µl PBS or clodronated liposomes to eliminate resident pulmonary macrophages via i.t. injection. After 3 d, the mice were injected with 5 mg/kg LPS 4 h prior to lung isolation, and lung edema was measured as described previously (19). Lungs were then isolated from mice anesthetized with 2.5% isoflurane, perfused via the pulmonary artery (REMPI 1640 solution with 5% albumin, 2 ml/min, 37°C), and ventilated via a tracheal cannula (peak inspiratory pressure of 7.5 and end expiratory pressure of 1 cm H2O). Left atrium was also cannulated, and pulmonary venous pressure was set to 3 cm H2O. The preparations underwent equilibration for 20 min. Then using a syringe drive, 4 × 107 neutrophils from HLL mice were injected into the arterial perfusate over a 20-min period. To measure the filtration rate, pulmonary venous pressure was elevated by 7.5 cm H2O for 20 s. Maximum slope of the resulting weight–gain curve indicates rate of lung edema formation. Horizontal axis displays the time from beginning of perfusion. Quantitative data for the filtration rate of edema were collected and analyzed.
**Statistical analysis**

Data were expressed as the mean ± SEM. Differences between groups of mice were evaluated with Student’s t test. A p value <0.05 was considered statistically significant. In the chimera mouse model, data are presented as mean ± SEM by one-way ANOVA between indicated groups. In lung edema experiments, statistical analysis was conducted using one-way ANOVA with Duncan’s multiple range post hoc test, and data are presented as mean ± SD.

**Results**

*p47<sup>phox</sup><sup>-/-</sup>/HLL mice exhibit enhanced NF-κB activation in response to LPS compared with HLL mice*

Luciferase expression in HLL transgenic reporter mice is driven by the NF-κB–dependent portion of the promoter. HLL mice and *p47<sup>phox</sup><sup>-/-</sup>/HLL mice were treated with a single i.p. injection of LPS (8 mg/kg), and bioluminescence was measured at 0, 8, and 24 h. To detect bioluminescence as a reflection of NF-κB activation in vivo, 100 μl 300 mg/ml luciferin solution was given by the i.p. route 10 min before measurement. Bioluminescence was measured by counting photon emission from a standardized region of the chest. The computer software generated a false-color image that reflects the intensity of photon emission (blue is the least intense, and red is the most intense). As shown in Fig. 1A, bioluminescent images illustrate a significant increase in luciferase activity over the chest in response to LPS treatment in both *p47<sup>phox</sup><sup>-/-</sup>/HLL and HLL mice. Bioluminescence in *p47<sup>phox</sup><sup>-/-</sup>/HLL mice is greater than in HLL mice at 8 and 24 h. In Fig. 1B, photon values from *p47<sup>phox</sup><sup>-/-</sup>/HLL mice exceed those of HLL mice at 8 and 24 h but not at 0 h. An increase in photon emission over the chest in *p47<sup>phox</sup><sup>-/-</sup>/HLL mice compared with the HLL mice indicates more NF-κB activation in the lungs and enhanced lung inflammation induced by LPS. To confirm the bioluminescence measurements, we examined the effects of LPS on NF-κB activation at an early time point. The *p47<sup>phox</sup><sup>-/-</sup>/HLL mice and HLL mice were sacrificed at 2 h after treatment with 8 mg/kg LPS, and luciferase activity was measured from lung homogenates. Luciferase activity in the lungs measured by luminometer shows significant increase in *p47<sup>phox</sup><sup>-/-</sup>/HLL mice compared with HLL mice (Fig. 1C). Because NF-κB activation is accentuated in *p47<sup>phox</sup><sup>-/-</sup>/HLL mice, these data suggest that *p47<sup>phox</sup><sup>-/-</sup> has an important role in modulating lung inflammation in this mouse model of noninfectious inflammation.

*p47<sup>phox</sup><sup>-/-</sup>/HLL bone marrow chimera mice have accentuated pulmonary inflammation in response to treatment with endotoxin*

We confirmed by PCR that only the genotype of the donor bone marrow was detected when irradiated HLL mice were rescued with FLC from *p47<sup>phox</sup><sup>-/-</sup>/HLL mice and vice versa at the end of the experiment (Fig. 2A). In our experiments, there was an increase in the luciferase activity of lung tissue in the HLL to HLL bone marrow chimera mice that were LPS treated, and this was markedly accentuated in the *p47<sup>phox</sup><sup>-/-</sup>/HLL to HLL and the *p47<sup>phox</sup><sup>-/-</sup>/HLL to *p47<sup>phox</sup><sup>-/-</sup>/HLL bone marrow chimera mice. In contrast,

![FIGURE 1](image1.png)

**FIGURE 1.** The *p47<sup>phox</sup><sup>-/-</sup>/HLL mice exhibit enhanced LPS-induced lung inflammation compared with HLL mice. HLL and *p47<sup>phox</sup><sup>-/-</sup>/HLL mice were treated with 8 mg/kg LPS, and luciferase activity was detected. (A) To reveal NF-κB activation patterns, in vivo luciferase activity was presented as bioluminescent images with artificial colors in *p47<sup>phox</sup><sup>-/-</sup>/HLL and HLL mice at 0, 8, and 24 h after LPS treatment (n = 3 each group). (B) Photon emission over chest area was determined by IVIS. (C) Luciferase activity assays measured by luminometer from lung homogenates of HLL and *p47<sup>phox</sup><sup>-/-</sup>/HLL mice at 2 h after LPS treatment (normalized for total protein). Results represent mean ± SEM (n = 6 each group). *p < 0.05.

![FIGURE 2](image2.png)

**FIGURE 2.** The *p47<sup>phox</sup><sup>-/-</sup>/HLL bone marrow chimera mice have accentuated LPS-induced lung inflammation. (A) PCR results showed that the *p47<sup>phox</sup><sup>-/-</sup>-knockout genotype was identified as a 900-bp band present only in the *p47<sup>phox</sup><sup>-/-</sup>/HLL chimera (lane 2), whereas the HLL genotype is identified as a 600-bp band present in the HLL:HLL and HLL:*p47<sup>phox</sup><sup>-/-</sup> bone marrow chimera mice (lanes 1 and 3). (B) Luciferase activity assays from lung homogenates of HLL and *p47<sup>phox</sup><sup>-/-</sup>/HLL bone marrow chimera mice with or without LPS treatment for 24 h (normalized for total protein). Results represent mean ± SEM (n = 3–7 each group); *p < 0.05. (C) MPO activity was measured from lung tissue in bone marrow chimera mice with or without LPS treatment for 24 h. Results represent mean ± SEM (n = 3–7 each group); *p < 0.05.
luciferase activity was decreased in the HLL to the p47phox/−/−/HLL bone marrow chimera mice (Fig. 2B). In these studies, luciferase activity was closely correlated with MPO activity (Fig. 2C). We interpret these data as indicating that the greatest proinflammatory activity tracks with the p47phox/−/−/HLL bone marrow cells, which suggests that either macrophages or neutrophils are involved.

p47phox-deficient macrophages are proinflammatory in LPS-induced lung inflammation

Because p47phox/−/−/HLL mice and p47phox/−/−/HLL to HLL bone marrow chimera mice displayed more inflammatory response to LPS treatment and macrophages play an important role in innate immunity as reported, we performed passive adoptive transfer of peritoneal macrophages from p47phox/−/−/HLL mice and HLL mice into HLL mice. Unstimulated peritoneal macrophages obtained by peritoneal lavage were injected directly into the trachea of anesthetized HLL mice to construct chimera mice. With this method, ~90% of macrophages in the bronchoalveolar lavage originated from the donor mice without any evidence of neutrophil infiltration at 16 h (Supplemental Fig. 1). As demonstrated in Fig. 3A, HLL mice that received the peritoneal macrophages from p47phox/−/−/HLL mice and HLL mice had similar bioluminescence at 0 h, but the mice that received peritoneal macrophages from p47phox/−/−/HLL mice had much more bioluminescence at 8 and 24 h following treatment with LPS than the mice received peritoneal macrophages from HLL mice. Quantified data as photon values in the area of the chest were reported in Fig. 3B. The extravasation of EBA into the tissue was an index of increased vascular permeability; therefore, we also quantitated in vivo lung edema by measuring EBA as a marker of lung injury. HLL mice that received p47phox macrophages showed more leakage of Evans blue dye in the lungs in response to LPS treatment, compared with the HLL mice that received wild-type macrophages (Fig. 3C). These data suggest that macrophages that are deficient in p47phox are capable of accentuating inflammation when administered to HLL mice.

To further dissect the effect of p47phox-deficient macrophages in LPS-induced lung inflammation, ex vivo lung edema studies were done. HLL and p47phox/−/−/HLL mice were treated with or without liposomal clodronate via i.t. injection to eliminate resident pulmonary macrophages. After 3 d, mice were challenged with a low dose of LPS (5 mg/kg) 4 h prior to the measurement of pulmonary edema formation. Both p47phox/−/−/HLL and HLL lungs were perfused with 4 × 10⁶ of wild-type neutrophils from HLL mice to isolate the effect of NOX deficiency in macrophages from that of the neutrophils. As shown, edema fluid did not accumulate in mice where the perfusate did not contain wild-type neutrophils (Fig. 4B). These data show a much greater rate of edema formation in lungs from p47phox/−/−/HLL mice compared with p47phox/−/−/HLL mice treated with liposomal clodronate (Fig. 4). In contrast to these data, there was more edema seen in the HLL mice that were treated with liposomal clodronate compared with the HLL mice that were treated with salinized liposomes. These data suggest that wild-type macrophages are anti-inflammatory, whereas macrophages from p47phox-deficient macrophages are proinflammatory during endotoxemia. This suggested to us that wild-type macrophages produce a counterinflammatory mediator, whereas this capacity is deficient in macrophages that lack NOX activity.

LPS-induced ROS production is p47phox dependent, and IL-10 secretion is decreased in p47phox/−/−/HLL macrophages

We observed that NOX deficiency resulted in a marked accentuation of lung inflammation in response to LPS and found that p47phox-deficient macrophages were proinflammatory during endotoxemia, which prompted us to focus on a mechanistic study of IL-10. To this end, we investigated macrophage functions by detecting the expression of anti-inflammatory cytokine IL-10 in vitro and in vivo. We found that both LPS-induced ROS generation and the production of IL-10 from macrophages was decreased when the cells were treated with the flavocytochrome inhibitor DPI (Fig. 5A, 5B, respectively). We also found DPI decreased the production of IL-6 and TNF-α (Supplemental Fig. 2), which suggested that DPI may globally inhibit cytokine production in macrophages. Furthermore, we measured IL-10 secretion in vitro and in vivo with using both macrophages and whole animals that are deficient in p47phox. In contrast to the studies using pharmacologic inhibition of NOX with DPI, the concentration of IL-10 was decreased in macrophage culture supernatants.
from p47phox−/−/HLL mice compared with supernatants from wild-type HLL mice (Fig. 5C). We also challenged HLL and p47phox−/−/HLL mice with i.p. injection of LPS (8 mg/kg), and after 2 h, blood was drawn by cardiac puncture. Similarly, serum IL-10 levels were decreased in p47phox−/−/HLL mice compared with that in HLL mice (Fig. 5D). However, in contrast to the studies using DPI, the production of proinflammatory cytokines TNF-α and IL-6 were not affected by genetic deficiency of NOX, suggesting that NOX is critical for the production of the anti-inflammatory cytokine IL-10 (Fig. 5E, 5F). These findings suggest that the p47phox−/−/HLL mice produced lower concentrations of anti-inflammatory mediator (IL-10) than HLL mice in response to LPS treatment. These results are supportive of our central hypothesis that activation of NOX in macrophages engages an anti-inflammatory program that involves regulation of IL-10 production.

**LPS phosphorylates GSK3-β via the PI3K-Akt pathway in macrophages**

We determined the effect of p47phox deficiency on Akt and GSK3-β phosphorylation, because these are critical for IL-10 production. Constitutively active kinase GSK3-β negatively regulates the production of IL-10. Phosphorylation of GSK3-β results in the inhibition of its own activity. LPS stimulation of BMDMs induced phosphorylation of Akt (Ser473) and GSK3-β (Ser9) at multiple time points (Fig. 6A). In Fig. 6A, we observed a decrease in the activation of Akt and GSK3-β in BMDM isolated from p47phox−/−/HLL mice compared with those from HLL mice. We next used a selective Akt inhibitor to determine whether Ser9 phosphorylation of GSK3-β was modulated by Akt activation (Fig. 6B). Blocking Akt phosphorylation with an Akt inhibitor attenuated LPS-induced phosphorylation of GSK3-3-β on Ser9 to nearly unstimulated level at 60 min. These indicate that LPS-induced phosphorylation of GSK3-β is Akt dependent and that phosphorylation of both GSK3-β and Akt are inhibited in p47phox−/−-deficient macrophages. We next investigated whether inactivation of GSK3-β mediated a functional effect on LPS-induced IL-10 production. Production of IL-10 was significantly increased when macrophages from wild-type HLL and p47phox−/−/HLL mice were stimulated by LPS in the presence of GSK3-β inhibitor (SB216763), compared with LPS alone. In contrast, inhibition of Akt using Akt inhibitor led to less phosphorylation of GSK3-β, which resulted in the accentuation of LPS-induced IL-10 production (Fig. 6B, 6C).

**Exogenous IL-10 reduces LPS-induced lung inflammation in p47phox−/−-deficient mice**

To further investigate the role of IL-10 in LPS-induced lung inflammation in p47phox−/−/HLL mice, we performed experiments to determine whether IL-10 mediated suppression of LPS-induced
lung inflammation. We injected p47phox−/−/HLL mice with recombinant mouse IL-10 (rIL10, 1 μg/mouse) by i.p. administration 2 h prior to LPS treatment and measured the in vivo NF-κB activation with IVIS-200 system. When mice received a LPS challenge, the bioluminescence was markedly increased at 8 and 24 h and also significantly decreased in mice that received LPS and rIL10 as compared with mice receiving LPS alone in a time-dependent manner (Fig. 7A). Quantitative data as photon values from chest area demonstrate that an LPS-induced increase in luciferase activity is significantly reduced in mice with rIL10 treatment (Fig. 7B). Our data show that exogenous IL-10 can substantially reduce excessive lung inflammation induced by LPS in p47phox−/−-deficient mice and decrease the levels of inflammation to that of wild-type mice.

Discussion

ROS, which are generated by the phagocytic NOX as a key component of antimicrobial defense, are commonly considered harmful mediators of acute inflammation, based on their highly reactive nature. However, recent studies in mice with targeted disruption of NOX components (gp91phox−/−, p47phox−/−) and autoimmune diseases suggest an inflammation-dampening role of ROS (20, 21). We hypothesize in this study that ROS are involved in counterregulation of inflammation in response to LPS treatment. Our hypothesis is supported by showing that deficiency of the p47phox component of NOX in macrophages was associated with a paradoxical accentuation of inflammation in a whole animal model of noninfectious sepsis induced by LPS and that this proinflammatory phenotype is associated with decreased production of anti-inflammatory cytokine IL-10. We generated p47phox−/−/HLL mice, which not only display impaired ROS generation, but also contain a convenient luciferase reporter gene that allows indirect detection of NF-κB–mediated luciferase activity as a surrogate measure of acute inflammation. We used four independent in vivo models that all point to a relationship between excessive acute inflammation and p47phox deficiency in macrophages. There are many reports that indicate that ROS have an important role in the generation of proinflammatory mediators that result in tissue damage and organ dysfunction (1, 22). In combination with our data, it appears that ROS have a dual role in regulating both pro- and anti-inflammation; one possibility is that abundant ROS cause tissue injury, whereas low levels of ROS prevent or limit inflammation and could possibly have a role in resolution of inflammation.

In p47phox−/−-deficient macrophages, we found anti-inflammatory cytokine IL-10 expression was decreased in response to stimulation with LPS, which indicates that the counterinflammatory component of the macrophage response is deficient. We found that the NOX inhibitor DPI totally blocked the LPS-induced IL-10 production, suggesting that NOX-dependent production of ROS is involved in the modulation of IL-10 expression. However, DPI also blocked IL-6 and TNF-α production in macrophages treated with LPS. In contrast, our data show that both macrophages and mice that lack the p47phox component of NOX have decreased production of IL-10 but not IL-6 or TNF-α production in response to treatment with LPS. This discrepancy in the results between the effect of pharmacologic and genetic ablation of NOX activity probably is related to other ROS-independent nonspecific inhibi-
tory effects of DPI (23). IL-10 has been shown to be critical for countering inflammation. For example, IL-10-deficient mice spontaneously develop inflammatory bowel disease or colitis (10–12), and neutralization of IL-10 by anti–IL-10 Ab or IL-10-deficient mice exhibit severe inflammation in response to infection that is associated with lung injury and brain edema (24, 25). Thus, decreased IL-10 expression in p47phox-deficient mice, by itself, could lead to enhanced pulmonary inflammation, and this is further supported by our observation that treatment with rIL-10 rescues the proinflammatory response seen in the p47phox-knockout mice.

During activation of macrophages with TLR agonists, several signal pathways including NF-κB, MAPK, and PI3K/Akt are activated and contribute to IL-10 expression (26, 27). In the PI3K/Akt/GSK3-β signal pathway, GSK3-β can be phosphorylated and inactivated, which in turn allows the nuclear transcription factors CREB and AP-1 to trigger IL-10 expression (26, 28, 29). In our studies, we showed that Akt activation was reduced, and GSK-3β phosphorylation was decreased in p47phox-deficient macrophages that probably contribute to decreased IL-10 production in p47phox-deficient mice.

In summary, our studies demonstrate that ROS have a counterc regulatory role in LPS-induced lung inflammation through modulation of macrophage production of IL-10. These data support the concept that ROS have a role in limiting inflammation and may be pivotal in resolving acute inflammation. On the basis of our data, it is also possible that deficiencies in ROS production contribute to the pathogenesis of chronic inflammatory lung disorders.

Disclosures
The authors have no financial conflict of interest.

References
Supplemental Figure 1. Flow cytometry shows efficacy of adoptive transfer technique. Peritoneal macrophages were resuspended in 1 ml of PBS and incubated with 1 μM CFSE (Sigma-Aldrich, MO, USA) for 15 min at 37 °C. Then washed cells for twice with PBS and resuspended. Cells (3 X 10^6/ml) were adoptively transferred to wild type HLL mice. After 16 h, cells collected from bronchoalveolar lavage (BAL) were stained with F4/80-APC or Ly6G-PE (Gr-1). A, Data presented CFSE positive cells in the F4/80 positive cell population. B, Data presented the percentage of Ly6G positive cells in the BAL.
Supplemental Figure 2. Inhibition of ROS by DPI decreases production of TNF-α and IL-6. Peritoneal macrophages from HLL mice were incubated with DPI for 30 min and the cells were left unstimulated or stimulated with LPS (100 ng/ml) for an additional 8 h. The production of TNF-α (A) and IL-6 (B) in the culture supernatant was detected by ELISA. Results represent mean ± SEM of three separate experiments and **, p < 0.01.