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

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Abstract

Reactive Oxygen Species (ROS) generated by NADPH oxidase are generally known to be pro-inflammatory, 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 p47^{phox} component of NADPH oxidase in macrophages was associated with a paradoxical accentuation of inflammation in a whole animal model of non-infectious sepsis induced by endotoxin. We have confirmed this observation by interrogating four separate *in vivo* models that employ complementary methodology including the use of p47^{phox}^{-/-} mice, p47^{phox}^{-/-} bone marrow chimera mice, adoptive transfer of macrophages from p47^{phox}^{-/-} mice, and an isolated perfused lung edema model that all point to a relationship between excessive acute inflammation and p47^{phox} 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-GSK3- β signal pathway and that it can be reversed by the administration of recombinant IL-10. Our data support the innovative concept that generation of ROS is essential for counter-regulation of acute lung inflammation.

Keywords

NADPH oxidase; p47^{phox}; NF-kB; macrophage; endotoxin; sepsis

Introduction

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

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includes the transmembrane proteins-gp91^{phox} and gp22^{phox}, the plasma proteins-p47^{phox}, p67^{phox} and the small GTPase-Rac2. Activation of NADPH oxidase requires the transfer of cytosolic proteins to membrane that bind to gp91^{phox} and gp22^{phox} 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 protect the host from pathogenic infection (4). Data are also reported that patients with chronic granulomatous disease (CGD), which is a genetic NADPH oxidase 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 non-infectious inflammation. Although ROS certainly contribute to a pro-inflammatory phenotype, paradoxically, leukocytes from CGD patients that lack NADPH oxidase, have a 'hyper-inflammatory phenotype' with increased production of pro-inflammatory cytokines in response to stimulation with Toll-like receptor (TLR) agonists (7, 8). Furthermore, studies using gp91^{phox}^{-/-} mice showed elevated concentrations of the pro-inflammatory chemokines and cytokines, including Monocyte Chemoattractant Protein-1 (MCP-1), Interleukin-6 (IL-6), Interleukin-1 β (IL-1 β), Tumor Necrosis Factor- α (TNF- α), and an increased myeloperoxidase (MPO) activity in lungs in response to treatment with endotoxin (9). 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 p47^{phox} component of NADPH oxidase have an exaggerated inflammatory response to endotoxemia, and this is associated with decreased activation of Akt/GSK3- β pathway that results in deficiency of IL-10 production.

Materials and methods

Mice

We have previously reported that the HLL transgenic and p47^{phox}^{-/-}/HLL mice express *Photinus* luciferase complementary DNA under the control of a proximal 5' human immunodeficiency virus-1 long terminal repeat (5' HIV-LTR) promoter, which is sensitive to NF- κ B-mediated signaling (13). Mice that were 8 to 12 weeks of age were used in this study. All experiments involving mice were conducted with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Chicago.

Preparation of bone marrow-derived macrophages (BMDMs)

Femoral and tibia bone marrow were isolated from HLL and p47^{phox}^{-/-}/HLL mice, by methods previously described (14). Mouse bone marrow cells flushed from femurs and tibias were washed with Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (HBSS). Then bone marrow progenitor cells were cultured in DMEM containing 10% FBS and supplemented with 10% L929 Cell Culture Media (LCCM). After 7 d, 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, MO, USA) and diphenylene iodonium (DPI) (5 μ M, EMD Biosciences, NJ, USA) for 8 h. Production of IL-10, IL-6, and TNF- α in culture supernatant was measured. IL-10 in

mouse blood serum was also measured after injecting with LPS (8 mg/kg, 2h) with enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., MN, USA) according to manufacturer's instructions.

Western-blot analysis

BMDMs were plated in 6-well plates at 2×10^6 /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 (SH X; EMD Biosciences, NJ, USA). The cells were then collected and lysed. The cell lysate was analyzed by immunoblot using antibodies against phospho-GSK3- β , total GSK3- β , phospho-Akt, and total Akt (Cell Signaling Technology, MA, USA).

Superoxide production assays

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

Mouse model of LPS-induced acute lung injury

Mice were injected intraperitoneally with 8 mg/kg of 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 of reporter lysis buffer (Promega, WI, USA) for luciferase activity assay. Blood was drawn by cardiac puncture for measurement of cytokines in serum. The other group of mice was used for measuring *in vivo* images with an In Vivo Imaging System (Xenogen IVIS-200, Caliper Life Sciences, MA, USA) at 8 and 24 h after LPS injection. In another group, each mouse was administered (i.p.) 1 μ g of recombinant IL-10 (R&D Systems Inc., MN, USA) 2 h prior to LPS treatment. Measurements of bioluminescent imaging were performed at 0, 8, and 24 h using IVIS.

Measurement of luciferase activity in lung tissue

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

Fetal Liver Cell (FLC) transplantation

FLC transplantation was performed as previously reported (15, 16). Briefly, FLCs were isolated from pregnant p47^{phox}^{-/-}/HLL and HLL mice. The recipient mice were irradiated by using 10 Gray radiations in two split doses of 5 Gray each where the first dose of radiation was followed by a second dose after 2 h. FLCs were transplanted by tail vein injection into recipient p47^{phox}^{-/-}/HLL and HLL mice. Four weeks following the transplantation, a single dose of liposomal clodronate was administered *via* intratracheal injection in order 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 p47^{phox}^{-/-}/HLL and HLL bone marrow chimera mice were injected intraperitoneally 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 of 0.5% hexadecyl trimethylammonium bormide (Sigma-Aldrich, MO, USA). 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 tetramethylbenzidine (Sigma-Aldrich, MO, USA) and 15 mM H₂O₂ (Sigma-Aldrich, MO, USA), and absorbance at 460 nm was determined. One unit of MPO activity was defined as the change in OD at 460 nm per milligram of protein per minute.

Adoptive transfer of mouse macrophages to HLL mice

Adoptive transfer of mouse macrophages was performed as previously reported (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 intratracheal (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 1/2 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 and 24 h after i.p. injection of LPS, mice were anesthetized with isoflurane inhalation and were subsequently i.p. injected with 100 µl of 300 mg/ml luciferin solution (Caliper LifeSciences, MA, USA). Bioluminescence imaging with a CCD camera of In Vivo Imaging Systems 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 photon value (p/sec/cm²/sr). 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 of formamide for 18 h at 60°C. The homogenate was centrifuged at 5000 *g* for 30 min, and the optical density of the supernatant was read at 620 nm. EBA concentration was calculated against a standard curve and presented as mg of EBA per g 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, AL, USA) and 86 mg egg-phosphatidylcholine (Avanti, AL, USA) was dissolved into chloroform solution and then evaporated under nitrogen. Chloroform was further removed under vacuum in a speedvac Savant concentrator. Dichloromethylene diphosphonic acid (1.2 g; Sigma-Aldrich, MO, USA) 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 μ l of 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 (19). Lungs were then isolated from mice anesthetized with 2.5% isoflurane, perfused *via* the pulmonary artery (RPMI 1640 solution with 3% 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 (Ppv) was set to 3 cm H₂O. The preparations underwent equilibration for 20 min. Then using a syringe drive, 4 $\times$ 10⁶ neutrophils from HLL mice were injected into the arterial perfusate over a 20 min period. To measure filtration rate, Ppv was elevated by 7.5 cm H₂O for 20 min. 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 $\pm$ SEM. Differences between groups of mice were evaluated with Student's *t* test. *P* value < 0.05 was considered statistically significant. In chimera mouse model, data are presented as mean $\pm$ 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 $\pm$ SD.

Results

p47^{phox}^{-/-}/HLL mice exhibit enhanced NF- κ B activation in response to LPS compared with HLL mice

Luciferase expression in HLL transgenic reporter mice is driven by the nuclear factor κ B (NF- κ B)-dependent portion of the promoter. HLL mice and p47^{phox}^{-/-}/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- κ B activation *in vivo*, 100 μ l of 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^{phox}^{-/-}/HLL and HLL mice. Bioluminescence in p47^{phox}^{-/-}/HLL mice is greater than in HLL mice at 8 and 24 h. In Fig. 1B, photon values from p47^{phox}^{-/-}/HLL mice exceed those of HLL mice at 8 and 24 h but not at 0 h. Increase in photon emission over the chest in p47^{phox}^{-/-}/HLL mice compared to the HLL mice indicate more NF- κ B activation in the lungs and enhanced lung inflammation induced by LPS. To confirm the bioluminescence measurements, we examined the effects of LPS on NF- κ B activation at an early time point. The p47^{phox}^{-/-}/HLL mice and HLL mice were sacrificed at 2 h after treatment with 8 mg/kg of LPS, and luciferase activity was measured from lung homogenates. Luciferase activity in the lungs measured by luminometer shows significant increase in p47^{phox}^{-/-}/HLL mice compared with HLL mice (Fig. 1C). Since NF- κ B activation is accentuated in p47^{phox}^{-/-}/HLL mice, these data suggest that p47^{phox} has an important role in modulating lung inflammation in this mouse model of non-infectious inflammation.

p47^{phox}-/-/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^{phox}-/-/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^{phox}-/-/HLL to HLL and the p47^{phox}-/-/HLL to p47^{phox}-/-/HLL bone marrow chimera mice. In contrast, luciferase activity was decreased in the HLL to the p47^{phox}-/-/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 pro-inflammatory activity tracks with the p47^{phox}-/-/HLL bone marrow cells which suggests that either macrophages or neutrophils are involved.

p47^{phox} deficient macrophages are pro-inflammatory in LPS-induced lung inflammation

As p47^{phox}-/-/HLL mice and p47^{phox}-/-/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 p47^{phox}-/-/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. Using this method, approximal 90% of macrophages in the BAL 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 p47^{phox}-/-/HLL mice and HLL mice had similar bioluminescence at 0 h, but the mice that received peritoneal macrophages from p47^{phox}-/-/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 p47^{phox} macrophages showed more leakage of Evans blue dye in the lungs in response to LPS treatment, compared to the HLL mice that received WT macrophages (Fig. 3C). These data suggest that macrophages that are deficient in p47^{phox} are capable of accentuating inflammation when administered to HLL mice. To further dissect the effect of p47^{phox} deficient macrophages in LPS-induced lung inflammation, *ex vivo* lung edema studies were done. HLL and p47^{phox}-/-/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 p47^{phox}-/-/HLL and HLL lungs were perfused with 4×10⁶ of wild-type neutrophils from HLL mice to isolate the effect of NADPH oxidase 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 p47^{phox}-/-/HLL mice compared with p47^{phox}-/-/HLL mice treated with liposomal clodronate (Fig. 4A and B). In contrast to these data, there was more edema seen in the HLL mice that were treated with liposomal clodronate compared to the HLL mice that were treated with salinated liposomes. These data suggest that wild-type macrophages are anti-inflammatory whereas macrophages from p47^{phox} deficient macrophages are pro-inflammatory during endotoxemia. This suggested to us that wild-type macrophages produce a counter-inflammatory mediator whereas this capacity is deficient in macrophages that lack NADPH oxidase activity.

LPS-induced ROS production is p47^{phox} dependent and IL-10 secretion is decreased in p47^{phox}^{-/-}/HLL macrophages

We observed that NADPH oxidase deficiency resulted in a marked accentuation of lung inflammation in response to LPS and found that p47^{phox} deficient macrophages were pro-inflammatory 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 diphenylene iodonium (DPI) (Fig. 5A and 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 p47^{phox}. In contrast to the studies using pharmacologic inhibition of NADPH oxidase with DPI, the concentration of IL-10 was decreased in macrophage culture supernatants from p47^{phox}^{-/-}/HLL mice compared to supernatants from wild type HLL mice (Fig. 5C). We also challenged HLL and p47^{phox}^{-/-}/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 p47^{phox}^{-/-}/HLL mice compared to that from HLL mice (Fig. 5D). However, in contrast to the studies using DPI, the production of pro-inflammatory cytokines, TNF- α and IL-6, were not affected by genetic deficiency of NADPH oxidase, suggesting that NADPH oxidase is critical for the production of the anti-inflammatory cytokine, IL-10 (Fig. 5E and F). These findings suggest that the p47^{phox}^{-/-}/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 NADPH oxidase in macrophages engages an anti-inflammatory program that involves regulation of IL-10 production.

LPS phosphorylates GSK3- β via the PI (3) K-Akt pathway in macrophages

We determined the effect of p47^{phox} deficiency on Akt and GSK3- β phosphorylation, since 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 (Ser 473) and GSK3- β (Ser 9) 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 p47^{phox}^{-/-}/HLL mice compared with those from HLL mice. We next used a selective Akt inhibitor to determine if Ser 9 phosphorylation of GSK3- β was modulated by Akt activation (Fig. 6B). Blocking Akt phosphorylation with an Akt inhibitor attenuated LPS-induced phosphorylation of GSK3- β on Ser 9 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 p47^{phox} 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 p47^{phox}^{-/-}/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 and C).

Exogenous IL-10 reduces LPS-induced lung inflammation in p47^{phox} deficient mice

To further investigate the role of IL-10 in LPS-induced lung inflammation in p47^{phox}^{-/-}/HLL mice, we performed experiments to determine if IL-10 mediated suppression of LPS-induced lung inflammation. We injected p47^{phox}^{-/-}/HLL mice with recombinant mouse IL-10 (rIL 10, 1 μ g/mouse) by i.p. administration 2 h prior to LPS treatment and measured

the *in vivo* NF- κ B 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 rIL 10 treatment (Fig. 7B). Our data show that exogenous IL-10 can substantially reduce excessive lung inflammation induced by LPS in p47^{phox} deficient mice and decrease the levels of inflammation to that of wild-type mice.

Discussion

Reactive oxygen species, which are generated by the phagocytic NADPH oxidase 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 NADPH oxidase components (gp91^{phox}, p47^{phox}) and autoimmune diseases suggest an inflammation-dampening role of ROS (20, 21). We hypothesize here that ROS are involved in counter regulation of inflammation in response to LPS treatment. Our hypothesis is supported by showing that deficiency of the p47^{phox} component of NADPH oxidase in macrophages was associated with a paradoxical accentuation of inflammation in a whole animal model of non-infectious sepsis induced by LPS, and that this pro-inflammatory phenotype is associated with decreased production of anti-inflammatory cytokine IL-10. We generated p47^{phox}^{-/-}/HLL mice which not only display impaired ROS generation, but also contain a convenient luciferase reporter gene that allows indirect detection of NF- κ B-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 p47^{phox} deficiency in macrophages. There are many reports that indicate that ROS have an important role in the generation of pro-inflammatory 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 p47^{phox} deficient macrophages, we found anti-inflammatory cytokine IL-10 expression was decreased in response to stimulation with LPS, which indicates that the counter-inflammatory component of the macrophage response is deficient. We found that the NADPH oxidase inhibitor, DPI totally blocked the LPS-induced IL-10 production suggesting that NADPH oxidase-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 p47^{phox} component of NADPH oxidase 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 NADPH oxidase activity probably is related to other ROS-independent non-specific inhibitory 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 antibody 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 p47^{phox} deficient mice, by itself, could lead to enhanced pulmonary inflammation and this is further supported by our observation that treatment with recombinant IL-10 rescues the pro-inflammatory response seen in the p47^{phox} knockout mice.

During activation of macrophages with TLR agonists, several signal pathways including NF- κ B, 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 GSK3- β phosphorylation was decreased in p47^{phox} deficient macrophages which probably contribute to decreased IL-10 production in p47^{phox} deficient mice.

In summary, our studies demonstrate that ROS have a counter 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. Based on our data, it is also possible that deficiencies in ROS production contribute to the pathogenesis of chronic inflammatory lung disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

The p47^{phox-/-}/HLL mice exhibit enhanced LPS-induced lung inflammation compared with HLL mice. HLL and p47^{phox-/-}/HLL mice were treated with 8 mg/kg of LPS and luciferase activity was detected. *A*, To reveal NF- κ B activation patterns, *in vivo* luciferase activity was presented as bioluminescent images with artificial colors in p47^{phox-/-}/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^{phox-/-}/HLL mice at 2 h after LPS treatment (normalized for total protein). Results represent mean $\pm$ SEM (n=6 each group) and *, $p < 0.05$.

FIGURE 2.

The p47^{phox}^{-/-}/HLL bone marrow chimera mice have accentuated LPS-induced lung inflammation. *A*, PCR results showed that the p47^{phox} knockout genotype was identified as a 900 bp band present only in the p47^{phox}^{-/-}: HLL chimera (Lane 2), whereas the HLL genotype is identified as a 600 bp band present in the HLL: HLL and HLL: p47^{phox}^{-/-} bone marrow chimera mice (Lane 1 and 3). *B*, Luciferase activity assays from lung homogenates of HLL and p47^{phox}^{-/-}/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) and *, *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) and *, *p* < 0.05.

FIGURE 3.

The p47^{phox} deficient macrophages are pro-inflammatory in LPS-induced lung inflammation. *A*, Adoptive transfer of peritoneal macrophages from p47^{phox}^{-/-}/HLL mice and HLL mice were performed *via* i.t. administration to HLL mice. At 0, 8, and 24 h after treatment with LPS, bioluminescent images were measured 10–15 min after i.p. injection of luciferin as the substrate of luciferase to show the *in vivo* luciferase activity. *B*, At 0, 8, and 24 h after treatment with LPS, photons over chest area were counted on the basis of bioluminescent images by IVIS (n=3 each group). Results represent mean ± SEM and *, $p < 0.05$. *C*, Recipient HLL mice underwent adoptive transfer of macrophages from either p47^{phox}^{-/-}/HLL mice or HLL mice for overnight and then were treated with LPS (8 mg/kg). 24 h later, Evans Blue Albumin was injected *via* tail vein into anesthetized recipient mice. After 1 h, mice were euthanized and lung vascular permeability was determined by quantifying EBA extravasation and *D*, left lung wet/dry weight ratio. Results represent mean ± SEM (n=3 each group) and *, $p < 0.05$.

FIGURE 4.

Lung edema formation induced by LPS increases in lungs of $p47^{phox}$ deficient mice and wild-type mice treated with liposomal clodronate. *A*, Maximum slope of resulting weight-gain curve indicated the rate of edema formation. Asymptotes in upper curve occurred when alveoli were flooded to the extent that most alveolar gas was displaced. Horizontal axis displays the time from beginning of perfusion. The measurement of lung edema formation was performed in HLL mice and $p47^{phox-/-}/HLL$ mice treated with or without liposomal clodronate and LPS. *B*, Quantitative data for filtration rates were measured from the slopes in lung edema formation figure. Results represent mean $\pm$ SD ($n=4$ per group) and *, $p < 0.05$.

FIGURE 5.

LPS-induced ROS production is p47^{phox} dependent in macrophages and ROS scavenger blocks IL-10 secretion from HLL mice. **A**, Representative tracing showed the generation of superoxide as counts per second (CPS) by peritoneal macrophages from p47^{phox-/-}/HLL mice and HLL mice, stimulated with 100 ng/ml of LPS. **B**, 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. IL-10 secretion in the culture supernatant was detected by ELISA. **C**, **E**, and **F**, Peritoneal macrophages from p47^{phox-/-}/HLL mice and HLL mice were treated with LPS (100 ng/ml) for 8 h. IL-10, IL-6, and TNF- α were measured in the culture supernatant. Results represent mean $\pm$ SEM of three separate experiments and *, $p < 0.05$. **D**, IL-10 in serum was measured by ELISA at 0 and 2 h after mice were given 8 mg/kg of LPS. Results represent mean $\pm$ SEM ($n=6$ each group) and *, $p < 0.05$.

FIGURE 6.

Macrophages from p47^{phox} deficient mice have decreased phosphorylation of Akt and GSK3-β induced by LPS, which modulates IL-10 production by inhibition of Akt and GSK3. Bone Marrow Macrophages were derived from p47^{phox}^{-/-}/HLL or HLL mice for 6 d. *A*, The cells were serum-starved for 4 h, then stimulated with 100 ng/ml LPS for 30 and 60 min or left unstimulated. *B*, Macrophages from HLL mice were starved for 4 h and then preincubated with 5 μM Akt inhibitor (Akt-i) for 1 h before stimulation with 100 ng/ml LPS or left unstimulated. Akt and GSK3-β phosphorylation were assessed using equal amounts of protein by Western blotting with antibodies against Akt-total and GSK3-β-total. Data are representative of 3 separate experiments. *C*, *D*, IL-10 production in macrophages isolated from HLL mice (*C*) and p47^{phox}^{-/-}/HLL mice (*D*) were preincubated for 1 h with PBS, DMSO, 20 μM Akt inhibitor or 10 μM SB216763 and then left unstimulated or stimulated with 100 ng/ml LPS for 8 h. Cell-free supernatants were analyzed by ELISA. Results represent mean ± SEM and *, *p* < 0.05.

FIGURE 7.

Recombinant IL-10 can substantially reduce the lung inflammation induced by LPS in $p47^{\text{phox-/-}}/\text{HLL}$ mice. **A**, Bioluminescent detection of NF- κ B activation in $p47^{\text{phox-/-}}/\text{HLL}$ mice was performed. After a single i.p. injection of recombinant IL-10 (1 μ g/mouse), LPS were administered by i.p. injection at 2 h and then bioluminescence was measured at 0, 8, and 24 h following luciferin injection. Images are representative of 3 mice per group. **B**, Photons were emitted over the chest in previous groups at 0, 8, and 24 h. Results represent mean $\pm$ SEM and **, $p < 0.01$.