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Monocyte- and Macrophage-Targeted NADPH Oxidase Mediates Antifungal Host Defense and Regulation of Acute Inflammation in Mice

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Chronic granulomatous disease, an inherited disorder of the NADPH oxidase in which phagocytes are defective in the generation of superoxide anion and downstream reactive oxidant species, is characterized by severe bacterial and fungal infections and excessive inflammation. Although NADPH oxidase isoforms exist in several lineages, reactive oxidant generation is greatest in neutrophils, where NADPH oxidase has been deemed vital for pathogen killing. In contrast, the function and importance of NADPH oxidase in macrophages are less clear. Therefore, we evaluated susceptibility to pulmonary aspergillosis in globally NADPH oxidase–deficient mice versus transgenic mice with monocyte/macrophage-targeted NADPH oxidase activity. We found that the lethal inoculum was >100-fold greater in transgenic versus globally NADPH oxidase–deficient mice. Consistent with these in vivo results, NADPH oxidase in mouse alveolar macrophages limited germination of phagocytosed Aspergillus fumigatus spores. Finally, globally NADPH oxidase–deficient mice developed exuberant neutrophilic lung inflammation and proinflammatory cytokine responses to zymosan, a fungal cell wall–derived product composed principally of particulate β-glucans, whereas inflammation in transgenic and wild-type mice was mild and transient. Taken together, our studies identify a central role for monocyte/macrophage NADPH oxidase in controlling fungal infection and in limiting acute lung inflammation. The Journal of Immunology, 2013, 190: 4175–4184.

The lung is an interface where host cells are regularly exposed to microbes and microbial products. Aspergillus-associated diseases encompass both invasive and allergic diseases in which pathogenesis is regulated by host factors (1). Alveolar macrophages are the first-line phagocytic cells that encounter inhaled fungi. Macrophages and other immune cells sense Aspergillus motifs by pathogen recognition receptors that include specific TLRs and dectin-1 (2) and initiate downstream inflammatory responses. The phagocyte NADPH oxidase generates reactive oxidant intermediates (ROIs) in response to specific microbial stimuli, which, in nature, would occur following direct contact with microbes (7). In contrast, neutrophil NADPH oxidase is activated by Aspergillus hyphae largely independent of dectin-1 (8). Ligation of dectin-1 can stimulate NADPH oxidase activity and proinflammatory cytokines and chemokines (7, 9–11). The ability of innate immune cells to recognize fungal products displayed at different stages of fungal growth is likely important in calibrating the immune response to control the growth of inhaled fungi while averting excessive inflammation. The important role of NADPH oxidase in host defense is demonstrated by chronic granulomatous disease (CGD), an inherited disorder of the NADPH oxidase characterized by severe bacterial and filamentous fungal infections. The phagocyte NADPH oxidase is the principal source of ROI generation in activated neutrophils and macrophages. Among CGD patients, residual neutrophil NADPH

Abbreviations used in this article: BAL, bronchoalveolar lavage; B/L, bronchoalveolar lavage fluid; CGD, chronic granulomatous disease; PPAR, peroxisome proliferator-activated receptor; ROI, reactive oxidant intermediate.

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oxidase activity correlates with less severe illness and improved survival (12). Infections by Aspergillus species and other filamentous fungi are major causes of mortality in CGD (13, 14). CGD patients are prone to developing inflammatory complications, such as inflammatory bowel disease and obstructive granulomatous inflammation of the genitourinary tract (15). Engineered NADPH oxidase–deficient mice have a hyperinflammatory phenotype to sterile products, including heat-killed Aspergillus hyphae (16, 17) and fungal cell wall–derived products (18, 19), emphasizing a key role of NADPH oxidase in limiting inflammation in addition to its antimicrobial activity.

NADPH oxidase activation requires translocation of cytosolic phox proteins (p47


phox, p67


phox, p40


phox) and rac to the membrane-bound flavocytochrome consisting of the gp91


phox and p22


phox heterodimer. NADPH oxidase activation results in conversion of oxygen to superoxide anion and generation of downstream reactive oxygen metabolites with antimicrobial activity, such as hydrogen peroxide, hydroxyl anion, and hypohalous acid. In neutrophils, NADPH oxidase activation is linked to activation of primary granule antimicrobial proteases and generation of neutrophil extracellular traps (20, 21). NADPH oxidase–generated ROS and activation of neutrophil proteases have distinct roles in host defense against bacterial and fungal pathogens (22).

Although NADPH oxidase is critical for neutrophil-mediated host defense, the importance of NADPH oxidase in macrophages is unclear. The strongest evidence for the role of macrophage NADPH oxidase in host defense is from the finding that mutations in gp91


phox that selectively affect macrophages lead to increased susceptibility to mycobacterial diseases (23). Prior studies have shown that alveolar macrophages ingest and kill Aspergillus spores, whereas neutrophils principally target the hyphal stage (24). However, there have been conflicting results as to the role of NADPH oxidase in macropahges in controlling the growth of Aspergillus spores (25, 26).

Our major goal was to delineate the specific role of NADPH oxidase in macrophages in mediating host defense against A. fumigatus and in regulating the inflammatory response to fungal components. Transgenic mice with CD68 promoter–driven gene expression have been widely used to study monocyte/macrophage lineage–restricted production of targeted proteins (27). To address the specific role of macrophage NADPH oxidase in mediating antifungal host defense and inflammation, we used mice with monocyte/macrophage-targeted NADPH oxidase activity that have a naturally acquired disabling mutation of Ncf1 (which encodes the p47


phox protein) and harbor a transgene containing wild-type Ncf1 under the control of a human CD68 promoter (28). We found that monocyte/macrophage-targeted NADPH oxidase conferred resistance to pulmonary aspergillosis. Consistent with these results, NADPH oxidase in isolated alveolar macrophages was required to limit the growth of phagocytosed spores. Additionally, globally NADPH oxidase–deficient mice developed exuberant neutrophilic inflammation in transgenic and wild-type mice was minimal. These results support a previously unappreciated role for macrophage NADPH oxidase both in mediating antifungal host defense and in controlling acute neutrophilic inflammation.

Materials and Methods

Mice

Mice with a targeted disruption of either the p47


phox or gp91


phox gene have a defective NADPH oxidase; rendering phagocytes incapable of generating measurable superoxide. NADPH oxidase–deficient mice have increased susceptibility to pathogens that afflict CGD patients, including Aspergillus species (29). We used p47


phox+/− mice (30) backcrossed to N14 in C57BL/6 and age- and sex-matched wild-type C57BL/6 controls.

Mice with a naturally occurring Ncf1 (which encodes the p47


phox protein) mutation (the mutation is Ncf1


+/− and is here abbreviated as Ncf1+/−) have a global NADPH oxidase deficiency (28, 31). The mutation was originally in a B6d2f1/12 strain and was extensively backcrossed to a C57BL/10.Q strain and ascertained to differ by only one mutation as checked with 10,000 single nucleotide polymorphism typing and selected typing around the Ncf1 gene (31). This strain is designated BQ.Ncf1+/−. The p47


phox protein variant expressed in mice Ncf1+/− mice results in defective assembly of the NADPH oxidase complex (32). To generate transgenic mice with monocyte/macrophage-restricted NADPH oxidase activity, the wild-type Ncf1 coding sequence was ligated downstream of the promoter and splice site in a vector containing the human CD68 promoter as previously described (28). The construct containing Ncf1 was named MN. The transgene was injected into the BQ.Ncf1+/− strain and maintained by backcrossing. Screening for Ncf1 mutant and the MN transgene was performed by PCR as described previously (28). Mice with mutant Ncf1 without the transgene (Ncf1−/−/−) are globally NADPH oxidase-deficient, whereas mutant mice that harbor the transgene (Ncf1+/−/−) have monocyte/macrophage-targeted expression of wild-type Ncf1 and NADPH oxidase activity (28, 33).

Mice were bred and maintained under specific pathogen-free conditions at the Animal Care Facility at Roswell Park Cancer Institute (Buffalo, NY). All procedures performed on animals in this study were approved by the Animal Care and Use Committee at Roswell Park Cancer Institute and complied with all state, federal, and National Institutes of Health regulations.

Administration of A. fumigatus

A clinical isolate of A. fumigatus was used in all in vivo studies. Conidial suspensions were prepared as previously described (29) and diluted to desired concentrations. Conidia were administered intratracheally as we described previously (29) or by oropharyngeal instillation. We found that oropharyngeal instillation leads to similar degrees of fungal pneumonia and mortality in p47


phox−/−/− mice compared with intratracheal administration, but avoids surgery. Oropharyngeal instillation therefore became our preferred approach for intrapulmonary instillations. Mice were anesthetized by isolurane inhalation using an approved chamber. Following anesthesia, mice were suspended by their upper incisors from a suture thread on a 90° incline board. The tongue was gently extended, and a liquid volume (maximum 50 μl) was delivered into the distal part of the oropharynx. With the tongue extended, the animal was unable to swallow, and the liquid volume was aspirated into the lower respiratory tract. Just prior to liquid delivery, the chest was gently compressed and then released just after deposition of liquid into the oropharynx to enhance aspiration of the liquid into the lung. Mice recovered within 5 min of the procedure and were observed until they resumed normal activity.

Cyclophosphamide administration

The alkylating agent cyclophosphamide was used to induce leukopenia. Cyclophosphamide (i.p. 250 mg/kg body weight) was administered on days −3 and +1 in relation to A. fumigatus challenge. Peripheral blood was collected in blood diluent (Delta Scientific, I Ivyclaw, PA) in K2 EDTA–coated tubes (Becton Dickinson, Franklin Lakes, NJ), and white cell counts and differentials were determined by analysis on the Advia 120 with Multi-Species software version 3.1.8-MS (Bayer HealthCare, Tarrytown, NY). This regimen leads to a total leukocyte count of <500/μl and absolute neutrophil count of <100/μl from day 0 until at least day 5 in relationship to fungal challenge in wild-type and p47


phox−/−/− mice.

Survival

Following A. fumigatus challenge, mice were monitored twice daily for death and moribundity until at least day 25. Mice with prespecified criteria for distress that included inability to eat or drink, labored breathing, or general moribund appearance were euthanized by CO2 asphyxiation. The primary end point was time to euthanasia.

Bronchoalveolar fluid collection and cytology

After sacrifice, bronchoalveolar lavage fluid (BALF) collection was performed as previously described (19). The trachea was cannulated with a 22-gauge i.v. catheter. Using a tuberculin syringe, 1 ml PBS was injected and withdrawn from the lung and again fresh 1 ml PBS was injected and withdrawn from the lung and both were pooled. Cells were pelleted by centrifugation at 1500 × g for 3 min. Supernatants were aliquoted and stored at −80°C. In the cell pellet, the RBCs were removed by one wash in ACK lysis buffer, and the cells were suspended in 1 ml PBS. The total
number of leukocytes per milliliter was counted using a hemocytometer. Cells were then cytocentrifuged onto clear glass slides and stained with the Hema 3 stain set (Fisher Scientific, Pittsburgh, PA), and cell differential counts were assessed blinded to genotype.

**Histopathology**

After sacrifice and BAL, mouse lungs were infused with 10% neutral buffered formalin via the trachea. Paraffin-embedded blocks were prepared and sections were stained with H&E. In mice administered *A. fumigatus*, Grocott–Gomori methenamine silver stain was used to visualize fungi. The percentage of lung involved by granulomatous or consolidative inflammation was scored in each mouse as follows: 0, 5, and 10% and then by 10% increments (e.g., 20, 30, 40%). The predominant inflammatory cell type was scored as neutrophilic, monocytic, or lymphocytic. In mice administered *A. fumigatus*, additional end points included necrosis (coagulative and inflammatory), hemorrhage, invasive parenchymal aspergillosis, and angioinvasive aspergillosis, as previously described (34). Coagulative necrosis was defined as acellular necrosis or necrosis with scant inflammation, whereas inflammatory necrosis was defined as necrosis occurring within inflammatory lesions (34). All slides were analyzed by one of us (B.H.S.) blinded to genotype and treatment.

**Analysis of antifungal activity of isolated alveolar macrophages**

Alveolar macrophages from unstimulated wild-type and *p47<sup>phox</sup>/<sup>−/−</sup>* mice were collected by BAL. The harvested cells were >95% macrophages, based on cytology. Cells were washed, resuspended in RPMI 1640 with 2 mM l-glutamine and 10% FBS, and 5 × 10<sup>5</sup> cells were seeded into each chamber of an eight-chamber glass slide (Thermo Scientific). Macrophages were allowed to adhere overnight at 37°C with 5% CO<sub>2</sub> and then seeded with 1 × 10<sup>5</sup> conidia of an *A. fumigatus* strain expressing GFP (35) (provided by Dr. Margo Moore, Simon Fraser University, Burnaby, BC, Canada). After 3 h, nonphagocytosed conidia were removed and fresh media were added to each well. Simultaneous imaging of wild-type and *p47<sup>phox</sup>/<sup>−/−</sup>* macrophages was performed at 30 min intervals using the Leica AF6000LX live cell imaging platform (Leica Microsystems, Silver Spring, MD) and was applied to the photographic images (Fig. 2) for contrast enhancement and for adjustments of the luminance and color channel settings.

The conidial activity of wild-type and *p47<sup>phox</sup>/<sup>−/−</sup>* alveolar macrophages was assessed, as previously described with modifications (36). Briefly, 2.5 × 10<sup>5</sup> alveolar macrophages were seeded in 24-well plates (Costar) in 2 ml complete media (RPMI 1640 with 2 mM l-glutamine and 10% FBS) and incubated at 37°C with 5% CO<sub>2</sub> overnight to allow macrophages to adhere. The following morning, 5 × 10<sup>5</sup> *A. fumigatus* conidia were added and the mixtures were incubated for 3 h. Wells were then gently washed and dilutions of supernatants were plated for nonphagocytosed conidia. At 6 h, sterile distilled H<sub>2</sub>O was added to lyse macrophages, cells were removed with vigorous pipetting, and dilutions were added to plates with corn meal agar plates (Becton Dickinson). All plates were incubated at 37°C for 24 h. Colonies were counted for both the nonphagocytosed conidia collected after 3 h and the number of phagocytosed conidia recovered at 6 h.

**Confocal laser-scanning microscopy**

We evaluated the ability of isolated alveolar macrophages from wild-type, *Ncf1<sup>+/+*</sup>*MN, and *Ncf1<sup>+/+*</sup>*MN mice to control growth of phagocytosed *Aspergillus* spores by confocal microscopy. Mice (*n = 5*/genotype) were administered i.v. 20 μM PKH26 (PKH26 red fluorescent cell linker kit; Sigma-Aldrich, St. Louis, MO), which stably accumulates in the membranes of alveolar macrophages but not in peripheral monocytes or narrow precursors (37,39). Ten days later, alveolar macrophages were harvested by BAL, and cells from the same genotype were pooled. Macrophages (8.8 × 10<sup>5</sup>) were seeded on 22 × 22-mm coverslips in six-well plates. Macrophages were allowed to adhere overnight at 37°C with 5% CO<sub>2</sub> and then were transfected with *p47<sup>phox</sup>* conidia of GFP-expressing *A. fumigatus*. Cells were fixed in 1% formaldehyde at 3, 7, and 14 h after addition of conidia. After fixation, coverslips were removed, gently washed in PBS, and mounted onto glass slides using Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA). Confocal images were acquired using a Leica TCS SP2 system with a laser base point scanner mounted on DMIRE2 fluorescence microscope. For all images a ×63 oil immersion lens was used and confocal microscopy was performed with 1 Airy unit. Z-stacks were acquired with an electronic zoom factor of 2.5 in bright field, DAPI (excitation, 405 nm; emission range collected, 410–499 nm), FITC (excitation, 488 nm; emission range collected, 500–537 nm), and PKH26 (excitation, 543 nm; emission range collected, 559–613 nm). The thickness for a Z-stack was determined using DAPI and bright field images. Z-stacks were in the range of 14–24 μm with individual slices ~1 μm thick. For quantification of macrophages, single scans of ×1 zoom were performed on at least six fields per cover slip. Intact macrophages were identified by confocal microscopy based on PKH26 and DAPI staining of the membrane and nucleus, respectively. Conidia were identified based on FITC staining and bright field image.

**Oropharyngeal zymosan administration**

Zymosan (Sigma-Aldrich) was diluted to a concentration of 2.5 mg/ml in saline, sonicated until the particles were suspended homogeneously, and frozen at ~20°C. Prior to use, the zymosan stock was diluted to 0.4 mg/ml and autoclaved to ensure sterility. Zymosan (50 μl) was administered by oropharyngeal instillation using the same technique as *A. fumigatus* administration.

**Statistical analysis**

GraphPad Prism 4.0 software was used for statistical analysis and to display graphical data. Kaplan–Meier curves were used to display time to euthanasia and analyzed using the log-rank method. Comparisons between two groups were made using the nonparametric Mann–Whitney method or Student t test. ANOVA with a Tukey posttest was used to compare the three genotypes when normal distribution was confirmed, and Kruskal–Wallis with Dunn’s multiple comparison test was used when normal distribution was not demonstrated. A p value <0.05 was considered statistically significant.

**Results**

NADPH oxidase–deficient mice are more susceptible to pulmonary aspergillosis than are leukopenic wild-type mice

We compared susceptibility to *Aspergillus* challenge in two well-defined immunocompromised mouse models: wild-type mice rendered leukopenic by cyclophosphamide administration (modeling anti-neoplastic chemotherapy-induced neutropenia) and nonleukopenic *p47<sup>phox</sup>/<sup>−/−</sup>* mice. In prior studies, we observed that immune intact wild-type mice rapidly clear respiratory *Aspergillus* challenge, and they were therefore not used in the current experiments (22). The rationale for cyclophosphamide is that it enables evaluation of antifungal host defense in the absence of recruited inflammatory cells. Although repeated cyclophosphamide administration gradually reduces the number of alveolar macrophages in mice (40), the most immediate effect is depletion of circulating neutrophils. Cyclophosphamide treatment led to a total leukocyte count of <500/μl and absolute neutrophil count of <100/μl from day 0 until at least day 5 in relationship to fungal challenge in wild-type and *p47<sup>phox</sup>/<sup>−/−</sup>* mice.

Cyclophosphamide-treated wild-type leukopenic mice were significantly more resistant to *Aspergillus* challenge than were nonleukopenic *p47<sup>phox</sup>/<sup>−/−</sup>* mice. Using a range of *Aspergillus* inocula (1.25 × 10<sup>5</sup>–2.5 × 10<sup>6</sup> spores/mouse), the LD<sub>50</sub> of *A. fumigatus* was >10-fold greater in wild-type leukopenic mice (>2.5 × 10<sup>5</sup> spores/mouse) compared with nonleukopenic *p47<sup>phox</sup>/<sup>−/−</sup>* mice (2.5 × 10<sup>5</sup> spores/mouse) (Fig. 1). In separate experiments, mice were challenged with an inoculum of 1.25 × 10<sup>5</sup> spores/mouse, an inoculum known to cause invasive aspergillosis in *p47<sup>phox</sup>/<sup>−/−</sup>* mice, and lungs were harvested on day 5 for histological analysis. Nonleukopenic *p47<sup>phox</sup>/<sup>−/−</sup>* mice developed discrete regions of consolidation principally composed of dense neutrophilic infiltrates surrounding invasive hyphae without evidence of hyphal angioinvasion. In contrast, lungs of cyclophosphamide-treated wild-type mice challenged with the same *Aspergillus* inoculum showed no evidence of fungal disease (Fig. 1). Taken together, both the survival data and lung histological analysis support the notion that NADPH oxidase in lung macrophages can confer protection against *Aspergillus* challenge in the absence of detectable circulating neutrophils.
In separate experiments, we evaluated the effect of cyclophosphamide treatment in susceptibility to aspergillosis in p47phox−/− mice. Pulmonary lesions in cyclophosphamide-treated p47phox−/− mice were characterized by coagulative necrosis and extensive hyphal vascular invasion, whereas angioinvasion was not observed in nonleukopenic p47phox−/− mice (Supplemental Fig. 1). These results suggest that recruited inflammatory cells can prevent hyphal angioinvasion through NADPH oxidase–independent pathways (Supplemental Fig. 1).

**Macrophone NADPH oxidase limits growth of phagocytosed conidia**

To investigate the role of macrophage NADPH oxidase in defense against Aspergillus, we evaluate the ability of alveolar macrophages from wild-type and p47phox−/− mice to restrain conidial germination. Alveolar macrophages constituted >95% of the harvested BALF cells based on cytology. Macrophages (5 × 10^5/well) from wild-type and p47phox−/− mice were seeded in parallel wells with GFP-producing *A. fumigatus* (1 × 10^6 spores/well). At 3 h, nonphagocytosed conidia were removed by gentle washing to enable unobstructed visualization of phagocytosed conidia. Conidial germination was then analyzed by simultaneous time-lapse imaging at 30-min intervals using GFP fluorescence to visualize fungi. We observed variable ability of wild-type macrophages to contain conidial growth. Even after 28 h following fungal seeding, we observed several intact macrophages with phagocytosed fungi. In contrast, fungal growth within p47phox−/− macrophages led to destruction of virtually all of the macrophages (Fig. 2, Supplemental Videos 1, 2).

In separate experiments, we found that NADPH oxidase increases the conidioidal activity of isolated alveolar macrophages. Alveolar macrophages from wild-type and p47phox−/− mice (n = 6/genotype) were harvested by BAL and seeded with *A. fumigatus* spores. Nonphagocytosed spores were removed by washing at 3 h, followed by lysis of macrophages at 6 h, and platting on agar. Whereas the recovery of nonphagocytosed spores was similar between genotypes (not shown), killing of phagocytosed conidia was increased in wild-type compared with p47phox−/− macrophages (Fig. 2). Taken together, our observations with isolated macrophages support a role for NADPH oxidase in restraining the growth of phagocytosed conidia and are consistent with the notion of NADPH oxidase in macrophages conferring protection in leukopenic mice.

**Monocyte/macrophage-targeted NADPH oxidase is protective in pulmonary aspergillosis**

To more definitively evaluate the contribution of macrophage NADPH oxidase to antifungal host defense in vivo, we used mice in the B10.Q lineage with a naturally acquired mutation in the Ncf1 gene (which encodes the p47phox protein) and transgenic mice (MN+) that harbor wild-type Ncf1 under the control of human CD68 promoter such that NADPH oxidase is functional in the monocyte/macrophage lineage (28). We previously found that the transgene expression is restricted to monocytes/macrophages and not detected in other cell types including neutrophils (28, 41). Whereas the PMA-stimulated respiratory burst in bone marrow–purified wild-type B10.Q neutrophils was robust, ROI generation in Ncf1+/+MN+ neutrophils was minimal (<1% of wild-type neutrophils), but it was distinguishable from ROI generation in Ncf1−/−MN− neutrophils (33). There was no detectable Ncf1 expression in Ncf1−/−MN+ neutrophils (33). However, expression of Ncf1 protein and NADPH oxidase activity occurred in DCs from Ncf1−/−MN+ mice (42); because myeloid dendritic cells and monocytes have a shared lineage, it is not unexpected that CD68 promoter activity would be present in both cells (43).

Wild-type, Ncf1−/−MN−, and Ncf1+/+MN+ mice were administered A. *fumigatus* by oropharyngeal instillation and monitored for morbidity requiring euthanasia. Based on prior studies, an inoculum of 1.25 × 10^6 spores/mouse results in lethal pulmonary aspergillosis in p47phox−/− mice within 7–15 d fungal challenge (29). We therefore used this fungal inoculum in globally NADPH oxidase–deficient mice and challenged transgenic mice and wild-type mice with fungal inocula ranging between 1.25 × 10^5 and 1.25 × 10^6 spores/mouse. All transgene-negative mice (Ncf1−/−MN−) administered 1.25 × 10^6 spores/mouse died by 13 d (Fig. 3A). In contrast, there was uniform survival in transgenic mice (Ncf1+/+MN+), administered up to a 100-fold greater inoculum (1.25 × 10^6 spores/mouse). An inoculum of 1.25 × 10^5 spores/mouse was lethal in both Ncf1+/+MN+ mice and wild-type B10.Q mice (data not shown). These results point to monocyte/macrophage-targeted NADPH oxidase being protective in pulmonary aspergillosis.

Because survival can be influenced by both fungal burden and inflammation, we compared airway inflammation and lung histology.
FIGURE 2. NADPH oxidase in alveolar macrophages limits growth of phagocytosed A. fumigatus spores. Alveolar macrophages (5 x 10^5/well) from wild-type (A-D) mice and p47^phox^−/− mice (E-H) were seeded in parallel wells with GFP-producing A. fumigatus (1 x 10^6 spores/well). At 3 h, non-phagocytosed conidia were removed by gentle washing, and conidial germination was analyzed by simultaneous time-lapse imaging at 30-min intervals. The full video clips are shown in Supplemental Videos 1 and 2. Time-lapse photos at 7-h intervals show that wild-type macrophages, to an extent, limit growth of phagocytosed spores. In contrast, fungal growth led to the destruction of p47^phox^−/− macrophages with phagocytosed fungi. Note the sparse numbers of p47^phox^−/− macrophages present at 14 h, most of which have ruptured. The green hue that was evident by 14 h in wells with p47^phox^−/− macrophages is probably related to the intensity of the GFP signal within Aspergillus hyphae, and likely spill-over to adjacent areas. Scale bars, 20 μm.

For alveolar macrophages from wild-type and p47^phox^−/− mice (n = 6/genotype) were harvested by BAL and seeded with A. fumigatus spores. Nonphagocytosed spores were removed by washing, followed by lysis of macrophages and assessment of viable fungal recovery by quantitative cultures. 

p < 0.004 by nonparametric Mann-Whitney method.
FIGURE 3. Transgenic mice with monocyte/macrophage-targeted NADPH oxidase are protected from lethal Aspergillus challenge. (A) Wild-type (B10.Q), Ncf1<sup>−/−</sup>MN<sup>+</sup>, and Ncf1<sup>−/−</sup>MN<sup>−</sup> were administered a range of A. fumigatus inocula by oropharyngeal instillation (1.25 × 10<sup>5</sup>–1.25 × 10<sup>7</sup> spores/mouse). All globally NADPH oxidase–deficient mice (Ncf1<sup>−/−</sup>MN<sup>−</sup>; n = 4) administered 1.25 × 10<sup>5</sup> spores/mouse died by 13 d, whereas there was uniform survival in transgenic mice (Ncf1<sup>−/−</sup>MN<sup>+</sup>; n = 5) administered a 100-fold greater inoculum (1.25 × 10<sup>6</sup> spores/mouse). p, <0.0001 by log-rank method. All wild-type mice administered 1.25 × 10<sup>6</sup> spores/mouse also survived, whereas an inoculum of 1.25 × 10<sup>7</sup> spores/mouse was fatal in both wild-type and Ncf1<sup>−/−</sup>MN<sup>+</sup> mice (data not shown). (B–D) In separate experiments, wild-type, Ncf1<sup>−/−</sup>MN<sup>+</sup>, and Ncf1<sup>−/−</sup>MN<sup>−</sup> mice (n = 5/genotype) were administered A. fumigatus (1.25 × 10<sup>5</sup> spores/mouse), sacrificed on day 5, and lungs and BAL exudate cells were analyzed. Lungs of wild-type (B) and Ncf1<sup>−/−</sup>MN<sup>+</sup> (C) mice were normal (H&E; original magnification ×40), with Grocott–Gomori methenamine silver stain showing no signs of fungal disease (not shown). (D) In contrast, all similarly treated Ncf1<sup>−/−</sup>MN<sup>−</sup> mice developed multifocal consolidative lesions (H&E; original magnification ×40) and invasive hyphae (inset, Grocott–Gomori methenamine silver stain; original magnification ×400; arrow points to hyphae). (E–G) Ncf1<sup>−/−</sup>MN<sup>−</sup> (Figure legend continues)
in wild-type, Ncf1*/*MN−, and Ncf1*/*MN+ mice following Aspergillus challenge (Fig. 3B–G). Mice were administered A. fumigatus (1.25 × 10⁴ spores/mouse) and sacrificed on day 5, a time that preceded morbidity in Ncf1*/*MN− mice. Ncf1*/*MN+ mice exhibited exuberant pulmonary consolidative lesions comprised of neutrophilic and monocytic cells. Hyphae invading the lung parenchyma, but not blood vessels, were observed. In contrast, lung inflammation in similarly treated wild-type and Ncf1*/*MN− mice was close to nil, and fungi were not observed. Ncf1*/*MN− mice had significantly greater neutrophilic airway inflammation compared with wild-type and Ncf1*/*MN+ mice. Thus, consistent with survival data, NADPH oxidase in the monocyte/macrophage lineage was protective against pulmonary aspergillosis in the context of NADPH oxidase deficiency in neutrophils.

We next evaluated whether isolated alveolar macrophages from Ncf1*/*MN+ phenotype wild-type macrophages in limiting growth of phagocytosed conidia. Although video imaging described in Fig. 2 has the advantage of sequential imaging of the same primary cell culture, confocal microscopy enables more detailed imaging of cells. Wild-type, Ncf1*/*MN−, and Ncf1*/*MN+ mice (n = 3/l genotype) were administered i.v. PKH26, which stably accumulates in the membranes of alveolar macrophages (37–39). Alveolar macrophages were harvested by BAL and seeded with conidia of GFP-expressing A. fumigatus. Cells were fixed at 3, 7, and 14 h after addition of conidia. The 3 and 7 h time points precede transition to the hyphal stage, and they therefore enable comparison of phagocytosis of conidia among the genotypes. In contrast, the 14 h time point enables comparison of the genotypes with regard to inhibiting transition of phagocytosed conidia to the tissue-invasive hyphal stage. Intact macrophages were identified by confocal microscopy based on PKH26 and DAPI staining of the membrane and nucleus, respectively, and phagocytosed conidia were identified based on GFP expression. We found that the number of total macrophages and macrophages with ≥1 phagocytosed conidia was similar among the three genotypes at 3 and 7 h, reflecting similar phagocytosis efficiency. At 14 h, the number of intact macrophages with and without phagocytosed conidia was similar between the wild-type and Ncf1*/*MN+ genotypes. In contrast, we did not observe any intact Ncf1*/*MN− macrophage with a phagocytosed conidium at 14 h; rather, remnants of ruptured Ncf1*/*MN− macrophages were observed in association with hyphae (Fig. 3H–L). Taken together, our in vivo and in vitro studies demonstrate that monocyte/macrophage NADPH oxidase defends against pulmonary Aspergillus infection.

Monocyte/macrophage-targeted NADPH oxidase can limit zymosan-induced lung inflammation

We next asked whether monocyte/macrophage NADPH oxidase regulates inflammation independent of its antimicrobial activity. We used oropharyngeal instillation of zymosan as a sterile model of lung inflammation. Zymosan is a fungal cell wall-derived product principally composed of β-glucan that activates the phagocyte NADPH oxidase through activation of dectin-1 (9). In prior studies using bone marrow chimeras, we showed that NADPH oxidase in the hematopoietic component restrained zymosan-induced lung inflammation (19). We therefore used Ncf1*/*MN− mice to evaluate whether monocyte/macrophage NADPH oxidase regulates neutrophilic inflammation in the lungs.

Wild-type (B10.Q), Ncf1*/*MN−, and Ncf1*/*MN+ mice were administered oropharyngeal zymosan, and BALF leukocytosis and lung histology were evaluated on days 1 and 5. On day 1 after zymosan administration, BALF neutrophilic leukocytosis was significantly greater in Ncf1*/*MN− mice compared with similarly treated Ncf1*/*MN+ and wild-type mice (Fig. 4A). On day 5, BALF neutrophilic leukocytosis persisted in Ncf1*/*MN− mice, whereas inflammatory cell recovery from BALF from Ncf1*/*MN+ and wild-type mice was close to unstimulated levels and composed principally of monocytes (Fig. 4B). Additionally, zymosan resulted in extensive lung consolidation composed of neutrophilic and monocytic cells in Ncf1*/*MN− mice, whereas lung inflammation in similarly treated Ncf1*/*MN+ mice and wild-type mice was close to nil (Fig. 4C–E). BALF from zymosan-stimulated Ncf1*/*MN− mice had significantly increased proinflammatory cytokine and chemokine levels compared with BALF from similarly treated Ncf1*/*MN+ and wild-type mice (Fig. 5). Taken together, these results point to NADPH oxidase in the monocyte/macrophage lineage limiting inflammation independent of its antimicrobial function.

Discussion

Our results show an important role of monocyte/macrophage NADPH oxidase in mediating antifungal host defense and in restraining acute neutrophilic inflammation. In the context of global NADPH oxidase deficiency, transgenic monocyte/macrophage-targeted NADPH oxidase was protective against pulmonary aspergillosis. The lethal inoculum in Ncf1*/*MN− mice was >100-fold greater compared with globally NADPH oxidase-deficient Ncf1*/*MN− mice. Transgenic mice developed no signs of histological fungal disease following a fungal inoculum that led to lethal fungal pneumonia in Ncf1*/*MN− mice. Consistent with these in vivo results, NADPH oxidase in isolated alveolar macrophages limited conidial growth. Additionally, monocyte/macrophage-targeted NADPH oxidase attenuated zymosan-induced neutrophilic lung inflammation. Taken together, these results support a model in which NADPH oxidase in the monocyte/macrophage lineage inhibits fungal growth and restrains inflammation induced by proinflammatory fungal products.

Lung macrophages ingest inhaled Aspergillus conidia and limit fungal growth (24). Early neutrophilic accumulation in the lungs is required to prevent fungal germination in mice following challenge with large inocula of A. fumigatus (44, 45). Depletion of
alveolar macrophages with clodronate prior to pulmonary A. fumigatus infection did not adversely affect neutrophil recruitment or control of fungal growth, whereas neutrophil depletion increased mortality in mice (45). Studies in isolated human neutrophils show that NADPH oxidase is important in controlling A. fumigatus hyphal growth but is dispensable for conidial activity (46). However, using a reporter system to evaluate fungal viability following pulmonary Aspergillus challenge, Jhingran et al. (47) showed that NADPH oxidase in neutrophils augments killing of phagocytosed conidia. Transgenic Ncf1*/* mice were able to defend against A. fumigatus, and they demonstrated no obvious impairment in antifungal host defense compared with wild-type mice. These results are consistent with our recent findings that monocyte/macrophage-targeted NADPH oxidase was protective against bacterial infections in mice (33).

Whereas isolated wild-type alveolar macrophages could limit fungal growth of phagocytosed conidia, NADPH oxidase–deficient macrophages failed to do so. These results are consistent with those of Philippe et al. (25) and differ from those of Cornish et al. (26). The approach used by Cornish et al. (26) involved evaluation of fungal germination in isolated alveolar macrophages at 8 h after fungal seeding. However, we observed that the most dramatic effects of NADPH oxidase in isolated alveolar macrophages in suppressing fungal growth were observed at later time points after fungal seeding (Figs. 2, 3, Supplemental Videos 1, 2). These results support a model in which NADPH oxidase in alveolar macrophages, either through the direct effect of ROIs or via downstream signaling, is sufficiently injurious to spores to limit their germination into tissue-invasive hyphae. Aspergillus species and other molds have a broad repertoire of antioxidative pathways that defend against phagocyte-derived ROIs, and inhibition of these antioxidative pathways can reduce fungal survival during in vivo infection (48). Further studies are required to evaluate the responses of phagocytosed conidia at the genomic and proteomic level to reactive oxygen species produced by macrophage NADPH oxidase. Such studies may identify important fungal stress response pathways that could be targets for drug development.

Although transgenic Ncf1*/* mice are a valuable tool to delineate the role of monocyte/macrophage NADPH oxidase in antimicrobial host defense and regulation of inflammation, we acknowledge a number of limitations. First, there is the potential for low-level CD68 promoter–independent Ncf1 expression in vivo that can lead to augmented neutrophil NADPH oxidase activity in transgenic mice. Second, Ncf1 expression in myeloid dendritic cells may influence the phenotype. Although NADPH oxidase in dendritic cells can mediate cross-presentation of fungal Ags and vaccine-induced immunity (49), it is unlikely that this effect would be relevant in our experiments that focus on early acute inflammatory responses to a single Aspergillus or zymosan challenge. Third, in transgenic mice, NADPH oxidase–derived oxidants from macrophages may diffuse to neighboring neutrophils; diffused H₂O₂ could potentially be a substrate for antimicrobial myeloperoxidase-dependent halide generation in NADPH oxidase–deficient neutrophils (50). Additionally, although NADPH oxidase is the major source of reactive oxidants in stimulated phagocytes, other oxidant-generating systems, such as xanthine oxidase (51) and mitochondrial respiration (52), can contribute to host defense.
Pathogen recognition receptors on macrophages sense fungal motifs displayed at different stage of fungal growth. Aspergillus cell wall β-glucans that are unmasked during germination activate macrophages (3–6). Dectin-1 signaling is only activated by particulate β-glucans that in nature would correspond to direct contact with microbe cell walls rather than soluble products (7). In mice, activation of dectin-1 by fungal β-glucans leads to NADPH oxidase activation and enhanced fungal clearance in experimental pulmonary aspergillosis (2). Dectin-1 induces proinflammatory cytokines and chemokines, including IL-17A (2, 11, 53, 54). In contrast, our current results and prior studies show that NADPH oxidase limits proinflammatory cytokine production in response to Aspergillus challenge (17) and to β-glucan preparations (18, 19). Consistent with our results, Deffert et al. (42) recently reported that intradermal administration of β-glucan resulted in prolonged neutrophilic inflammation in Ncf1−/−/Mx1−/− mice, but self-limited inflammation in wild-type and Ncf1−/− MN+ mice. Seen in this light, our results point to macrophage NADPH oxidase both limiting fungal growth and counterregulating the proinflammatory responses induced by fungal cell wall products.

Neutrophils and macrophages comingle in the inflammatory milieu, where cross-signaling occurs whereby macrophages recognize and remove dying neutrophils (55, 56). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate neutrophil apoptosis (57, 58). Additionally, ROIs produced by neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60). Depending on the nature of the stimulus, neutrophil NADPH oxidase can stimulate the NADPH oxidase of macrophages (60).

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Disclosures

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Supplemental Figures

Figure 1. Leukopenia renders \( p47^{phox-/-} \) (CGD) mice more susceptible to \textit{Aspergillus fumigatus} challenge. A) Kaplan-Meier survival curves of cyclophosphamide (Cyp)-treated CGD mice (n=13) and non-leukopenic CGD (n=10) mice administered a low inoculum of intratracheal \textit{A. fumigatus} (1.25 \( \times \text{10}^3 \) spores/mouse). Log-rank (Mantel-Cox) test, \( p < 0.0001 \). In separate experiments, Cyp-treated and non-leukopenic CGD mice were administered intratracheal \textit{A. fumigatus} (1.25 \( \times \text{10}^4 \) spores/mouse), and sacrificed on day 5 (\( n = 5 \) mice per group). B) Lung histology in non-leukopenic mice was characterized by pyogranulomatous lesions with central neutrophilic infiltrates (H&E, 400x) and invasive hyphae (inset, GMS 400x). Note sparing of the blood vessel (white arrow) within the consolidative lesion. C) In contrast, lung histology in leukopenic CGD mice showed extensive acellular necrosis (H&E, 400x), and D) hyphal vascular invasion (GMS, 400x). Arrows demarcate the blood vessel wall. Results were highly consistent among similarly treated mice.

Movies. \textit{NADPH oxidase in alveolar macrophages is required to limit} \textit{A. fumigatus} growth. Time-lapse imaging of wildtype and \( p47^{phox-/-} \) alveolar macrophages (5 \( \times \text{10}^5 \)/well) seeded with GFP+ \textit{A. fumigatus} spores (1 \( \times \text{10}^6 \) spores/well). At 3h after fungal seeding, non-phagocytosed conidia were removed by gentle washing to enable unobstructed visualization of phagocytosed conidia. Fungal growth was then analyzed by simultaneous time lapse imaging at 30-min intervals. The video clips correspond to 6-29h after initial conidial seeding. Movie 1) Wildtype macrophages were variably able to contain conidial growth. Movie 2) In contrast, fungal growth led to the rupture of \( p47^{phox-/-} \) macrophages with phagocytosed fungi. The green hue is probably related to the intensity of the GFP signal within \textit{Aspergillus} hyphae, and likely spill-over to adjacent areas.
Supp Fig. 1