HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2023 November 15.

Published in final edited form as:

J Immunol. 2022 November 15; 209(10): 1960–1972. doi:10.4049/jimmunol.2200543.

Neutrophil and macrophage NADPH oxidase 2 (NOX2) differentially control responses to inflammation and to *Aspergillus fumigatus* in mice

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Abstract

Aspergillus fumigatus (AF) is an important opportunistic fungal pathogen and causes invasive pulmonary aspergillosis in conditions with compromised innate antifungal immunity, including chronic granulomatous disease, which results from inherited deficiency of the superoxide-generating leukocyte NADPH oxidase 2 (NOX2). Derivative oxidants have both anti-microbial and immunoregulatory activity, and in the context of AF, contribute to both fungal killing and dampening inflammation induced by fungal cell walls. As the relative roles of macrophage vs neutrophil NOX2 in the host response to AF are incompletely understood, we studied mice with conditional deletion of NOX2. When NOX2 was absent in alveolar macrophages (AM) as a result of LysM-Cre-mediated deletion, germination of inhaled AF conidia was increased. Reducing NOX2 activity specifically in neutrophils via S100a8 (MRP8)-Cre also increased fungal burden, which was inversely proportional to the level of neutrophil NOX2 activity. Moreover, diminished NOX2 in neutrophils synergized with corticosteroid immunosuppression to impair lung clearance of AF. Neutrophil-specific reduction in NOX2 activity also enhanced acute inflammation induced by inhaled sterile fungal cell walls. These results advance understanding into cell-specific roles of NOX2 in the host response to AF. We show that alveolar macrophage NOX2 is a non-redundant effector that limits germination of inhaled AF conidia. In contrast, reducing NOX2 activity only in neutrophils is sufficient to enhance inflammation to fungal cell walls as well as to promote

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Disclosures
The authors have no financial conflicts of interest.

invasive AF. This may be relevant in clinical settings with acquired defects in NOX2 activity due to underlying conditions, which overlap risk factors for invasive aspergillosis.

Introduction

Aspergillus fumigatus (AF) conidia are ubiquitous in the environment, and humans inhale up to thousands of AF conidia daily, which are readily cleared in immunocompetent hosts (1). However, in patients with suppressed fungicidal innate immunity, failure to eliminate inhaled conidia and the hyphae that emerge upon germination results in invasive pulmonary aspergillosis (IPA). Mortality remains high despite development of more potent antifungal agents (1, 2). Risk factors include prolonged neutropenia during treatment of myeloid leukemia, impaired leukocyte function from corticosteroid use following allogeneic marrow or solid organ transplant, and primary immunodeficiencies with phagocyte function defects, particularly chronic granulomatous disease (CGD), where NADPH oxidase 2 (NOX2)-dependent reactive oxygen species (ROS) are not produced (1–3). Invasive aspergillosis is increasing in new groups of patients, which include COPD, critically ill patients with underlying co-morbidities or following influenza or SARS-CoV2, and patients with lymphoid malignancies receiving kinase inhibitors (1, 3–5).

Alveolar macrophages and neutrophils are crucial for the host response to inhaled conidia (6–8). Dormant conidia are detected by lung epithelial cells and resident alveolar macrophages (AM) to trigger ingestion and early production of inflammatory mediators, initiating neutrophil recruitment to the lung within hours. In mice, alveolar macrophages are sufficient to eliminate conidia unless their function is compromised, for example, due to corticosteroids (9, 10). However, large inocula exceed the capacity for AM control even in immunocompetent mice (9), and after inhalation of millions of conidia, an influx of neutrophil in the first hours is crucial to prevent IPA (11). Neutrophils ingest and kill conidia(12) as well as eradicate emerging hyphae. Moreover, if functional neutrophils are present, AM are dispensable to prevent IPA (11).

The role of NOX2 in the host response to AF has long been recognized because of its association with CGD, where IPA remains a major clinical problem (13). CGD results from inactivating X-linked or recessive mutations in genes encoding subunits of the NOX2 enzyme complex (14). The absence of NOX2-derived ROS results in recurrent bacterial and fungal infections as well as aberrant inflammation, reflecting their importance not only for killing microbes but their influence on redox-regulated cellular processes that limit inflammation (14, 15). Even sterile fungal cell walls induce neutrophilic hyperinflammation in CGD mice (16–19), and fungal infections in CGD are often accompanied by pyogranulomatous abscesses that can complicate treatment (13, 15).

As a first line of defense, alveolar macrophages and neutrophils possess both non-oxidative and oxidative pathways to eliminate AF. Conidia are ingested and killed within phagosomes. The highly acidic environment of macrophage phagosomes is conidiacidal (20), augmented by NOX2-derived ROS (10, 12, 21). Mitochondria- and copper-derived ROS may also contribute (22, 23). In neutrophils, ingested conidia can be eradicated by non-oxidative mechanisms, including sequestration of iron (24–26); NOX2 can also enhance neutrophil

killing of conidia (12, 27). Due to their size, hyphae are killed by extracellular means. This is strongly dependent on neutrophils and NOX2 ROS, which synergizes with MPO and other granule proteins to kill hyphae (26, 28–30).

Despite the importance of NOX2 in the host response to AF, the relative contributions of neutrophil and macrophage NOX2 are not fully resolved. Some suggest effective killing of conidia by murine CGD AM (16, 31, 32), but others found conidiacidal activity was markedly reduced in vitro and/or in vivo (10, 33). Neutrophil NOX2 ROS are essential to kill AF hyphae and prevent progressive infection in mouse cornea or zebrafish (28, 34). However, control of AF in these tissues may not fully reflect the host response in the lung, where neutrophils are rapidly deployed and can eliminate conidia in the absence of NOX2 (12, 35). The impact of reduced but not absent NOX2 activity is also not well understood. Diminished neutrophil NOX2 activity from loss of type 1 interferon signaling or following ethanol exposure increases susceptibility to IPA in mice, but other associated functional defects may also contribute (36, 37). Excessive lung inflammation elicited by fungal cell walls is promoted by inflammatory mediators produced by CGD neutrophils (17, 18) but may also involve other dysregulated leukocytes.

In this study, we took a genetic approach to better define cell-specific roles of NOX2 in responses to pulmonary AF and developed mice with conditional deletion of NOX2 in macrophages and/or neutrophils. We showed that alveolar macrophage NOX2 had a non-redundant role to limit germination of AF conidia in the first 24 hours after inhalation when conidia numbers were not overwhelming. The level of neutrophil NOX2 activity was a key determinant for clearance of larger inocula, and even without complete loss of NOX2, reducing NOX2 activity was sufficient to increase lung AF burden. Deficient neutrophil NOX2 activity also impaired clearance of AF in corticosteroid-immunosuppressed mice. Finally, we showed that neutrophil rather than macrophage NOX2 played a dominant role in limiting excessive lung inflammation to fungal cell walls. That even partial loss of NOX2 activity results in impaired control of AF may have relevance to clinical settings where NOX2 activity is depressed, which could increase the risk of developing invasive or chronic AF infection.

Methods.

Mice

Mice were maintained in specific pathogen-free conditions and all experiments were approved by the Institutional Animal Care and Use Committee at Washington University in St. Louis. Age-matched male and female mice were used in experiments between the ages of 10 to 20 weeks. Wild type (WT) C57BL/6J were purchased from Jackson Laboratory or bred in-house. X-linked CGD (*Cybb*^{KO}) (38) and *Ncf2*$^{-/-}$ (*Ncf2*^{KO}) (39) CGD mice were from in-house colonies, as were *Ncf2*$^{fl/fl}$ mice that have loxP sites flanking *Ncf2* exon 3, and *Ncf2*$^{fl/fl}$*LysM*$^{WT/Cre}$ mice, referred to as *Ncf2*^{LysMCre} (40).

We also bred Ncf2$^{fl/fl}$ mice to a neutrophil-directed Cre strain, B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw/J (41) (JAX stock 021614) (Figure S1A). We took advantage of transient embryonic expression of Cre from the S100A8-Cre transgene (42) to identify mice

with global deletion of exon 3 to generate Ncf2$^{-Ex3/-Ex3}$ S100A8-Cre mice. These were crossed with Ncf2$^{fl/fl}$ mice to produce NCF2$^{fl/-Ex3}$S100A8-Cre mice, denoted as *Ncf2*^{S100A8Cre}. To generate mice with an X-linked *Cybb* allele with loxP sites flanking exon 5, C57BL6/J oocytes were fertilized with C57BL/6N-Cybb$^{tm2a(KOMP)Wtsi}$/H sperm (UKRI-MRC Harwell) and heterozygous female offspring were bred to B6.129S4-Gt(ROSA)26Sortm1(FLP1)Dym/JRainJ mice(43) (JAX stock 009086). These mice were crossed with B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw/J to produce *Cybb*$^{fl/Y}$S100A8-Cre and *Cybb*$^{fl/fl}$S100A8-Cre mice (*Cybb*^{S100A8Cre} mice) (Figure S1B). Ncf2$^{fl/fl}$ and Cybb$^{fl/fl}$ mice were crossed to generate *Ncf2*$^{fl/fl}$ *Cybb*$^{fl/fl}$ mice. These were bred to B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw/J to produce "double-floxed" *Ncf2*$^{fl/fl}$*Cybb*$^{fl/fl}$S100A8-Cre mice, referred to as *Ncf2Cybb*^{S100A8Cre} and maintained by crossing *Ncf2*$^{fl/fl}$ *Cybb*$^{fl/fl}$ and *Ncf2Cybb*^{S100A8Cre} mice.

Reagents and Cell Isolation

Reagents were from Sigma Aldrich unless indicated. Flow cytometry antibodies were from BD Biosciences unless noted, and data collected on Cytek-modified FACScan instrument and analyzed with FlowJo. Neutrophils were purified from bone marrow cells by non-adherence, and in some experiments, followed by the EasySep Mouse Neutrophil Enrichment Kit (STEMCELL Technologies)(18, 39). Bone marrow monocytes, peripheral blood mononuclear cells, resident peritoneal macrophages, and alveolar macrophages were also isolated as described (39, 40).

NADPH oxidase activity

Neutrophil NADPH oxidase activity was monitored by flow cytometry using dihydrorhodamine 123 (DHR) with phorbol 12-myristate 13-acetate (PMA) stimulation, or by Cytochrome C reduction on a Spectramax 340PC (Molecular Devices) (39, 44). The DHR assay was used to calculate the stimulation index of peripheral blood Ly6C$^+$CD115$^+$CD11b$^+$ monocytes as the ratio of the MFI for PMA-stimulated cells to unstimulated cells (45). In resident alveolar and peritoneal macrophages, NADPH oxidase activity was assessed following stimulation with serum-opsonized zymosan in the presence of nitroblue tetrazolium (NBT) (40).

Western Blots

Cell lysates were electrophoresed for Western blots and probed with antibodies to assess expression of mouse NCF2 (p67^{phox})(Abcam), mouse CYBB (clone 54.1(46), Santa Cruz Biotechnology) and β-actin (Cell Signaling Technologies), as described (39, 47, 48).

LTB4 production by zymosan-stimulated neutrophils

Purified bone marrow neutrophils at 4×10^6/ml were stimulated for one hour with zymosan (MOI=1:2) in RPMI1640 (0.4 mM Ca^{2+}) and LTB4 measured by ELISA (Cayman Chemicals) (18).

Sterile inflammation

Zymosan (20 mcg) was administered by intranasal (IN) instillation and mice euthanized after 18hours for analysis of BAL cell counts, leukocyte differential on cytospins, and lung histology (18). Sterile peritoneal inflammation was induced by intraperitoneal (IP) injection of 1 mL of 5mM sodium meta-periodate (48) and mice euthanized after 72 hours. Peritoneal cavities were lavaged to determine cell counts and leukocyte differential by cytospin. For IL-1α neutralization experiments, mice received 0.5mg of anti-IL-1α or isotype control antibody (BioXCell) retro-orbitally one hour prior to induction of peritonitis (48).

A. fumigatus pneumonia

A. fumigatus strains AF10 (clinical isolate 90240; ATCC) or AF293 (MYA-4609, ATCC; obtained from Nancy Keller, University of Wisconsin) were grown on minimal glucose media (MGM) plates using an overlay method for 3 days at 37°C (22) and conidia were harvested in PBS by gentle scraping with a cell spatula, passed through two layers of sterile Miracloth and a 40μM strainer (39). Conidia were enumerated using a hemocytometer and numbers subsequently verified by plate culture for CFU. Mice were anesthetized by IP ketamine and challenged IN with indicated doses of conidia in 25μl of sterile PBS. In some experiments, mice received 200mg/kg freshly prepared cortisone acetate IP on day −3, 0 (day of challenge) and day 4. Mice were euthanized at the indicated times and 1mL BAL collected for cell counts, cytospins for leukocyte differential and colony forming unit (CFU). The left lung was processed for histology and staining with H&E and with Gomori methamine silver (GMS) (39). Images were captured with a NanoZoomer digital slice scanner and NDP.view2 software (Hamamatsu Photonics, Japan). The right lung was homogenized in 500 μl PBS. For fungal burden, aliquots of BAL and lung homogenates were each plated on MGM for CFU analysis. ELISAs on BAL samples were performed using ProcartaPlex panels from Thermo Fisher Scientific.

Analysis of in vivo germination of *A. fumigatus* conidia

Mice were challenged with 5×10^4 AF293 conidia by IN administration. Mice were treated by IP injection with either a double-antibody-based strategy using 200 μg anti-Ly6G (day −2 and just before challenge on D0) and 100 μg anti-rat-kappa immunoglobulin (day −1) to produce neutropenia or with 100 μg isotype control on Day −2 and D0 (clones 1A8, MAR18.5, or clone 2A3, respectively; BioXcell) (18, 49, 50). Peripheral blood was obtained by retro-orbital puncture at time of challenge (day 0) and at 24 hpi to determine the absolute neutrophil count (ANC), based on the complete blood count determined by a Hemavet 950S (Drew Scientific) and the percentage of neutrophils on Wright-Giemsa-stained blood smears. At 24 hpi, 1mL BAL was collected for cell counts, Wright-Giemsa-stained cytospins for leukocyte differential and analysis of hyphae, and CFU. In some experiments, the right lung was homogenized and also analyzed for CFU. BAL cytospins were photographed with a Zeiss AxioSkope (Carl Zeiss), and hyphae lengths determined using the Zen Black software (Carl Zeiss) measurement tool. To assess hyphae number in BAL samples, 700uL from each sample was centrifuged in an Eppendorf tube, resuspended in 900ul PBS with two drops of ORTHO BSA (ORTHO Clinical Diagnostics), and equal volumes cytospun onto 3 slides per

animal. Hyphae numbers were hand-counted on each slide under 40X magnification, and the total number of hyphae per mouse from 1 ml BAL fluid calculated.

Phagocytosis of *A. fumigatus* conidia

Mice were challenged IN with either saline or 5×10^6 conidia prepared from the YFP-expressing AF293.1 strain TBK1.1 (51) (obtained from Nancy Keller). After 2 hours, mice were euthanized and BAL was obtained by five sequential one ml lavage with ice cold PBS with 2mM EDTA and 2% FBS. AM were identified as CD45+(BV 450, clone: 30-F11) SiglecF+ (PE, clone: E50–2440) and CD11c+(PE Cy™7clone: HL3). To quench any extracellular conidia adherent to AM, 0.1% Trypan blue was added, and AM gated for YFP to assess the percentage of AM with ingested conidia.

Statistics

Statistical analyses used GraphPad Prism 9.0 (GraphPad Software). A P value of <0.05 was considered statically significant. For normally distributed data, either the Student's t-test to compare two groups or one-way ANOVA with Tukey's multiple comparisons test were used. Frequency testing used the Chi-squared test. Non-parametric data was evaluated using the Kolmogorov-Smirnov test and Mann-Whitney U test.

Results

Development of mice with macrophage and/ or neutrophil conditional deletion of NOX2 subunits

To investigate the impact of cell-type-specific NOX2 deficiency, we developed mice with conditional deletion of NOX2 subunit genes *Ncf2* and/or *Cybb*. We previously produced *Ncf2*^{LysMCre} mice by crossing mice with a conditional loss-of-function *Ncf2* allele (*Ncf2*$^{fl/fl}$) to LyzM (LysM)-Cre(52), which deletes *Ncf2* and NOX2 activity in ≈95% of *Ncf2*^{LysMCre} resident alveolar and peritoneal macrophages (40). *Ncf2* deletion in *Ncf2*^{LysMCre} neutrophils is less efficient, with only a ≈two-fold reduction in expression and NOX2 activity (40). To target NOX2 expression specifically in neutrophils, we crossed mice with a S100A8-Cre (MRP8-Cre) transgene (41) to *Ncf2*$^{fl/fl}$ (Figure S1A) or *Cybb*$^{fl/fl}$ mice (Figure S1B). *Ncf2*^{S100A8Cre} neutrophils retained partial NOX2 activity by dihydrorhodamine (DHR) fluorescence, although more reduced compared to Ncf2^{LysMCre} (Figure 1A). Reflecting these results, superoxide production measured by cytochrome c assay averaged 50% and 20% of WT for Ncf2^{LysMCre} and *Ncf2*^{S100A8Cre} neutrophils, respectively (Figure 1B). *Cybb*^{S100A8Cre} neutrophils also had residual NOX2 activity (Figure 1A). We therefore developed "double-floxed" *Ncf2Cybb*^{S100A8Cre} mice that harbored floxed alleles of both *Ncf2* and *Cybb*. DHR fluorescence was further reduced in *Ncf2Cybb*^{S100A8Cre} neutrophils (Figure 1A), and by cytochrome c assay, superoxide production was only ≈10% of WT (Figure 1C). These results correlated with reduced NCF2 and CYBB levels on Western blots (Figure S1C, D). As anticipated, resident macrophages had intact NOX2 activity and subunit expression in S100A8-Cre mice, in contrast to *Ncf2*^{LysMCre} (Figure 1D, Figure S1E). Finally, *Ncf2*^{S100A8Cre} and *Ncf2Cybb*^{S100A8Cre} monocytes had normal NOX2 activity (Figure 1E) and expression of NCF2 and CYBB (Figure S1F), respectively, similar to Ncf2^{LysMCre} monocytes (40).

Although we succeeded in producing mice with absent NOX2 activity in resident macrophages, we were unable to entirely eliminate NOX2 specifically in neutrophils. Floxed alleles in self-renewing resident macrophages are deleted by LysM-Cre during fetal development. In neutrophils, *Ncf2* and *Cybb* are expressed early in their differentiation (53), and some could be produced before S100A8- or LysM-Cre-mediated gene deletion is complete. Nevertheless, these mice allowed us to interrogate how reduced but not absent NOX2 neutrophil activity impacts host responses. Table 1 summarizes NOX2 activity in among the different mouse genotypes. The majority of our studies compared $Ncf2^{LysMCre}$ and $Ncf2^{S100A8Cre}$ mice, but we also used double-floxed $Ncf2Cybb^{S100A8Cre}$ mice in some experiments.

Responses to infectious *A. fumigatus* conidia in mice with conditional NOX2 deletion

As NOX2 is an essential effector in the host response to AF, we examined whether conditional deletion of NOX2 activity would differentially impair control of inhaled conidia. CGD mice show markedly increased susceptibility compared to immunocompetent mice, with progressive neutrophilic lung infiltrates containing hyphae (16, 38, 54, 55). Since $Ncf2^{LysMCre}$, $Ncf2^{S100A8Cre}$ and $Ncf2Cybb^{S100A8Cre}$ mice all have residual neutrophil NOX2 activity, we reasoned that the control of AF would display more modest defects compared to CGD mice. As using mortality as an endpoint could require large numbers of mice to achieve significant differences, we instead compared responses in the first few days following conidia challenge.

Variation among AF strains can influence the host response in both wild-type and immunosuppressed mice (56–59). The widely used AF293 strain is considered less virulent because of slower germination and reduced induction of pro-inflammatory cytokines compared to CEA10, another commonly used strain (56, 59, 60). Our prior studies in CGD mice used AF10, which resulted in high mortality, even with administration of only a few hundred conidia into the lung (16, 55). AF10 is reported to be similar or even identical to CEA10 (61, 62). We compared AF10 and AF293 in $Ncf2^{KO}$ CGD mice. At 48 hours following challenge with 1-million conidia of either strain, $Ncf2^{KO}$ mice had multiple lung foci containing scattered hyphae (Figure S2A); hyphae were seen in almost all AF10 foci but in only ≈ 50% of foci in AF293-challenged mice. AF10 also induced greater inflammation, with significantly higher bronchoalveolar lavage (BAL) neutrophils and CXCL2, TNF-α, IL-1α and IL-1β levels (Figure S2B, C). Although both strains produced IPA in CGD mice, the results are consistent with differential virulence attributes, and thus we studied both AF10 and AF293 in selected experiments.

Early control of A. fumigatus following inhalation of conidia requires alveolar macrophage NOX2 activity—As sentinels of the airways, alveolar macrophages play a major role in early elimination of inhaled AF conidia, especially when inocula do not exceed AM numbers, estimated to be $\approx 5 \times 10^5$ in mice (63). We investigated whether early control of inhaled AF conidia in vivo was defective in $Ncf2^{LysMCre}$ mice, which lack NOX2 in 95% of AM. We challenged $Ncf2^{LysMCre}$ mice with 5×10^4 AF293 conidia, along with mice with WT NOX2 activity, global knockout of NOX2, or neutrophil-specific reduction in NOX2 for comparison (Figure 2A). As recruited neutrophils can ingest and kill conidia, we also

examined the impact of anti-Ly6G-mediated neutrophil depletion (18, 49) initiated 24 hours prior to challenge, which significantly reduced peripheral blood neutrophils at the time of challenge (Figure 2B).

To assess responses, we analyzed BAL fluid at 24 hours-post-infection (hpi). Notably, BAL samples from isotype-treated *Ncf2*^{LysMCre} mice lacking AM NOX2 had significantly higher numbers of neutrophils and hyphae compared to *Ncf2*$^{fl/fl}$ or to *Ncf2*^{S100A8Cre} mice, where AM NOX2 activity is intact (Figure 2C, D). This supports the importance of AM NOX2 to eradicate conidia before germination in vivo. BAL hyphae numbers were further increased in neutropenic *Ncf2*^{LysMCre} mice (Figure 2D), showing that reducing neutrophil assistance further unmasks the conidiacidal defect in NOX2-deleted AM. Neutropenia in *Ncf2*^{LysMCre} mice also led to a significant increase in BAL CFU compared to *Ncf2*$^{fl/fl}$ or to *Ncf2*^{S100A8Cre} mice and lung CFU compared to *Ncf2*$^{fl/fl}$ (Figure 2E). Longer hyphae were present in neutropenic WT and *Ncf2*^{LysMCre} mice compared to isotype-treated mice, a difference that was statistically significant for *Ncf2*^{LysMCre} (Figure 2F). This result is consistent with the importance of neutrophils to damage and kill hyphae. Representative examples of hyphae with different lengths in BAL cytospins from mice treated with either isotype or anti-Ly6G are shown in Figure 2G. Finally, to compare phagocytosis of conidia by AM among different mouse genotypes, we challenged mice with YFP-AF293, but ingestion by *Ncf2*^{LysMCre} mice was similar to others (Figure 2H). Taken together, these findings establish that alveolar macrophage NOX2 activity has a non-redundant role in vivo to prevent germination of inhaled conidia in the first 24 hours, which becomes even more important in the setting of neutropenia.

Impaired control of A. fumigatus following inhalation of larger numbers of conidia when NOX2 activity is reduced only in neutrophils—We also challenged mice with conditional deletion of NOX2 with much higher numbers (5 – 8 million) of either AF293 or AF10 conidia, a range where neutrophils are required to prevent IPA in WT mice and AM are dispensable (11). Mice were euthanized at 72 hpi, and appeared mildly ill, but weights and activity scores (39) were not significantly different among the study groups for either AF strain (not shown). For comparison, CGD mice challenged with 5 million AF10 conidia were very ill by 72 hpi, with lungs showing extensive inflammatory infiltrates containing hyphae (Figure S2D).

We first compared inflammation and fungal burden in response to AF293 (Figure 3A). *Ncf2*^{S100A8Cre} mice, where NOX2 activity is reduced 5-fold specifically in neutrophils, had significantly higher numbers of neutrophils and CFU in BAL and lung homogenates (Figure 3B–D) compared to *Ncf2*$^{fl/fl}$ mice with WT NOX2 or to *Ncf2*^{LysMCre} mice with only a two-fold reduction in neutrophil NOX2 activity. Pulmonary histology (Figure 3E) showed scattered peribronchial and alveolar infiltrates that were more prominent in *Ncf2*^{S100A8Cre} mice, and neutrophil-filled bronchi were seen in two of the seven mice in this group. AF hyphae by GMS-staining were not seen in *Ncf2*$^{fl/fl}$ or *Ncf2*^{LysMCre} samples, but were present within a neutrophil-filled airway in a *Ncf2*^{S100A8Cre} mouse (Figure 3E). Taken together, these results show increased inflammation with higher fungal burden in *Ncf2*^{S100A8Cre} mice at 72 hpi, consistent with delayed AF293 clearance by mice with reduced neutrophil NOX2 activity.

Next, we studied the response to AF10 (Figure 4A). We compared *Ncf2*$^{fl/fl,}$ *Ncf2*^{LysMCre}, *Ncf2*^{S100A8Cre} mice, as well as double-floxed *Ncf2Cybb*^{S100A8Cre} mice, which have ten-fold less NOX2 activity than WT. *Ncf2*^{LysMCre} mice were similar to *Ncf2*$^{fl/fl}$ mice, except for a modest but a significant increase in lung CFU. The S100A8Cre cohorts, with more greatly reduced neutrophil NOX2 activity, had significantly higher numbers of BAL neutrophils as well as BAL and lung CFU compared to *Ncf2*$^{fl/fl}$ mice (Figure 4B–D). Notably, inflammation and fungal burden were the highest in the *Ncf2Cybb*^{S100A8Cre} mice. BAL and lung CFU in the double-floxed S100A8Cre mice were also significantly greater than for the single-floxed S100A8Cre mice. Lung histology for all four genotypes showed scattered peribronchial and alveolar infiltrates (Figure 4E). Hyphae were not found in *Ncf2*$^{fl/fl}$ or *Ncf2*^{LysMCre} mice, but one *Ncf2*^{S100A8Cre} mouse had a few hyphae detected (Figure 4E). In the double-floxed *Ncf2Cybb*^{S100A8Cre} mice, lung infiltrates were the most prominent and hyphae were seen in all mice (Figure 4E). The presence of hyphae among *Ncf2Cybb*^{S100A8Cre} mice (7 of 7 mice) was significantly greater than for *Ncf2*^{S100A8Cre} (1 of 8 mice) ($p = 0.0014$). As for AF293, these results show that reduced NOX2 activity specifically in neutrophils impairs clearance of AF10 in proportion to NOX2 activity. The impact of neutrophil NOX2 may be greater following AF10 challenge, as lung CFU in *Ncf2*^{S100A8Cre} mice were ≈40-fold higher than WT compared to only ≈2-fold higher with AF293 challenge. Finally, *Ncf2Cybb*^{S100A8Cre} mice, which have the lowest neutrophil NOX2 activity, had the most extensive disease at 72 hpi, with hyphal forms in lung sections and significantly greater CFU.

Reduced neutrophil NOX2 activity further impairs control of A. fumigatus in mice immunosuppressed by cortisone acetate—Immunosuppression by corticosteroids is a well-recognized risk factor for developing invasive AF. In addition to suppressing transcription of pro-inflammatory cytokines that activate immune cells, corticosteroids compromise alveolar macrophage function and reduce their conidiacidal activity, in part by suppressing NOX2 ROS and LC3-mediated phagosome maturation (9, 10, 64–66). Neutrophil-mediated damage to AF hyphae and neutrophil NOX2 activity are also depressed by corticosteroid treatment (67, 68). Cortisone acetate-treated mice challenged with inhaled AF develop invasive infection characterized by accumulation of dysfunctional phagocytes surrounding only a few hyphae (65).

We investigated the impact of NOX2 conditional deletion in mice immunocompromised by cortisone acetate and challenged with AF293 conidia (Figure 5A). We used small numbers (5000–8000) of conidia, which produced little disease in the majority of corticosteroid-treated *Ncf2*$^{fl/fl}$ mice with WT NOX2 activity. We analyzed the mice seven days after inhalation of conidia (Figure 5B–D). In the cortisone acetate-treated *Ncf2*$^{fl/fl}$ cohort, 5 of 6 mice had normal lung histology, with few BAL neutrophils or AF cultured from the lung. One cortisone acetate-treated *Ncf2*$^{fl/fl}$ mouse developed a parenchymal abscess with numerous hyphae, accompanied by increased BAL neutrophils and lung CFU. In comparison, cortisone acetate-treated *Ncf2*^{LysMCre} and *Ncf2*^{S100A8Cre} cohorts (8 and 7 mice, respectively) had significantly higher numbers of BAL neutrophils and lung CFU (Figure 5B, C). AF was cultured from the lungs of all 13 conditional deletion mice for which CFU were available. Abscesses were present in three *Ncf2*^{LysMCre} and three *Ncf2*^{S100A8Cre}

mice, and focal infiltrates in others (Figure 5D). One *Ncf2*^{S100A8Cre} mouse died at day 4, and one *Ncf2*^{LysMCre} mouse died at day 7 where post-mortem analysis revealed extensive *Aspergillus* pneumonia (not shown). We conclude that a reduction in neutrophil NOX2 activity is sufficient to synergize with corticosteroid-mediated immunosuppression and significantly further impair the eradication of inhaled AF. In the *Ncf2*^{LysMCre} mice, absence of AM NOX2 may also enhance susceptibility.

Conditional deletion of NOX2 in macrophages and/or neutrophils differentially impacts responses to sterile inflammation

Increased neutrophilic inflammation even in the absence of infection is a hallmark of CGD (14), and NOX2-deficient macrophages and neutrophils may each contribute to dysregulated responses (18, 48). To separate the effects of immunoregulatory ROS from infection, we studied two different models of sterile inflammation to determine whether conditional NOX2 deletion could reveal cell-type specific roles.

Inhalation of the fungal particle zymosan produces increased acute lung inflammation in CGD mice, and is initiated by increased LTB4 production by CGD neutrophils (18). We therefore compared this response in mice with conditional deletion of NOX2 to CGD (*Ncf2*^{KO}) mice and mice with WT NOX2 (*Ncf2*$^{fl/fl}$). Mice were challenged with 20 μg zymosan (≈1–2 million particles) and analyzed after eighteen hours (Figure 6A). CGD (*Ncf2*^{KO}) mice had the highest numbers of inflammatory neutrophils in BAL (Figure 6B). *Ncf2*^{LysMCre} mice lacking alveolar macrophage NOX2 along with a partial reduction in neutrophil NOX2 activity had a small but significant increase in BAL neutrophils compared to *Ncf2*$^{fl/fl}$ mice (Figure 6B). *Ncf2*^{S100A8Cre} and *Ncf2Cybb*^{S100A8Cre} mice, which had a greater reduction in neutrophil NOX2 activity, had even higher numbers of BAL neutrophils, which were similarly elevated and more than two-fold higher compared to *Ncf2*$^{fl/fl}$ mice (Figure 6B). By histology (Figure 6C), *Ncf2*$^{fl/fl}$ mice had minimal lung inflammation and, rarely, very small neutrophil foci were seen in alveoli of *Ncf2*^{LysMCre} mice. However, S100A8Cre cohorts had scattered alveolar neutrophil infiltrates, albeit smaller in size than in *Ncf2*^{KO} mice. *Ncf2*^{S100A8Cre} neutrophils stimulated with LTB4 in vitro also produced significantly higher amounts of LTB4 compared to WT neutrophils, although not as elevated as *Ncf2*^{KO} neutrophils (Figure 6D). We conclude that reducing NOX2 activity specifically in neutrophils is sufficient to enhance acute zymosan-induced lung inflammation, and that even a partial loss of NOX2 activity suffices to increase production of the key inflammatory mediator LTB4.

CGD mice also have increased acute inflammation in DAMP (damage-associated molecular pattern)-induced tissue injury, as studied following intraperitoneal injection of periodate, a mild oxidizing agent (48). CGD macrophages release higher amounts of IL-1α in response to necrotic cells and are implicated in driving this augmented response. We therefore evaluated this model in mice with conditional NOX2 deletions. Of note, peritoneal inflammation induced by zymosan is not increased in *Ncf2*^{LysMCre} mice, which lack NOX2 in resident macrophages (40). However, at 72 hours following periodate-induced injury, *Ncf2*^{LysMCre} mice, similar to *Ncf2*^{KO} mice, displayed significantly increased leukocyte numbers in the peritoneal lavage and the fraction that were neutrophils, compared to

mice with WT NOX2 activity or with neutrophil-specific reduction of NOX2 (Figure 6E, F). Moreover, administration of an antibody blocking IL-1α significantly reduced the neutrophilic peritoneal inflammation in *Ncf2*$^{fl/fl}$, *Ncf2*^{KO} mice and *Ncf2*^{LysMCre} mice (Figure S3), consistent with importance of IL-1α as a key mediator of DAMP-induced inflammation. While we cannot rule out that the partial reduction in neutrophil NOX2 activity contributes to increased DAMP-induced inflammation in *Ncf2*^{LysMCre} mice, these results strongly implicate NOX2 activity in resident macrophages as critical for regulating the response to tissue injury.

Discussion

These results advance understanding into cell-specific functions of NOX2 in two different innate immune cells that are key effectors in the host response to inhaled AF. Analysis of mice with conditional genetic deficiency of NOX2 highlight distinct but complementary roles in alveolar macrophages and neutrophils. Prior studies on the importance of alveolar macrophage NOX2 for killing ingested conidia yielded conflicting results, likely related to different endpoints and methodologies (1). Here, we show that AM NOX2 has a non-redundant role to reduce germination of inhaled conidia in vivo and the early emergence of hyphae when conidia numbers are not overwhelming. Hyphae numbers in BAL at 24 hpi were significantly increased in *Ncf2*^{LysMCre} mice that lack AM NOX2 activity. Numbers of germinated hyphae increased still further in *Ncf2*^{LysMCre} mice made neutropenic, which illustrates the importance of cooperation between these two cell types for early control of inhaled AF conidia. Hyphae numbers at 24 hpi were little affected in *Ncf2*^{S100A8Cre} mice, where neutrophil NOX2 activity is even more impaired than in *Ncf2*^{LysMCre} mice. This is congruent with recent study showing that early non-oxidative killing of conidia by CGD neutrophils could protect CGD mice from IPA when additional neutrophils were pre-recruited to the airways by stimulation of Type I interferon (35). Our results also show that even with intact NOX2 in monocytes and macrophages, robust neutrophil NOX2 activity is critical to limit AF progression following inhalation of larger numbers of conidia that overload the capacity of AM or to smaller inocula when there is additional immunosuppression from corticosteroids. This is consistent with the recognized importance of neutrophil NOX2 activity to kill emerging hyphae (26, 28–30, 34). Impaired control of AF as a consequence of depressed neutrophil NOX2 activity was observed for two AF strains of differing relative virulence. Moreover, our results demonstrate experimentally for the first time that there is a “dose dependence” of neutrophil NOX2 activity to eliminate AF, which was defective even when neutrophil NOX2 activity was not completely absent, with fungal burden inversely proportional to the level of NOX2 activity.

Our study also identified non-overlapping roles of NOX2 in neutrophils and macrophages for limiting acute inflammation in response to fungal cell walls and to DAMPs, respectively. The results reinforce the importance of NOX2 activity to modulate immune responses, and show that in this context, NOX2 activity can act in a cell-specific manner and depend on the inciting agent. That acute inflammation elicited by fungal cell walls is regulated by neutrophil NOX2 activity is consistent with prior work demonstrating that increased production of LTB4 by CGD neutrophils instigates an over-amplified feed-forward loop to recruit neutrophils into the lungs following zymosan challenge. (18). An important result

was that even a partial reduction in NOX2 activity promoted lung inflammation induced by zymosan and increased neutrophil production of LTB4. While augmented recruitment of neutrophils, which are able to kill AF conidia by non-oxidative means, might be beneficial in NOX2-deficient hosts for controlling infection, ultimately, overexuberant inflammation can be detrimental and worsen disease outcomes.

In the clinical setting, there are many variables that can interact and lead to failed eradication of AF, especially for those patients that are not severely immunocompromised. As evidenced in the current study, NOX2 activity in macrophages and neutrophils are each important for the innate response to AF and if either is impaired, could contribute to a “net state of immunosuppression“ (3) that increases the risk of invasive or chronic aspergillosis. Corticosteroids are well-known to impair both neutrophil and macrophage NOX2 activity (9, 10, 64–68). Neutrophil NOX2 activity can also be depressed by intercurrent co-morbidities, including diabetes, liver disease, chronic alcohol use, chronic obstructive pulmonary disease, post-influenza infection, and with aging, which overlap risk factors for invasive aspergillosis (36, 69–73). Invasive aspergillosis was also discovered to be an unexpected complication in lymphoma patients being treated with the Bruton tyrosine kinase (BTK) inhibitor ibrutinib (3). While the importance of BTK in lymphocytes is well understood, little is known about its role in innate immune cells. However, a recent study found that human neutrophils treated with ibrutinib show impaired killing of AF hyphae in vitro (74). Influenza infection is another risk factor for IPA, and reduced neutrophil ROS post-influenza is reported in mice (70). Other than corticosteroids, little is known regarding how drugs or co-morbidities associated with invasive AF impact NOX2 function in alveolar macrophages, but may warrant investigation. Regardless, recognizing impaired NOX2 activity as a predisposing factor for aspergillosis may help in developing mitigating strategies for at-risk patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Tina McGrath for assistance with manuscript preparation, Hongjie Gu in the Division of Biostatistics at Washington University School of Medicine in St. Louis (WUSM) for assistance with statistical analysis, and Wandy Beatty and the Imaging Core of the Molecular Microbiology Department at WUSM. These studies used the WUSTL Mouse Embryonic Stem Cell Core (affiliated with the Siteman Cancer Center at the time the work was performed) for providing ES cell culture services, the Mouse Genetics Core at WUSM for their support with animal production and care, the Andrew M. and Jane M. Bursky Center for Human Immunology and Immunotherapy Programs (CHiiPs), and the Hope Center Alafi Neuroimaging Laboratory.

Grant Support:

This work was supported by grants from the National Institutes of Health (NIH) National Heart, Lung, and Blood Institute (R01HL140837 to M.C.D), NIH National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01AR072212 to M.C.D.), the Children’s Discovery Institute of Washington University and St. Louis Children’s Hospital (M.C.D.), National Institute of Allergy and Infectious Diseases (5R01AI150669-01A1 to N.P.K.), National Institute of General Medical Sciences (R35GM118027-01 to AH), and by a NIH Shared Instrumentation grant (S10 RR027552)

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Key Points

- Alveolar macrophage NOX2 limits germination of inhaled *A. fumigatus* conidia in mice
- Reduced neutrophil NOX2 activity promotes invasive AF and enhances inflammation

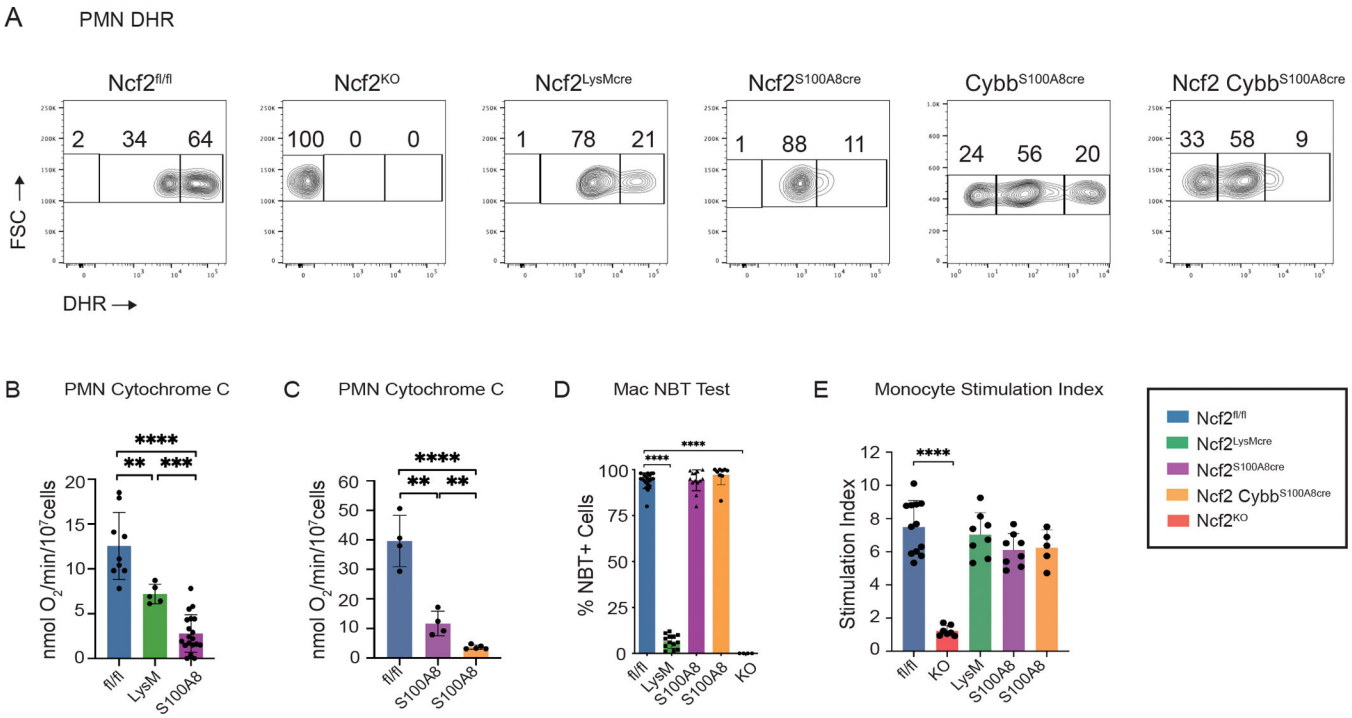

Figure 1. Conditional deletion of *Ncf2* and *Cybb* NOX2 subunits and characterization of expression and NOX2 activity in phagocytes.

(A**)** Dihydrorhodamine (DHR) assay for NOX2 activity in peripheral blood PMN. Percent of DHR-high, low DHR-low and DHR-negative cells as indicated in *Ncf2*$^{fl/fl}$, *Ncf2*^{KO}, *Ncf2*^{LysMcre}, *Ncf2*^{S100A8Cre,} *Cybb*^{S100A8Cre} and *Ncf2Cybb*^{S100A8Cre} mice. Representative samples shown. (B, C**)** Superoxide production by cytochrome C assay of marrow neutrophils from *Ncf2*$^{fl/fl}$, *Ncf2*^{LysMcre} and *Ncf2*^{S100A8Cre} mice (B) and *Ncf2*$^{fl/fl}$, *Ncf2*^{S100A8Cre} and *Ncf2Cybb*^{S100A8Cre} mice (C). (D) Percent of oxidase positive (NBT-positive) RPM and AM in the indicated mice. 200 cells scored per mouse. (E) Monocyte stimulation index calculated from DHR+ bone marrow monocytes in the indicated mice. Each data point represents one mouse with n≥4 per group. Bar graph data shown as mean ± SD. Student's 't' test was performed for comparisons between 2 groups and **P<0.01, ***P<0.001 ****P< 0.0001 were considered as significant. Data represents at least 2 independent sets of experiments.

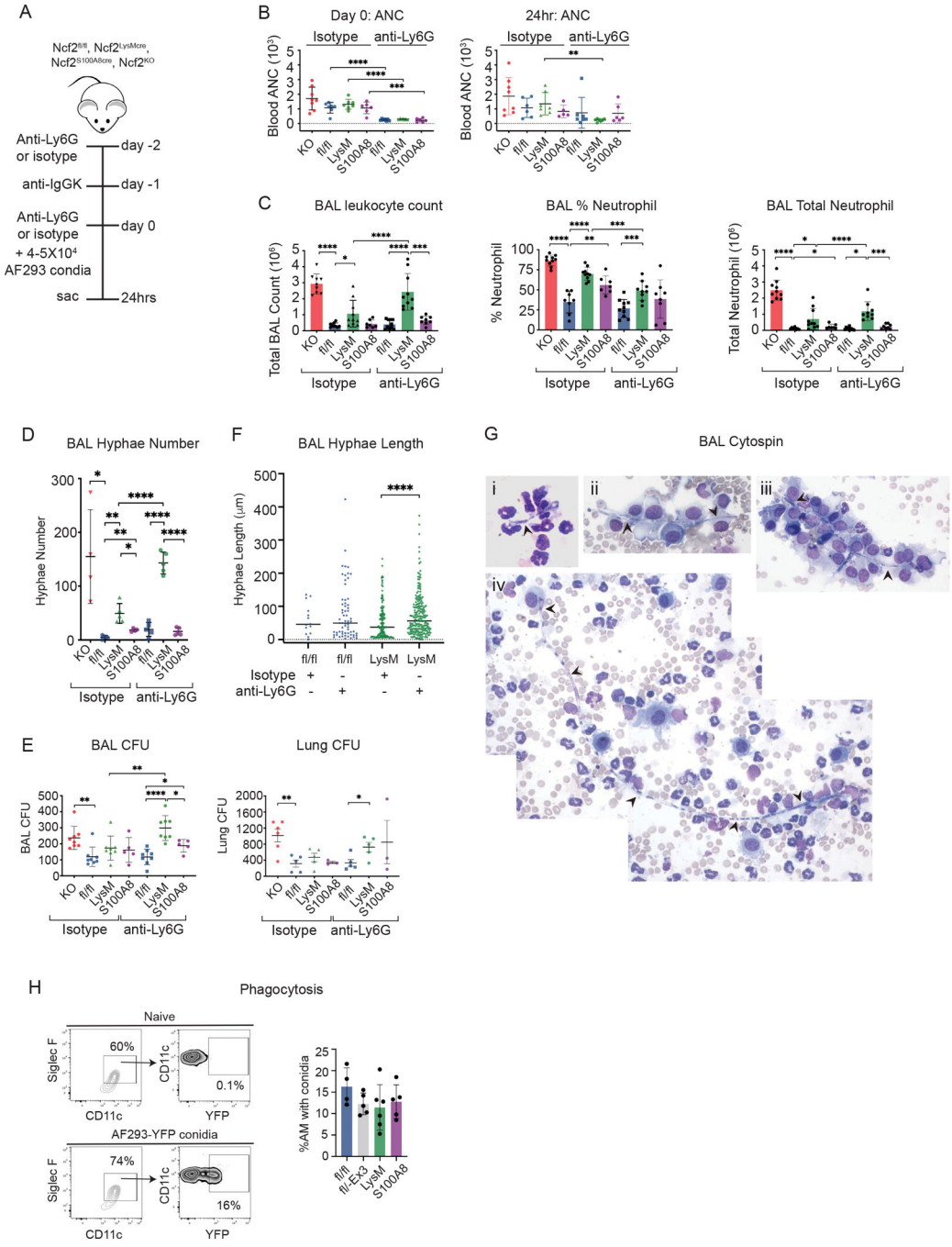

Figure 2. *A. fumigatus* germination at 24 hours following inhalation of conidia is increased in absence of alveolar macrophage NOX2, especially with neutropenia.

(A) Schema to assess germination into hyphae of inhaled AF293 conidia at 24 hpi without or with antibody-mediated neutrophil depletion. (B) Peripheral blood obtained by retro-orbital puncture was used to determine the absolute neutrophil count (ANC) at time of challenge (day 0) and at 24 hpi. (C) Total leukocyte counts from 1 ml BAL fluid. The percentage of neutrophils were identified by cytospin and used to calculate total BAL neutrophils. (D) Enumeration of hyphae in 1 ml BAL fluid. Each point represents an individual mouse. (E) Aspergillus CFU in 1 ml BAL fluid and in homogenate of right lung for the indicated

mice. (F) Hyphae length measured on BAL cytospins from indicated groups, combined data from at least three mice per group. (G) Photomicrographs of hyphae (indicated with black arrowheads). i. 28 μm hypha from *Ncf2*^{LysMCre} mouse treated with isotype (63X). ii. 44 μm hypha from *Ncf2*^{LysMCre} mouse treated with anti-Ly6G (100X). iii. 66 μm hypha from *Ncf2*$^{fl/fl}$ mouse treated with anti-Ly6G (100X); iv. tiled photomicrographs showing 244 μm hypha from *Ncf2*^{LysMCre} mouse treated with isotype (40 X). (H) Phagocytosis of YFP-labeled AF293 conidia by AM from indicated *Ncf2* genotypes at two hours after inhalation. *Ncf2*$^{fl/-Ex3}$ mice are heterozygous for a floxed and an Exon 3-deleted *Ncf2* allele. CD45^{+} AM were identified as SiglecF^{+}CD11c^{+} and gated for YFP using BFL1 channel to identify AM with ingested conidia. Data represents at least 2 independent sets of experiments with n≥4 per group. Graphical data shown as mean ± SD. Student's 't' test was performed for comparisons between 2 groups and *P<0.05, **P<0.01, ***P<0.001 ****P< 0.0001 were considered as significant. For (F), distribution of hyphae length is non-normal per the Kolmogorov-Smirnov test and statistical comparisons between 2 groups used the Mann-Whitney test.

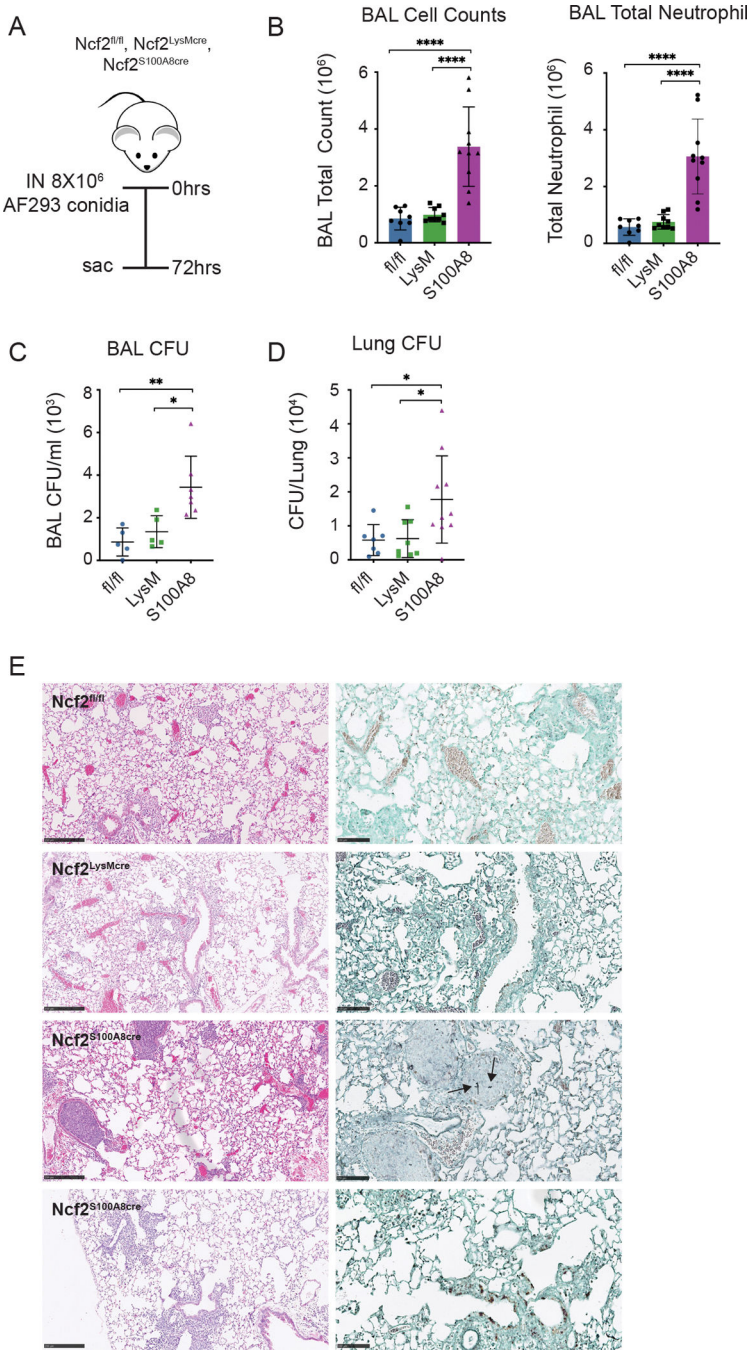

Figure 3. Reduced NOX2 activity in neutrophils increases *A. fumigatus* burden following challenge with AF293.

(A) Schema for AF293 challenge in indicated mice, with analysis at 72 hpi. (B) Total leukocyte and neutrophil counts from 1 ml BAL fluid. (C, D) Aspergillus CFU in 1 ml BAL fluid (C) and in homogenate of right lung (D) for the indicated mice. (E). Representative histology of lung sections for the indicated genotypes. From left to right, panels show H&E (bar 250 μm), and GMS (bar 100 μm). Arrows indicate hyphae. Data represents three independent sets of experiments with n≥5 per group. Graphical data shown as mean ± SD.

Student's 't' test was performed for comparisons between 2 groups and *P<0.05, **P<0.01, ***P<0.001 ****P< 0.0001 were considered as significant.

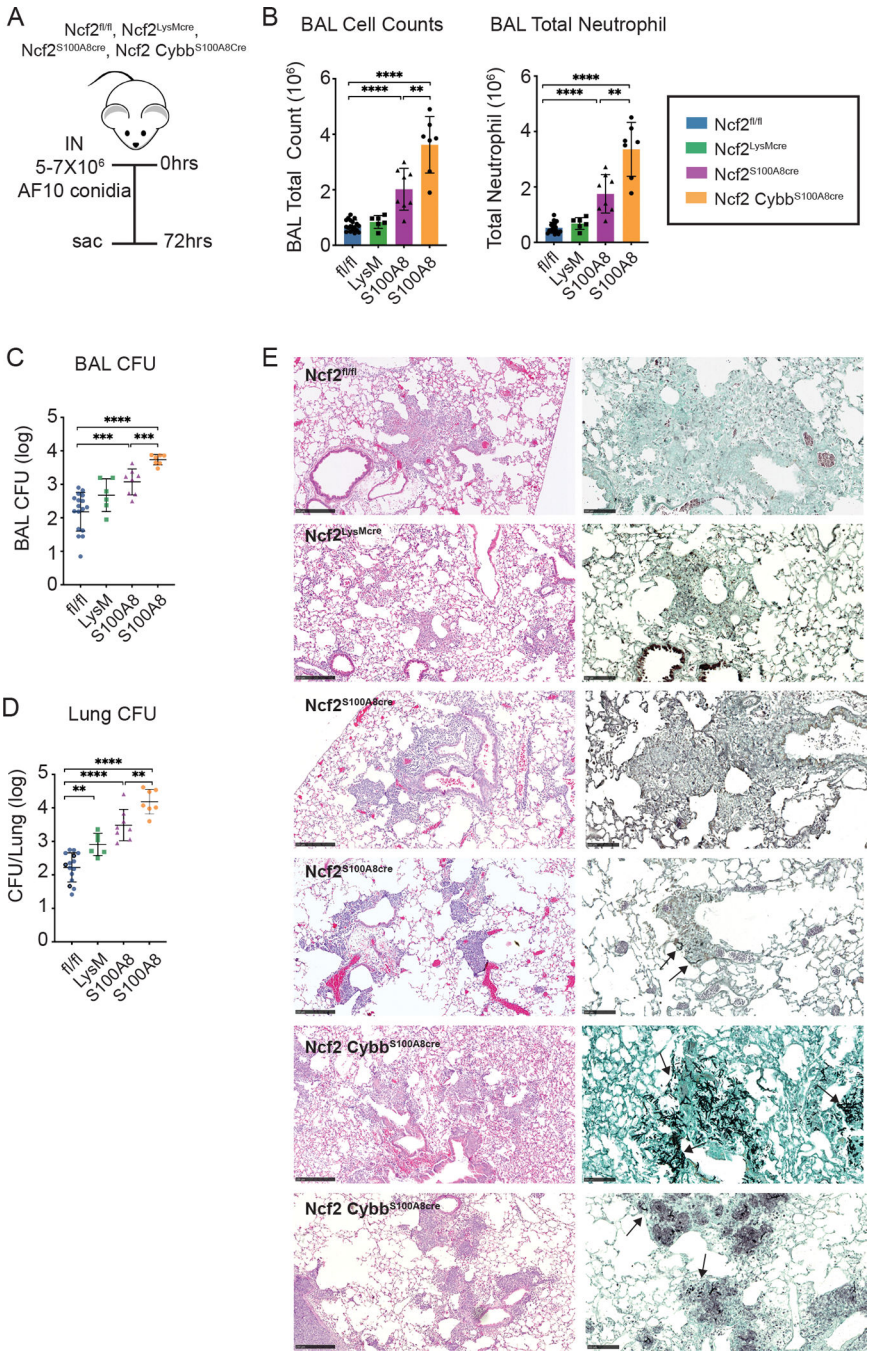

Figure 4. Reduced NOX2 activity in neutrophils increases *A. fumigatus* burden following challenge with AF10 conidia.

a) Schema for AF10 challenge in *Ncf2*$^{fl/fl}$, *Ncf2*^{LysMCre}, *Ncf2*^{S100A8Cre} and *Ncf2Cybb*^{S100A8Cre} mice, with analysis at 72 hpi. B) Total leukocyte and neutrophil counts from 1 ml BAL fluid. (C, D) Aspergillus CFU in 1 ml BAL fluid (C) and in homogenate of right lung (D) for the indicated mice. (E). Representative histology of lung sections for the indicated genotypes. From left to right, panels show H&E (bar 250 μm), and GMS (bar 100 μm). Arrows indicate hyphae. Data represents 2 – 4 independent sets of experiments with n≥4 per group. Graphical data shown as mean ± SD. Student's 't' test was performed for

comparisons between 2 groups and *P<0.05, **P<0.01, ***P<0.001 ****P< 0.0001 were considered as significant.

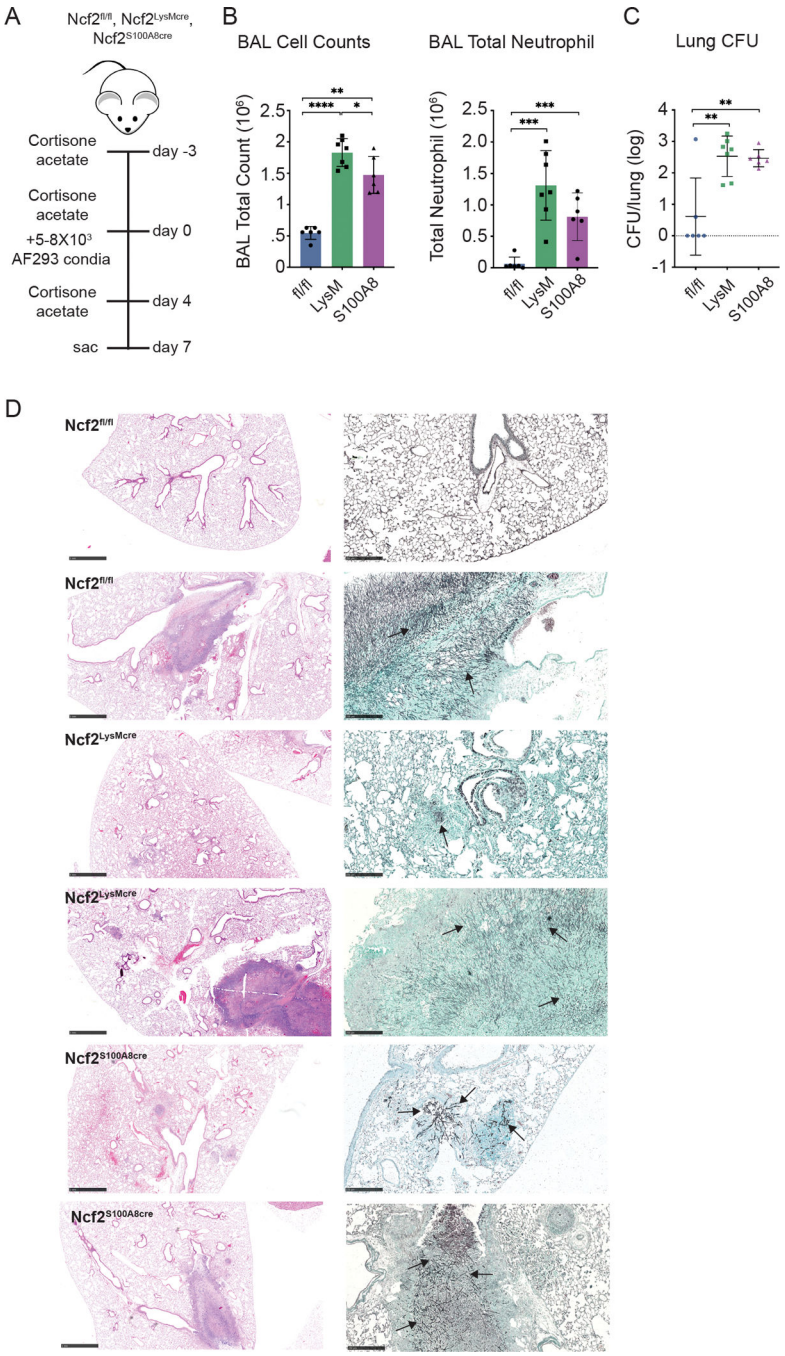

Figure 5. Reduced neutrophil NOX2 activity impairs control of *A. fumigatus* in corticosteroid-treated mice.

a) Schema for AF293 challenge of cortisone acetate-treated *Ncf2*$^{fl/fl}$, *Ncf2*^{LysMCre}, and *Ncf2*^{S100A8Cre} mice, with analysis at 7 days after challenge. B) Total leukocyte and neutrophil counts from 1 ml BAL fluid. (C) Aspergillus CFU in homogenate of right lung for the indicated mice. (D) Representative histology of lung sections for the indicated genotypes. From left to right, panels show H&E (bar 1 mm), and GMS (bar 100 μm). Arrows indicate hyphae.

Data represents 3 independent sets of experiments with n≥5 per group. Graphical data shown as mean ± SD. Student's 't' test was performed for comparisons between 2 groups and *P<0.05, **P<0.01, ***P<0.001 ****P< 0.0001 were considered as significant.

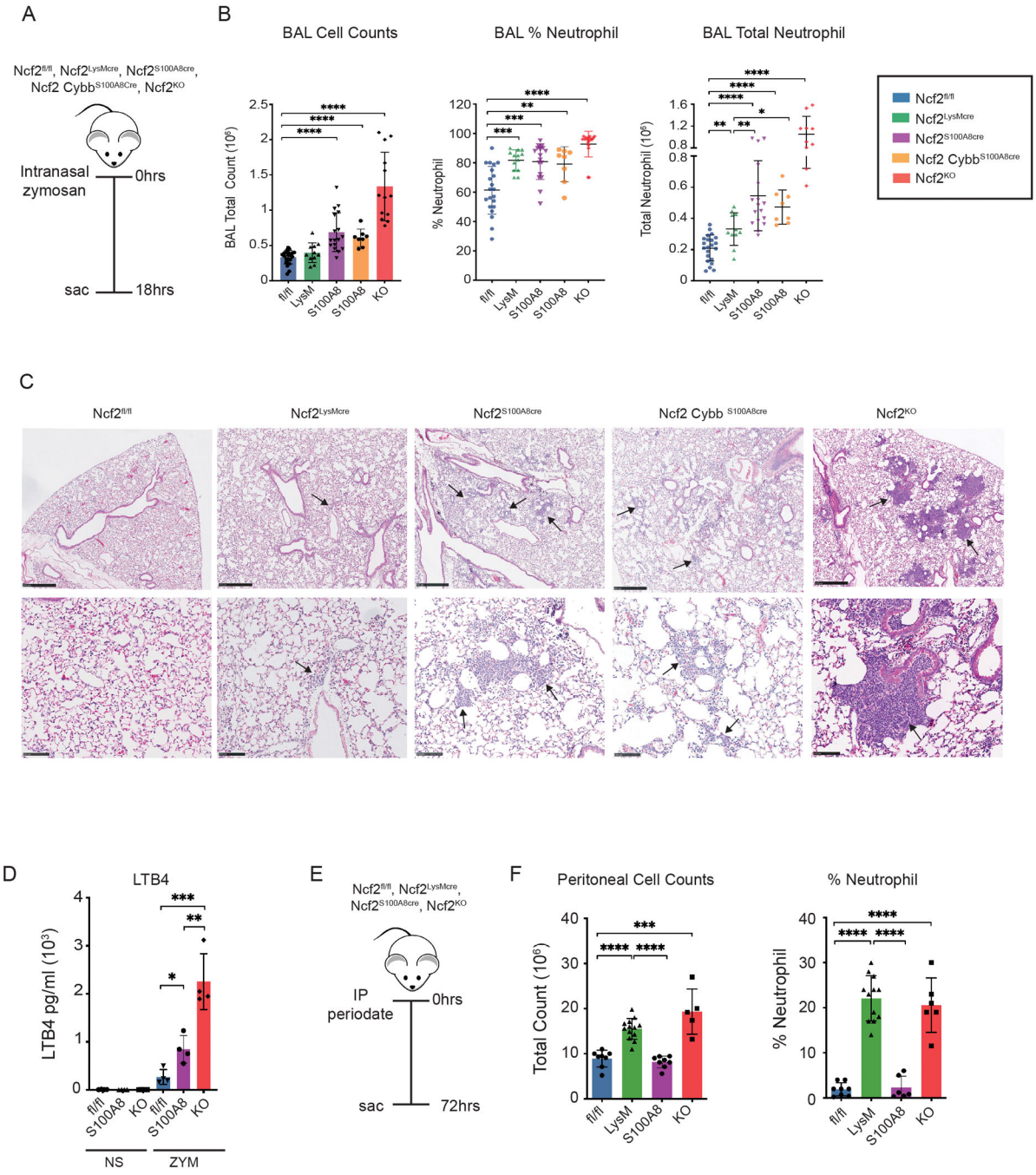

Figure 6. Sterile inflammation is increased in conditional NOX2-deleted mice and differentially dependent on neutrophil or macrophage NOX2 activity.

(A) Schema for zymosan-induced lung inflammation in indicated mice. Mice were challenged with 20 μg zymosan IN and studied after 18 hours. (B) Total leukocyte counts from 3 ml BAL fluid. The percentage of neutrophils were identified by cytospin and used to calculate total BAL neutrophils. (C) Representative histology of H&E-stained lung sections for the indicated genotypes. Scale bar for top row of panels is 500 μm and 100 μm for the lower panels. Arrows indicate neutrophil foci. (D) LTB4 ELISA from supernatants obtained from purified bone marrow neutrophils incubated for one hour either with no

stimulus (NS) or with zymosan (1 neutrophil:2 zymosan particles). *Ncf2*$^{fl/fl}$, *Ncf2*^{S100A8Cre} and *Ncf2*^{KO} mice were studied as indicated. (E) Schema for periodate-induced peritonitis in indicated mice and (F) peritoneal lavage cell counts and % neutrophils at 72 hours following IP injection of periodate. Data represents 3 independent sets of experiments with n≥4 per group. Graphical data shown as mean ± SD. Student's 't' test was performed for comparisons between 2 groups and *P<0.05, **P<0.01, ***P<0.001 ****P< 0.0001 were considered as significant.

Table 1.

Summary of mouse genotypes and NOX2 activity*

Genotype	Resident Macrophage NOX2	Neutrophil NOX2	Monocyte NOX2
Ncf2 **fl/fl**	WT	WT	WT
Ncf2 **LysMCre**	5%	50%	WT
Ncf2 **S100A8Cre**	WT	20%	WT
Ncf2Cybb **S100A8Cre**	WT	10%	WT
Ncf2 **KO**	0	0	0

* For resident alveolar and peritoneal macrophages, the percentage of macrophages that were nitroblue tetrazolium-positive following stimulation with serum-opsonized zymosan is shown. Wild-type (WT) corresponds to ≥95% nitroblue tetrazolium-positive. For neutrophils, the percent reduction of NOX2 activity compared to wild-type neutrophils is shown, as determined using the cytochrome C assay. Monocyte NOX2 activity was determined by flow cytometry and was at WT levels, expect for *Ncf2*^{KO}