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Absence of phagocyte NADPH oxidase 2 leads to severe inflammatory response in lungs of mice infected with *Coccidioides*☆

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ABSTRACT

Production of reactive oxygen species (ROS) resulting from phagocytic NADPH oxidase (NOX2) activity has been reported to contribute to host defense against numerous microbial pathogens. In this study we explored the role of NOX2 production in experimental coccidioidomycosis, a human respiratory disease caused by a soil-borne fungal pathogen. Activated and non-activated macrophages isolated from either *NOX2*$^{-/-}$ knock-out or wild type (WT) mice showed comparable ROS production and killing efficiency *in vitro* when infected with parasitic cells of *Coccidioides*. Both mouse strains also revealed similar fungal burden in their lungs and spleen at 7 and 11 days after intranasal challenge with *Coccidioides* spores, although the *NOX2*$^{-/-}$ mice died earlier than the WT strain. Immunization of the *NOX2*$^{-/-}$ and WT mice with a live, attenuated vaccine strain of *Coccidioides* also resulted in comparable reduction of the fungal burden in both lungs and spleen. These combined results initially suggested that NOX2 activity and ROS production are not essential for protection against *Coccidioides* infection. However, the reduced survival of non-vaccinated *NOX2*$^{-/-}$ mice correlated with high, sustained numbers of lung-infiltrated neutrophils on days 7 and 11 postchallenge, an expansion of the regulatory T cell population in infected lungs in the knock-out mice, and elevated concentrations of pro-inflammatory cytokines and chemokines in lung homogenates compared to infected WT mice. Although NOX2-derived ROS appeared to be dispensable for both innate and acquired immunity to pulmonary *Coccidioides* infection, evidence is presented that NOX2 production plays a role in limiting pathogenic inflammation in this murine model of coccidioidomycosis.

1. Introduction

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), represent important constituents of the arsenal of chemical defenses produced by the mammalian host to combat microbial pathogens [1,2]. Phagocytic leukocytes generate ROS by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2), which consists of two membrane-associated components (gp91phox and p22phox) and four cytosolic regulatory subunits (p40phox, p47phox, p67phox, and Rac2), where phox represents phagocyte oxidase [3]. Rac2 is a member of a family of GTPases involved in regulating the activation of protein kinases. Assembly of the NADPH complex following phosphorylation of the cytosolic subunits is essential for respiratory burst oxidase activity in neutrophils and macrophages. Chronic granulomatous disease (CGD) is an inherited condition in which a deficiency of NOX2 results in the inability of phagocytes to generate microbicidal superoxide anion and its metabolites. Patients with CGD have recurrent, life-threatening bacterial and fungal infections as well as chronic inflammatory diseases due to dysregulated inflammatory pathways [4]. Introduction of a mutation in the gp91phox gene in mice has been shown to result in loss of function of the 91 kDa membrane glycoprotein and inability of phagocytes to generate superoxide by the respiratory burst oxidase complex [5]. This was further shown to result in a significant increase in susceptibility to microbial infection [6] and onset of chronic granulomatous disease [7]. Loss of NOX2 activity in this murine model of CGD has been shown to be associated with exceptional vulnerability to

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experimental infection with *Mycobacterium tuberculosis* [8], *Acinetobacter baumannii* [9], *Chlamydia trachomatis* [10], *Candida albicans* [11], and *Aspergillus fumigatus* [12]. Coccidioidomycosis, also referred to as San Joaquin Valley fever, is a systemic fungal disease typically contracted by inhalation of airborne spores of *Coccidioides immitis* or *Coccidioides posadasii*. In spite the genetic diversity revealed by comparative genomic sequence analyses of the two species, laboratory studies have shown no significant difference between their virulence in mice [13]. A peculiarity of this fungal pathogen is that the mature parasitic cells (spherules) are too large to be engulfed by phagocytes (typically >100 μm diam.), which poses a potential problem for host defense. Coccidioidomycosis is endemic to regions of Southwestern United States, Northern Mexico, and certain areas of Central and South America [14]. It is estimated that more than 100,000 new infections of *Coccidioides* occur annually in the United States alone, but the majority of these are asymptomatic or result in self-limited pneumonia. On the other hand, approximately 40% of infected individuals develop symptomatic disease which can progress to involve extrapulmonary organs and become life-threatening [15]. Vaccine immunity against coccidioidomycosis has been reported to be primarily mediated by $CD4^+$ and $CD8^+$ T cell activation [16] with concomitant stimulation of T helper (Th)1, Th2 and Th17 signal pathways [17]. Macrophages are believed to be the pivotal effector cells in the host innate response to infection. The general paradigm for granulomatous diseases applies to coccidioidomycosis: activated T-lymphocytes secrete cytokines, which activate macrophages, inducing the formation of a granuloma that kills or contains the organism [18]. However, the nature of the interactions between macrophages and *Coccidioides* is still not well defined. We recently demonstrated that primary macrophages isolated from genetically-engineered mice lacking inducible nitric oxide synthase (iNOS) activity and nitric oxide (NO) production revealed phagocytic and fungicidal activities which were comparable to macrophages isolated from wild type mice. We concluded that the observed *in vitro* fungicidal response of host phagocytes was not dependent on NO production [19]. Although phagocyte secretion of ROS has been implicated in host defense against certain fungal infections [4,11], a recent study has shown that NOX2 is dispensable in murine defense against *Coccidioides* [20]. In this study, we have further confirmed that NOX2 activity is not essential for macrophage killing of *Coccidioides* parasitic cells, but plays a potentially important role in limiting pathogenic inflammation of infected lung tissue.

2. Results

2.1. Primary macrophages isolated from WT and $NOX2^{-/-}$ mice showed comparable phagocytic and fungicidal activities against spherule initials in vitro

Primary macrophages isolated from peritoneal fluids of WT or $NOX2^{-/-}$ mice were incubated for 3 h in the presence of live spherule initials of the virulent strain of *C. posadasii*. In each case the macrophages were either activated with IFN-γ plus LPS or untreated (controls). A near-identical phagocytic index was recorded for both activated and non-activated peritoneal macrophages isolated from the two mouse strains (Fig. 1A; data for non-activated macrophages not shown). Intracellular killing was evaluated in co-cultures of the same host and fungal cells after incubation for 12 h. The residual numbers of viable fungal cells (CFUs) engulfed by activated or non-activated macrophages derived from the WT and $NOX2^{-/-}$ mouse strains showed no statistically significant difference when results of three separate experiments were compared (Fig. 1B). These data suggest that macrophage NOX2 activity *in vitro* is not essential for intracellular killing of *Coccidioides* spherule initials.

2.2. ROS production by activated peritoneal macrophages was not altered by Coccidioides infection

To test whether levels of macrophages production of highly reactive oxygen species were altered upon *Coccidioides* infection, relative amounts of hROS detected in co-cultures of host and fungal cells were measured after 12 h of incubation (Fig. 1C). Activated WT macrophages produced significantly higher and comparable concentrations of hROS in the presence or absence of spherule initials compared to non-activated macrophages. The basal levels of hROS produced by non-activated $NOX2^{-/-}$ macrophages were comparable to that of IFN-γ+LPS-activated $NOX2^{-/-}$ macrophages, and showed no difference in hROS production in the presence or absence of spherule initials. These results indicate that *Coccidioides* spherule initials do not induce ROS production by primary murine macrophages even when the phagocytes are activated prior to exposure to the live fungal cells.

2.3. $NOX2^{-/-}$ mice challenged intranasally with Coccidioides died earlier than WT mice but showed comparable fungal burden

To assess the effect of phagocytic NOX2 activity on the outcome of pulmonary coccidioidomycosis, we compared the survival plots of vaccinated or non-vaccinated WT and $NOX2^{-/-}$ mice challenged intranasally with a potentially lethal inoculum of *C. posadasii* spores. Vaccinated WT and $NOX2^{-/-}$ mice showed 100% survival after 25 days postchallenge (data not shown). The mean survival times for the non-vaccinated WT and $NOX2^{-/-}$ mice was 12.5 and 10.5 days, respectively (Fig. 2), which was determined to be a statistically significant difference ($p = 0.0012$). On the other hand, comparison of the fungal burden between lungs of separate, non-vaccinated groups of WT and $NOX2^{-/-}$ mice, or vaccinated groups of the same two mouse strains after 7 and 11 postchallenge revealed no significant difference (Fig. 3A,B). Viable fungal cells were not detected in the spleen at 7 days postchallenge, but comparable CFUs in spleen homogenates were recorded between non-vaccinated WT and $NOX2^{-/-}$ mice, and between vaccinated mice of the same two strains at 11 days after intranasal challenge (Fig. 3C). NOX2 activity appeared to have no effect on the fungal burden, irrespective of whether the mice were vaccinated or non-vaccinated.

2.4. Absence of NOX2 resulted in increased numbers of pulmonary leukocytes after intranasal challenge with Coccidioides spores compared to WT mice

Leukocyte recruitment into the lungs of *Coccidioides*-infected WT and $NOX2^{-/-}$ mice was compared by flow cytometry. An increase in total number of lung-infiltrated leukocytes (LIL; $CD45^+$ cells) was observed in $NOX2^{-/-}$ mice compared to WT mice at both 7 and 11 days postchallenge (Fig. 4). The actual numbers of LIL detected in the lungs of $NOX2^{-/-}$ and WT mice at day 7 were $17.4 \pm 0.93 \times 10^6$ versus $2.46 \pm 0.21 \times 10^6$ ($p < 0.05$) and at day 11 postchallenge were $43.65 \pm 6.0 \times 10^6$ versus $14.27 \pm 1.13 \times 10^6$ cells ($p < 0.001$), respectively.

2.5. Increased infiltration of PMNs and expansion of the $CD4^+CD25^+FoxP3^+$ Treg population were observed in lungs of Coccidioides-infected $NOX2^{-/-}$ mice compared to WT mice

We further characterized the subpopulations of leukocytes that had infiltrated the lungs of *Coccidioides*-infected WT and $NOX2^{-/-}$ mice on days 7 and 11 postchallenge by flow cytofluorometry. A striking difference and sustained high numbers of neutrophils (PMNs) were observed in the lungs of $NOX2^{-/-}$ mice compared to