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Oxidative burst and neutrophil elastase contribute to clearance of *Aspergillus fumigatus* pneumonia in mice

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ABSTRACT

Polymorphonuclear neutrophils (PMN) are important for the control of invasive aspergillosis (IA), a major threat to immunocompromised individuals. For clearance of *Aspergillus fumigatus* infections, PMN employ their potent oxidative and non-oxidative mechanisms. To clarify the relative contribution of these mechanisms, we analyzed $p47^{phox-/-}$, $gp91^{phox-/-}$ and elastase (ELA) deficient mice (ELANE) after intratracheal infection with *A. fumigatus*. Infected $p47^{phox-/-}$ and $gp91^{phox-/-}$ mice died within 4 days and had a significant higher fungal burden in the lungs compared to wild-type controls. Interestingly, the survival of ELANE mice after infection was unimpaired suggesting that ELA is not essential here. Nevertheless, *A. fumigatus* clearance was delayed in ELANE mice indicating a partial contribution of ELA to fungal immunity. Comparing $p47^{phox-/-}$, $gp91^{phox-/-}$ or ELANE mice for PMN activation and recruitment to the lungs, we were unable to detect significant differences *in vitro* or *in vivo* among mutant or wild-type strains suggesting intact PMN functionality of basic effector mechanisms. Fungal killing *in vitro* by ELA deficient PMN was comparably reduced as in $p47^{phox-/-}$ and $gp91^{phox-/-}$ deficient PMN corroborating the importance of oxidative and non-oxidative PMN mechanisms for the control of fungal outgrowth. Taken together, this suggests that intact oxidative as well as non-oxidative PMN effector functions are highly relevant for the control of *A. fumigatus* infections *in vitro* and *in vivo*. While ELA contributes to clearance of *A. fumigatus*, the oxidative functions are essential for survival.

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Introduction

Invasive aspergillosis (IA) is caused by *Aspergillus fumigatus* (*A. fumigatus*) and a major threat to immunocompromised patients (Bodey et al., 1992; Kuhns et al., 2010). Patients requiring immunosuppressive drugs for organ or allogeneic hematopoietic stem cell transplantation (HSCT) (Bodey et al., 1992; Cornely, 2008; Cornely et al., 2009; Kuhns et al., 2010). Despite the availability of potent antifungal drugs, IA remains a significant problem in the daily clinical patient care (Cornely et al., 2009).

Prolonged neutropenia is a major risk factor for IA (Dillon et al., 2006; Post et al., 2007) underscoring the importance of polymorphonuclear neutrophils (PMN) in the innate host defense against fungal pathogens. Antifungal activities of PMN include the killing of germinating *Aspergillus* conidia (Bonnett et al., 2006; Diamond and Clark, 1982; Kuhns et al., 2010; Levitz and Farrell, 1990; Post et al., 2007; Roilides et al., 1990) as well as hyphae (Cornely et al., 2009; Diamond and Clark, 1982; Sheppard et al., 2005). While the size of hyphae may prevent phagocytosis by PMN, they are still in direct contact and cause hyphal damage both by oxidative and non-oxidative mechanisms (Braedel et al., 2004; Diamond and Clark, 1982; Dillon et al., 2006; Klebanoff, 2005; Post et al., 2007; Roilides et al., 1990). Oxidative PMN effector functions depend on the assembly of the phagocytic NADPH oxidase, a multi component complex that consists of the cytoplasmic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G-proteins Rac2, Cdc42 and p29 peroxiredoxin (Kuhns et al., 2010; Sheppard et al., 2005). This enzyme complex converts molecular oxygen to superoxide anion that is further converted into cytotoxic metabolites, including H_2O_2, hydroxylanion and peroxinitrite. In PMN,

Abbreviations: BAL, bronchoalveolar lavage; CFU, colony forming units; CGD, chronic granulomatous disease; ELA, neutrophil elastase; IA, invasive aspergillosis; NADPH, nicotinamide dinucleotide phosphate; NET, neutrophil extracellular trap; PMN, polymorphonuclear neutrophils.

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myeloperoxidase catalyzes the conversion of H_2O_2 to hypochlorous acid, which is highly microbicidal (Klebanoff, 2005; Pollock et al., 1995). Chronic granulomatous disease (CGD) patients frequently suffer from opportunistic IA infections which is linked to impaired oxidative functions of PMN (Jackson et al., 1995; Kuhns et al., 2010). This is also reconciled by mouse models for X-linked or autosomal CGD in gp91$^{phox-/-}$ (Madan et al., 1997; Pollock et al., 1995) or p47$^{phox-/-}$ mice (Cornish et al., 2008; Jackson et al., 1995). Beyond this, p47$^{phox-/-}$ macrophages phagocytize conidia in a regular manner, but are unable to kill them (Levitz et al., 1986; Philippe et al., 2003).

PMN also utilize non-oxidative mechanisms to mediate antifungal activities (Park and Mehrad, 2009; Zarember et al., 2007). Primary as well as secondary granule contents are fungistatic, *e.g.* defensins (Levitz et al., 1986; Reeves et al., 2002) or lactoferrin (Reeves et al., 2002; Tkalcevic et al., 2000; Zarember et al., 2007). Also serine proteases, as another secondary granule constituent, contribute to microbial killing by PMN (Papayannopoulos et al., 2010; Reeves et al., 2002). In this instance, PMN elastase (ELA) has been also implicated in host defense against microbial or fungal pathogens using cathepsin G or ELA deficient mice (ELANE) (Bianchi et al., 2011; McCormick et al., 2010; Reeves et al., 2002; Tkalcevic et al., 2000). In addition, ELA are an important component of neutrophil extracellular traps (NET) (Jackson et al., 1995; Papayannopoulos et al., 2010) with a role in immunity against *A. fumigatus* (Bianchi et al., 2011; McCormick et al., 2010; Pollock et al., 1995).

While oxidative and non-oxidative PMN effector mechanisms clearly contribute to the immune response against *A. fumigatus*, the impact of distinct effector functions *in vivo* is only partially clear. Therefore we employed a mouse model of pulmonary IA utilizing gp91$^{phox-/-}$, p47$^{phox-/-}$ or ELANE mice to reveal the relative importance of various PMN effector mechanisms. Only in NADPH oxidase deficient mice, IA had a lethal course, while ELANE mice able to clear the infection. Evaluating distinct PMN effector functions *in vitro* or *ex vivo*, we were unable to detect significant differences among gp91$^{phox-/-}$, p47$^{phox-/-}$ or ELA deficient PMN. For fungal killing *in vitro*, we found that ELA and oxidative PMN functions were required for conidia killing. These results clearly suggest that oxidative and non-oxidative PMN effector mechanisms are involved in immunity against *A. fumigatus*, while a functional NADPH oxidase is required for survival.

Materials and methods

Materials

Glucose was from Carl Roth (Karlsruhe, Germany), Ketamin from Ratiopharm (Ulm, Germany) and Rompun 2% from Bayer Health Care (Leverkusen, Germany). PMA, XTT and coenzyme Q_0 was purchased from Sigma–Aldrich (Taufkirchen, Germany), WKYMVm (Merck Millipore, Darmstadt, Germany) and PE-labeled beads from Polyscience (Eppelheim, Germany). For flow cytometry, the following antibodies were used: PB-labeled anti-CD11b, FITC-labeled anti-CD45.2 and PE-labeled anti-Gr-1 (all from eBioscience, San Diego, CA, USA), FITC-labeled anti-CD62L (BioLegend, San Diego, CA, USA) or PECy7-labeled anit-CD62L (Becton Dickinson, Heidelberg, Germany). Cell counting beads were from Beckman-Coulter (Krefeld, Germany).

Mice

C57BL/6 mice at 8 weeks were obtained from the local animal facility of the University of Mainz. p47$^{phox-/-}$ mice (Jackson et al., 1995; Tkalcevic et al., 2000) and gp91$^{phox-/-}$ (Nox2 knockout) (Behnsen et al., 2007; Brakhage and Van den Brulle, 1995; Pollock et al., 1995) mice on C57BL/6 background were kindly provided by P. Wenzel (Center for Thrombosis and Hemostasis, University Medical Center Mainz, Germany). ELANE mice (Behnsen et al., 2007; Jahn et al., 1997; Tkalcevic et al., 2000) were on 129/Sv/Pas background. All animal procedures were performed in accordance with the institutional guidelines and approved by the responsible national authority (National Investigation Office Rheinland-Pfalz, Appoval ID: AZ 23 177-07/G11-1-034).

Fungal strains and cultivation conditions

The *A. fumigatus* strain ATCC 46645 was kindly provided by M. Gunzer (Molecular Immunology, University of Duisburg-Essen, Germany) and cultured in *Aspergillus* minimal medium (AMM) with 1% (w/v) glucose as carbon source as described previously (Behnsen et al., 2007; Brakhage and Van den Brulle, 1995; Hasenberg et al., 2011). Briefly, conidia were incubated on AMM agar plates for four days at 37 °C and 5% CO_2. For preparation of spore suspensions, plates were washed with sterile water containing a small amount of glass pearls (Behnsen et al., 2007; Jahn et al., 1997; Rowe et al., 2006). The obtained spore suspension was filtered twice through a sterile 40 μm nylon mesh and stored at 4 °C in sterile water until required.

Mouse model of IA

Mice received 10^7 *A. fumigatus* conidia intratracheally (i.t.) as described in detail elsewhere (Gladigau et al., 2012; Hasenberg et al., 2011). Briefly, a 22 G indwelling venous catheter (B. Braun AG, Melsungen, Germany, Vasofix Braunüle) was gently inserted into the trachea of anesthetized animals. 100 μl spore-suspension or sterile water was added into the catheter and inhaled without additional help. To enhance dispersion of fungal suspension in the lungs, mice were ventilated mechanically for two minutes using an animal respirator (MiniVent, Hugo Sachs, March-Hugstetten, Germany, 250 breaths/min, 300 μl/breath). After infection, mice were monitored for 14 days to determine overall survival. The severity of systemic infection was examined daily using a scoring system adopted from a Graft-*versus*-Host disease model (Rowe et al., 2006; Weber et al., 2013) including 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity. Mice with severe symptoms determined by clinical scores equal or greater than 6, were immediately euthanized by CO_2, as required by the institutional animal ethics guide lines and the day subsequent to death determined as the following day. Where indicated, PMN depletion was induced by i.p. injection of anti-Gr-1 antibody (150 μg, day-1, clone RB6-8C5 or clone 1A8).

Lung histology

After euthanasia, mouse lungs were filled with 10% formalin *via* the trachea. Paraffin embedded blocks were prepared, sections were taken and stained with hematoxylin and eosin (H&E) to assess inflammatory responses and Grocott-Gomori silver stain to visualize fungi. Tissues were examined by microscope for pulmonary injury, vascular invasion, and *A. fumigatus* hyphae. All slides were reviewed by the pathologist (A.K.) using 40× or 200× magnification without formal morphometric analysis.

Analysis of blood and BAL fluid

Mice were sacrificed 24 h after infection. Blood samples were collected by retro-orbital incision and lungs were flushed with 1 ml PBS. The blood samples were analyzed by flow cytometry after a hypotonic lysis step. Total cell counts in the BAL fluid

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