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Original article

Decreased inflammatory response in Toll-like receptor 2 knockout mice is associated with exacerbated *Pneumocystis* pneumonia

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Abstract

Pneumocystis pneumonia (PcP) is marked by substantial inflammatory damage to the lung. We have found that Toll-like receptor 2 (TLR2) mediates macrophage inflammatory responses to *Pneumocystis* and hypothesized that TLR2 deficiency would lead to less severe inflammation and milder lung injury during PcP. Histopathology examination showed that TLR2−/− mice with PcP indeed exhibited milder pulmonary inflammation. TLR2−/− mouse lungs contained less TNF-α and displayed lower levels of NF-κB activation during PcP. However, TLR2−/− mice with PcP displayed increased severity in symptoms and organism burden. The increased organism burden is likely due to defects in protective mechanisms in TLR2−/− mice. mRNA levels of the inducible nitric oxide synthase and NADPH oxidase p47phox, as well as nitric oxide levels in the lungs, were decreased in TLR2−/− PcP mice. Taken together, this study shows that TLR2-mediated inflammatory responses contribute to a certain degree to the clearance of *Pneumocystis* organism in mice.

Keywords: *Pneumocystis*; Toll-like receptor 2; Inflammation

1. Introduction

Pneumocystis pneumonia (PcP) is the most common opportunistic disease in immunocompromised hosts, such as patients with AIDS [1]. *Pneumocystis* organisms that infect humans are now referred to as *Pneumocystis jirovecii* [2], and those that infect rats and mice are called *Pneumocystis carinii* and *Pneumocystis murina*, respectively [2,3]. Although there has been a significant decline in incidence of PcP due to the use of highly active antiretroviral therapy (HAART) [4], PcP remains a significant cause of morbidity and mortality in human immunodeficiency virus (HIV)-infected patients and a life-threatening disease in other immunocompromised patients.

Alveolar macrophages play an important role in the clearance of organism from the lungs, and the phagocytosis is mediated by interactions between the pathogen and various types of receptors. Several surface molecules on alveolar macrophages have been shown to interact with *Pneumocystis*, including Fc-gamma receptor [5], complement receptor [6], scavenger receptor [7], mannose receptor [8], and dectin-1 [9]. TLR2 is a pattern recognition receptor recognizing a large variety of ligands such as peptidoglycan, lipoprotein, lipopeptide, and zymosan [10]. Activated TLR2 transduces signals through a myeloid differentiation factor 88 (MyD88)-dependent pathway, inducing nuclear translocation of the transcription factor NF-κB [10]. NF-κB activation is crucial for the production of proinflammatory cytokines and chemokines that initiate host inflammatory response. We have recently demonstrated that TLR2 mediates the alveolar macrophage inflammatory response to *Pneumocystis* in vitro [11]. The role of TLR2 response against *Pneumocystis* in the host remains undefined.

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In this study, we show that TLR2 plays a major role in the inflammatory response to *Pneumocystis* infection in vivo.

2. Materials and methods

2.1. Mouse model of PcP

TLR2-deficient mice (TLR2−/−, C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Wild type C57BL/6 mice were obtained from Harlan (Indianapolis, IN). All mice were female, 6–8 weeks of age, and 18–20 g in weight at arrival. Animal studies were approved by the Indiana University Animal Care and Use Committee. Animals were housed in the Indiana University Laboratory Animal Resource Center, an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility. All mice were immunosuppressed by weekly intraperitoneal injection of 0.3 mg anti-CD4 mAb (clone GK1.5, Harlan, Indianapolis, IN). Flow cytometry analysis indicated that 0.3 mg of the antibody achieved 95% depletion of CD4+ cells after a single injection. This result was consistent with that of Zheng et al. [12]. Immunosuppressed mice were transtracheally instilled with 2×10^6 *P. murina* organisms. Immunosuppressed, non-infected mice were used as controls. Tetracycline was added to the drinking water (9.2 g/L) to prevent bacterial infections. Mice were sacrificed at 8 weeks after infection or when they displayed four of the following criteria: weight loss below 65% of arrival weight, ability to palpate bony structures such as the spine, dark appearance of the eyes, dried blood around the nares, extremely inactive and labored breathing.

2.2. Histopathology examination

Mice were anesthetized and sacrificed by cardiac exanguination. The lungs were fixed and embedded in paraffin. Histologic sections were stained with H&E and evaluated for the following features: perivascular and peribronchiolar mononuclear infiltration, neutrophil infiltration, foamy exudates, fibrosis, and effacement of alveolar structure. Pulmonary inflammation was graded blindly by two experienced microscopists using a semi-quantitative scale described by Rudmann et al. [13]. Scores of 1–5 were based upon grading of the entire lung tissue present on the sections. To determine the organism burden, lung sections were stained with the GMS silver staining kit (Sigma Chemical Co., St. Louis, MO) and examined under light microscope.

2.3. Single cell preparation from mouse lungs

Mice were anesthetized, and pulmonary vasculature was perfused via right ventricle with 10 ml cold Dulbecco's phosphate buffered saline (D-PBS). Lungs were inflated with 2 ml of Dispase II (Sigma) at 5 mg/ml with an intratracheal catheter. Trachea was ligated with suture silk to prevent leakage of Dispase II. Lungs were incubated in D-PBS for 45 min at 37 °C and then minced well with scissors and scalpels. Minced lungs were incubated in 5 ml D-PBS containing 1 mg/ml of Collagenase/Dispase (Roche, Indianapolis, IN) and 0.08 mg/ml of DNase I Type II (Sigma) for 10 min at 37 °C with shaking. Digested cells were filtered through a 70 μm nylon mesh (BD Biosciences, Franklin Lakes, NJ), followed by a 40 μm nylon mesh (BD Biosciences).

2.4. Analysis of inflammatory cells in single cell suspensions by flow cytometry

Approximately 1 million cells were stained with fluorescence labeled antibodies for monocytes/macrophages (Phycoerythrin-labeled anti-Mac3, R&D systems, Minneapolis, MN), neutrophils (Fluorescein isothiocyanate-labeled anti-Ly-6G, eBioscience, San Diego, CA), and CD8+ lymphocytes (Fluorescein isothiocyanate-labeled anti-CD8, eBioscience). The stained cells were analyzed by a BD FACScan flow cytometer (BD Biosciences). Percentages of monocytes/macrophages, neutrophils, and CD8+ lymphocytes in total lung cells were determined.

2.5. Determination of arterial blood oxygen tension

After gentle heating with a lamp, the mice were restrained and heparin was swabbed onto the skin to prevent clotting. The ventral artery of the tail was nicked by plunging a scalpel blade diagonally into the artery. Approximately 100 μl of blood was collected in a heparinized syringe and immediately placed on ice. The samples were analyzed on a clinical blood gas machine within 60 min of collection.

2.6. RNA isolation and real-time RT-PCR assay

Total RNA was isolated from lung tissue using the TRIzol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time RT-PCR for TNF-α, MIP-2, iNOS, and p47phox were performed using the Assays-on-Demand™ gene expression kits (Applied Biosystems, Foster City, CA) as previously described [11]. The mRNA level of RPS8 was used as an internal control since mRNA levels transcribed from this gene are not altered by *Pneumocystis* infection [11].

2.7. Quantification of P. murina organism burden by real-time RT-PCR

Organism burdens in the lungs were estimated based on the rRNA levels of *P. murina* mitochondrial large subunit ribosomal RNA gene (GenBank accession no. AF257179) using the method described by Zheng et al. [12]. A portion of the *P. murina* mitochondrial large subunit ribosomal RNA gene was cloned, and Pc rRNA was produced by in vitro transcription. A standard curve was generated with the known copy numbers of *P. murina* rRNA. Total RNA from infected lungs was purified and real-time RT-PCR for Pc rRNA was performed using the TaqMan® One-Step RT-PCR Master