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Short communication

Clearance of *Pneumocystis murina* infection is not dependent on MyD88

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Abstract

To determine if myeloid differentiation factor 88 (MyD88), which is necessary for signaling by most TLRs and IL-1Rs, is necessary for control of *Pneumocystis* infection, MyD88-deficient and wild-type mice were infected with *Pneumocystis* by exposure to infected seeder mice and were followed for up to 106 days. MyD88-deficient mice showed clearance of *Pneumocystis* and development of anti-*Pneumocystis* antibody responses with kinetics similar to wild-type mice. Based on expression levels of select genes, MyD88-deficient mice developed immune responses similar to wild-type mice. Thus, MyD88 and the upstream pathways that rely on MyD88 signaling are not required for control of *Pneumocystis* infection.
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Keywords: *Pneumocystis*; PCP; MyD88; Innate immunity; TLR

1. Introduction

Pneumocystis is a fungal pathogen of immunosuppressed hosts that also causes infection in immunocompetent hosts [1]. Although the organism can cause severe disease in the former, it is cleared by a robust immune response in immunocompetent hosts without causing significant disease [2–6]. While CD4 cells have been shown to be critical to the clearance of *Pneumocystis*, the early innate immune mechanisms responsible for control and clearance of infection are not well defined. Studies have suggested that interaction of *Pneumocystis* with the mannose receptor or dectin 1 may be important for innate responses [7,8]. In addition, toll-like receptors (TLRs) have also been implicated through studies of TLR deficient mice [9–11].

MyD88 is an adaptor molecule that is required for signaling for all TLRs except TLR3 and, in part, TLR4, as well as most IL-1Rs [12]. MyD88-deficient mice have been extensively used to explore the role of this signaling pathway in host defenses against a variety of pathogens, including fungal pathogens such as *Candida*, *Aspergillus*, and *Cryptococcus* species [13,14]. Most studies with *Pneumocystis* have utilized cells from MyD88-deficient mice and explored short-term immune responses [8,15,16]. The lack of susceptibility of MyD88-deficient mice to *Pneumocystis* infection, using a bolus intratracheal inoculation model, has very recently been reported [17]. The current study was undertaken to address the role of MyD88 in a natural infection model, which more closely mimics human disease, by exposing MyD88-deficient but otherwise immunocompetent mice to *Pneumocystis*-infected seeder mice and comparing the kinetics of infection to wild-type mice as well as CD40-deficient mice. The goal of the current study was to understand the role of Myd88 and related pathways in control of *Pneumocystis* infection in the immunocompetent host, rather than in a host with immunodeficiency-associated *Pneumocystis* pneumonia, which represents a different clinical entity.

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2. Methods

2.1. Animals

Healthy C57 black (C57bl/J6) mice were obtained from the National Cancer Institute, and MyD88-deficient (strain B6) mice were kindly provided by Dr. Alan Sher (NIAID, NIH) with the permission of Dr. Shizuo Akira, Osaka University. CD40-deficient mice (B6.129P2-*Tnfrsf5*^{tm1Kik}/J) were obtained from The Jackson Laboratory (Bar Harbor, Me). MyD88-deficient and CD40-deficient mice were subsequently bred at the NIH. Because of poor survival of offspring, MyD88-deficient mice were bred with C57bl/J6 mice, and the heterozygous F1 mice were bred together to obtain homozygous and heterozygous MyD88-deficient mice. Genotyping was performed to determine if mice were homozygous, heterozygous, or wild-type. Genotyping primers were kindly provided by Sarah Hieny and Dr. Alan Sher, with the following sequences: Myd88 left 5′TGGCATGCCTCCATCATAGTTAACC-3′, Myd88 right 5′GTCAGAAACAACCACCACCATGC-3′, and Myd88 neo 5′ATCGCCTTCTATCGCCTTCTTGACG-3′. The left primer is common for both PCR reactions, while the right is specific for the wild-type gene and the neo is specific for the deficient gene. Mice were housed in microisolator cages and kept in ventilated racks. All animal work was performed under an NIH Clinical Center Animal Care and Use Committee-approved protocol.

2.2. Co-housing experiments

Susceptibility of MyD88-deficient mice to *Pneumocystis* infection was examined in 2 experiments. To reproduce natural infection as closely as possible, homozygous and (as controls) heterozygous MyD88± mice and C57bl/J6 wild-type mice (10 total mice per cage) were co-housed with an immunodeficient (CD40L-deficient or *scid*) mouse with active *Pneumocystis* pneumonia. This has previously been shown to result in infection in healthy animals that peaks ∼35 days after exposure and is subsequently cleared by approximately 60–75 days, while immunodeficient mice have progressive infection throughout this period [2]. Seeder mice (one per cage) were co-housed for the entire experiment and were replaced if they developed respiratory distress. In the current study animals were sacrificed at days 35 and 75 (exp. 1) or days 35, 75 and 106 (exp. 2) after beginning exposure to the seeded animal, and lungs and serum were removed. Similarly, CD40-deficient mice were exposed to a seeder and lungs were examined at days 35 and 150 following exposure. Approximately 20–40 mg of lung tissue was placed in PBS for Q-PCR, and a similar amount in RNAlater for quantitation of expression levels of select genes. Lung and serum samples were stored at −80 °C until analysis. *Pneumocystis* organisms were quantified using a real-time quantitative PCR (Q-PCR) assay that quantitates the number of *Pneumocystis murina dhfr* gene copies/mg lung tissue as previously described [2]. Anti-*P. murina* serum antibodies were measured by ELISA utilizing a crude *Pneumocystis* antigen preparation as previously described [2]. The secondary antibody was an HRP-conjugated goat anti-mouse IgG that is heavy and light chain specific (Jackson ImmunoLabs) and thus would cross-react with IgM.

2.3. QuantiGene multiplex assay

To compare the immune response in healthy animals to MyD88-deficient animals, we utilized a customized QuantiGene Plex assay (Panomics) targeting genes that had been previously identified in microarray experiments as being upregulated in *Pneumocystis*-infected animals following exposure to seeder mice [3]. Targeted genes included T-cell receptor gamma chain (TCRg), granzyme G (Gzmg), colony stimulating factor 3 receptor (granulocyte) (Csf3r), killer cell lectin-like receptor, subfamily a, member 4 (Klra4), interleukin 12b (IL12b), chemokine (c–x–c motif) ligand 9 (Cxcl9), tumor necrosis factor receptor superfamily, member 4 (Tnfrsf4), chemokine (c–c motif) receptor 5 (Ccr5), CD86 antigen (CD86), CD3 antigen, epsilon polypeptide (CD3e), CD68 antigen (CD68), chemokine (c–c motif) ligand 9 (Ccl9), tumor necrosis factor receptor superfamily, member 18 (Tnfrsf18), interferon-gamma induced gtpase (Igtp), tumor necrosis factor (ligand) superfamily, member 9, (Tnfsf9), chemokine (c–c motif) ligand 8 (Ccl8), interleukin 18 binding protein (IL18bp), chemokine (c–c motif) ligand 12 (Ccl12), chemokine (c–x–c motif) receptor 3 (Cxcr3), chemokine (c–c motif) ligand 7 (Ccl7), integrin alpha l (Itgal), chemokine (c–x–c motif) ligand 1 (Cxcl1), chemokine (c–c motif) receptor 6 (Ccr6), interferon-gamma inducible protein 30 (Ifi30), inducible T-cell co-stimulator (Icos), immunoglobulin kappa chain, constant region (Igk-c) and Ig heavy chain v region (loc380804); interleukin 13 (IL13) was included as a control gene. Peptidylprolyl isomerase A (Ppia) was included as a housekeeping gene. Samples were analyzed in triplicate and normalized to Ppia. Results are expressed as fold change compared to the levels of expression for each gene in uninfected C57bl/J6 mice.

2.4. Statistics

Results are reported as geometric mean (*Pneumocystis* Q-PCR) or arithmetic mean (ELISA). Comparison of Q-PCR and ELISA results between MyD88-deficient and control mice were performed using unpaired Student's *t*-test.

3. Results

To help understand the role of MyD88 in control of *Pneumocystis* infection in the immunocompetent host, we utilized a mouse model in which animals are co-housed with immunosuppressed seeder animals that are infected with *Pneumocystis* [2–6]. This mimics natural infection that occurs by the respiratory route and avoids direct inoculation with a large bolus of organisms that may provide a skewed immune response.

We have previously characterized the course of infection in both immunocompetent (C57bl/J6) and immunodeficient