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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.

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].

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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.

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 off-spring, 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 ~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 to 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 *P. 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 (Cxl9), 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 (Igtg), 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 1 (Itgal), chemokine (c-x-c motif) ligand 1 (Cxl1), chemokine (c-c motif) receptor 6 (Ccr6), interferon gamma inducible protein 30 (Ifi30), and 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 house-keeping 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 (CD40L knock-out) mice [2]. At ~day 35 after beginning of co-housing, all animals are typically infected, with similar organism loads as measured by Q-PCR of <1,000 *dhfr* copies/mg lung tissue. By ~day 75, immunocompetent mice have cleared *Pneumocystis* infection, while immunodeficient animals have had a steady increase in organism burden to levels typically >100,000 *dhfr* copies/mg. We thus examined organism burden in MyD88-deficient mice as well as controls (heterozygous MyD88 +/- and wild-type) over time following co-housing with a *Pneumocystis*-infected seeder in 2 separate experiments. Both MyD88-deficient mice and controls were included in each cage to ensure similar levels of exposure. In both experiment 1 (n=10 mice; data not shown) and experiment 2 (n=26; Fig. 1), *Pneumocystis* infection was documented at day 35 with organism loads similar to that seen in prior studies; there were no significant differences in organism load between the 2 groups of mice ($p > 0.05$). By day 75 (experiments 1 and 2) and day 106 (experiment 2), all animals in both groups had cleared *Pneumocystis*. In contrast, in a separate experiment, CD40-deficient animals (n=7; Fig. 1), which had similar levels of *Pneumocystis* infection at day 35, had an increased organism load to over 1,000,000 *dhfr* copies/mg lung tissue by day 150. In all studies the genotype of individual animals was confirmed by PCR assays.

A seeder animal that was heavily infected with *Pneumocystis* (verified after completion of the study by Q-PCR) was co-housed in each cage to transmit infection. To verify that infection was successfully transmitted to all animals, and that the MyD88-deficient animals had in fact been infected with *Pneumocystis* during co-housing, an ELISA was used to detect anti-*Pneumocystis* antibodies. As shown in Fig. 2, and similar to prior studies [2], antibodies were not detected at day 35, a time at which *Pneumocystis* could be detected by PCR, but antibodies were subsequently detected in both MyD88-deficient and control mice at days 75 and 106. There were no significant differences in ELISA optical densities between the 2 groups at either time-point ($p > 0.05$).

To see if immune responses in MyD88-deficient mice were similar to controls, we utilized a multiplex assay to examine expression of a number of genes previously found to be up-regulated in healthy mice infected with *Pneumocystis*, primarily at ~day 35 [3]. As shown in Fig. 3, MyD88-deficient mice at day 35 showed upregulation of multiple genes in a pattern similar to that of healthy C57bl/J6 mice, while CD40-deficient mice showed little upregulation of these genes. The upregulated genes in MyD88-deficient mice are reflective of a robust adaptive immune response, and include interferon-gamma induced genes such as *Igtp* and *Ifi30*, a broad range of chemokines and chemokine receptors including *CCR5*, *CCL7* (MCP3), *CCL8* (MCP2), and *CXCL9* (MIG), monocyte/macrophage markers such as

CD68, and markers of T cell entry and activation, including CD3E, CD86, TNFRSF4, and TNFRSF18. Similar to the findings in CD40L-deficient mice [3], none of these genes were upregulated in CD40-deficient mice.

4. Discussion

The present study has demonstrated that MyD88 is not required for control of naturally acquired *Pneumocystis* infection in otherwise immunocompetent mice. By extension, signaling by the TLRs and IL-1Rs that are dependent on MyD88's role as an adapter molecule, are similarly not required for clearance of *Pneumocystis*. Our results are similar to those recently reported by Bello-Irizarry et al., using a different model of *Pneumocystis* infection [17]. In contrast, like the CD40L-deficient mouse, the CD40-deficient mouse is also highly susceptible to *Pneumocystis* infection [3, 18].

MyD88-deficient mice have been used extensively to explore innate host responses to a variety of pathogens, and have been reported to permit higher growth rates for 46 pathogens, and increased mortality for 33 pathogens, including 4 fungal species [19]. *Cryptococcus neoformans*, for example, is associated with greater mortality in MyD88-deficient as well as TLR2-, TLR9-, and IL-18R-deficient mice [20, 21].

Our study found no significant difference in *Pneumocystis* organism load at day 35, which is approximately the time of peak infection in our co-housing model. In contrast, Bello-Irizarry et al. found that MyD88-deficient animals had increased numbers of organisms at day 14 after inoculation, though not at subsequent time-points [17]. This may be related to differences in the models, in that bolus inoculation introduces large numbers of organisms, including presumably both live and dead organisms, which would include a substantial amount of organism-associated β -glucans that may result in activation of MyD88-dependent innate responses (see below). These responses may not be activated in our natural infection model, which likely transmits infection through inhalation of a very small number of organisms [22]. Alternatively, this difference may be related to the timing and frequency of sampling during the acute infection.

Using a multiplex assay targeting select genes that were previously shown to be differentially expressed in wild-type vs. CD40L-deficient mice, we demonstrated in MyD88-deficient mice that at day 35 following exposure there is increased expression of genes associated with effective anti-*Pneumocystis* immunity, including genes induced by interferon- γ , as well as those associated with macrophage and T cell activation. This demonstrates that MyD88 is not required for induction of the adaptive immune responses necessary for clearance of *Pneumocystis* infection. Consistent with results in our microarray studies using CD40L-deficient animals, CD40-deficient animals were also unable to upregulate expression of these same genes, strongly suggesting that the CD40-CD40L interaction is a critical and non-redundant upstream event in the development of anti-*Pneumocystis* immunity and other receptors or ligands cannot be substituted.

Prior studies with *Pneumocystis* have shown that MyD88 plays a role in release of TNF- α by macrophages in response to *P. carinii*-derived β -glucans [16]. In addition, both MyD88

and IL-1R were found in an earlier study by Bello-Irizarry et al. to be important for an inflammatory response by alveolar epithelial cells to intratracheally inoculated *Pneumocystis*, as measured by CCL2 and CXCL2 production, while TLR2 and TLR4 were not necessary for this response [15]. The latter study also found that in MyD88-deficient mice had decreased levels of CCL2 and CXCL2 in BAL fluid at 3 but not 8 or 24 hours after intratracheal inoculation of 1×10^6 *Pneumocystis* cysts. In their more recent study, this group found increased levels of CCL2, IL-4, IL-5, and IL-17 at some but not all time-points after intratracheal inoculation of *Pneumocystis*, in MyD88-deficient compared to wild-type mice, with no significant differences in TNF-alpha or interferon-gamma levels [17]. It is difficult to determine if these findings are related only to MyD88 deficiency, or to the bolus inoculation of *Pneumocystis* organisms in the setting of MyD88 deficiency. We cannot compare their results with our findings since we focused on different readouts for host immune responses.

In another paper that focused primarily on the role of dectin-1 in control of *Pneumocystis* infection in mice, macrophages from MyD88-deficient mice had decreased production of TNF- α and IL-12, but not reactive oxygen species, following challenge with *Pneumocystis* cysts [8]. It is noteworthy that while the latter study found a 3- to 5-fold increase in cyst burden in dectin 1-deficient compared to wild-type mice at 1 to 2 weeks after inoculation, in fact all dectin 1-deficient but otherwise immunocompetent mice cleared infection by 20 days.

Taken together with our study, these data suggest that there is an interaction between *Pneumocystis* and receptors that are dependent on MyD88 for signaling, but that MyD88-associated pathways are not needed for control of *Pneumocystis* infection, either because they represents a non-critical component of the immune response to *Pneumocystis*, or there are redundancies or alternative pathways that can function to control *Pneumocystis* infection in their absence. It is noteworthy that neither of two sets of pattern recognition receptors, dectin 1 [8] and those dependent on MyD88 ([17] and current study), which are felt to play an important role in control of fungal infections, are in fact critical to the clearance of *Pneumocystis* infection. Similarly, mannose-receptor deficient mice are also able to clear *Pneumocystis* [23]. Supporting this, patients with MyD88-deficiency and dectin 1-deficiency do not appear to have increased susceptibility to *Pneumocystis* infection [24, 25]. Thus, while innate responses presumably play a role in clearance of *Pneumocystis* infection, additional studies are needed to better define which pathways are critical to the clearance of this pathogen.

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Fig. 1. Clearance of *Pneumocystis* infection is similar in MyD88-deficient and control mice MyD88-deficient and control (wild-type or heterozygous MyD88 +/-) mice in experiment 2 were co-housed in 3 cages together with a seeder mouse and animals from each group were sacrificed at the indicated time-points (n= 2 for controls at day 35, and otherwise n=4 to 6 per group per time-point). *Pneumocystis* infection in the lung tissue was quantitated by Q-PCR using the single-copy *dhfr* gene as the target. For comparison, *Pneumocystis* burden in lungs from CD40-deficient mice from a separate co-housing experiment are shown. MyD88-deficient mice showed kinetics similar to control animals, while CD40-deficient mice were unable to control the infection. Results represent the geometric mean and error bars indicate the standard deviation.

Fig. 2. Antibody responses to *Pneumocystis* infection are similar in MyD88-deficient and control mice

To document that the MyD88-deficient and control mice were infected with *Pneumocystis*, anti-*Pneumocystis* antibodies in experiment 2 were quantitated by ELISA using a crude *Pneumocystis* antigen (n= 2 for controls at day 35, and otherwise n=4 to 6 per group per time-point). Antibody responses were detected using an HRP-conjugated goat anti-mouse IgG that is heavy and light chain specific. Antibody responses to *Pneumocystis* developed in both groups between day 35 and day 75. Results represent the mean optical density and error bars indicate the standard deviation. No significant differences in antibody titers were seen between the two groups at any of the time-points.

Fig. 3. Gene expression of select genes at day 35 following exposure to *Pneumocystis*

A multiplex assay (QuantiGene Plex assay) was developed to examine gene expression in 28 genes that were selected based on prior microarray studies in wild-type mice [3]. Results represent the mean fold-change, relative to unexposed C57bl/J6, of 2 to 4 mice per group, and are shown as a heat map, with upregulation indicated in red and down-regulation in green; the scale is shown above the heat-map. There is upregulation of most of the genes in the MyD88-deficient animals, similar to what is seen in control animals. In contrast, the CD40-deficient animals which are unable to control infection, showed little upregulation at the same time-point, similar to what was previously seen by microarray in CD40L-deficient animals [3], which are also highly susceptible to *Pneumocystis* infection. In each animal, expression levels were normalized to a house-keeping gene (PPIA) prior to analysis. The full names of the individual genes are provided in the methods. MyD88-deficient mice (MyD88 -/-), CD40 deficient mice (CD40 -/-), and C57bl/J6 (C57BL-E) were all exposed to a seeded animal for 35 days and were positive in the lungs for *Pneumocystis* by Q-PCR. C57BL-U are unexposed C57bl/J6 mice.