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MyD88 Signaling Regulates Both Host Defense and Immunopathogenesis during *Pneumocystis* Infection

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Abstract

The immune response protects against *Pneumocystis* infection, but is also a key component of PcP-related immunopathogenesis. Signaling through MyD88 is critical for activation of immune pathways downstream of TLRs and IL-1 receptor. To determine whether MyD88 regulates normal host defense against *Pneumocystis*, non-immunosuppressed wild-type (WT) and MyD88 deficient mice were infected. MyD88^{-/-} mice had higher early *Pneumocystis* burdens than WT mice, but mounted an effective adaptive immune response and cleared *Pneumocystis* similar to WT. However, MyD88^{-/-} mice displayed a more intense and prolonged pulmonary immune response than WT mice. To determine the role of MyD88 in the development of PcP-related immunopathogenesis, WT and MyD88^{-/-} mice were rendered susceptible to PcP by depletion of CD4⁺ T cells. At 4 weeks post-infection, CD4-depleted WT and MyD88^{-/-} mice harbored similar organism burdens, but MyD88^{-/-} mice were protected from the PcP-related respiratory impairment observed in WT mice. Improved pulmonary physiology in MyD88^{-/-} mice correlated with lower lung CCL2 levels, and reduced cell recruitment. However, by 5 weeks post-infection the overall health of MyD88^{-/-} mice began to deteriorate rapidly relative to WT, with accelerated weight loss, impaired lung function, and exacerbated alveolar inflammation. This physiological decline of MyD88^{-/-} mice was associated with increased TNF- α and IFN- γ in the lung, and by the inability to control *Pneumocystis* burden. Thus, MyD88 is not required for resistance to *Pneumocystis* infection, but limits the adaptive immune response in immunocompetent mice. In the setting of active PcP, MyD88 signaling contributes to both immunopathogenesis and control of fungal burden.

Introduction

Pneumocystis is respiratory fungal pathogen which causes pneumonia (PcP) in immunocompromised individuals. PcP-related morbidity and mortality continues to be a major health concern for HIV patients, as well as for non-HIV patients who are undergoing

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immunosuppression as a consequence of chemotherapy or organ transplant (1, 2). New immunosuppressive therapies, such as anti-TNF- α therapy for Crohn's disease and rheumatoid arthritis, are increasing the pool of "at risk" patients (3). In addition, *Pneumocystis* frequently colonizes COPD patients, which appears to exacerbate disease severity (4). Therefore, a better understanding of the mechanisms of PcP-related immunopathogenesis is key to improving upon current treatments.

Clinical observations and animal studies have indicated that lung injury during PcP is caused primarily by the host's immune-mediated inflammatory response, and is not absolutely related to *Pneumocystis* burden (5–8). For example, in the CD4⁺ T cell-depleted model of PcP physiological deterioration is associated with an increase in lung chemokine and cytokine levels, and the recruitment of large numbers of CD8⁺ T cells and neutrophils to the lung. Interestingly, when CD4⁺ and CD8⁺ T cells are depleted simultaneously, there are fewer signs of inflammation, less cell recruitment, and improved lung function, suggesting that CD8⁺ T cells are responsible for lung injury and respiratory impairment in this model of PcP (9).

Recent studies have focused on characterizing the mechanisms involved in generating pathogenic immune and inflammatory responses that damage the lung and other tissues. The Toll-Like Receptor (TLR) system is one of the most important host defense machineries involved in recognition of invading pathogens. Upon recognition pathogens, TLRs activate downstream kinases and transcription factors that induce the expression of genes involved in innate and adaptive immune responses. All TLRs, with the exception of TLR3, signal through the adaptor molecule myeloid differentiation factor 88 (MyD88). MyD88 is also critical for signaling through cytokine receptors that belong to the IL-1 receptor (IL-1R) family (10). A protective role for MyD88 in the control fungal infections such as *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Paracoccidioides brasiliensis* has been reported (11–14). Moreover, our laboratory and others have demonstrated that MyD88-dependent signaling is required for optimal alveolar epithelial cell (AEC) and alveolar macrophage (AM) cytokine responses to *Pneumocystis* or *Pneumocystis* cell wall components (15, 16). TLRs, including TLR2 and TLR4, have also been linked to *Pneumocystis*-stimulated AM cytokine responses (17, 18) but neither TLR2 nor TLR4 are required for AEC chemokine responses to *Pneumocystis*. Rather, the IL-1 receptor is the upstream molecule required for the MyD88-dependent AEC response (16).

Although *in vitro* studies suggest that TLR-, IL-1R-, and MyD88-dependent responses are involved in the AEC and AM responses to *Pneumocystis*, the *in vivo* role of MyD88-dependent signaling events during active *Pneumocystis* infection remain undefined. In the current study, we utilized WT and MyD88 deficient mice to assess the role of MyD88 in host defense against *Pneumocystis* infection, and/or the immunopathogenesis of PcP.

Materials and Methods

Mice

CB.17 severe combined immunodeficient (SCID) and C57BL/6 wild type (WT) mice were bred at the University of Rochester. C57BL/6 MyD88^{-/-} mice were generously provided by

Dr. S. Akira (Osaka University, Japan) (19, 20). All animal protocols were approved by the University Committee for Animal Research at the University of Rochester Medical Center.

Isolation and enumeration of mouse *Pneumocystis*

Pneumocystis was isolated from the lungs of heavily infected SCID mice and enumerated by Gomori's methenamine silver (GMS) staining as previously described (21). Each *Pneumocystis* preparation was tested to insure there was no bacterial contamination.

Mouse models of *Pneumocystis* infection

For the immunocompetent mouse model, 6 to 8 week old mice were anesthetized and 1e6 freshly isolated *Pneumocystis* (based on cyst count) were inoculated directly into the lungs via the trachea. For the immunosuppressed mouse model of PcP, CD4⁺ T cells were depleted by intraperitoneal injections of 250µg per mouse of monoclonal antibody specific for mouse CD4 (clone GK1.5, ATCC TIB207). Injections were given twice per week, starting one week prior to infection and continuing throughout the experiment. Depleted mice were inoculated with 5e5 freshly isolated *Pneumocystis* (based on cyst count).

Physiologic assessment of pulmonary function in live, ventilated mice

Dynamic lung compliance and lung resistance were measured in live ventilated mice using a whole body plethysmograph (BUXCO Electronics Inc., Wilmington, NC) connected to a Harvard rodent ventilator (Harvard Apparatus, Southnatic, MA) as previously described (22). Dynamic lung compliance was normalized to the peak body weight of each animal. Respiratory rates were measured using whole body unrestrained chambers (BUXCO Electronics Inc). Data was collected and analyzed using the Biosystems XA software package (BUCXO Electronics Inc.).

Lung tissue preparation and Bronchoalveolar lavage (23)

At indicated time points, mice were anesthetized, exanguinated and perfused through the heart. Right lung lobes were lavaged with three, one-ml aliquots of 1X Hank's balanced salt solution (HBSS) (GIBCO[®], Invitrogen), and then snap frozen in liquid nitrogen and stored at -80°C. The BAL fluid was centrifuged for 10 minutes at 150 × g at 4°C, and supernatants were frozen at -80°C. Recovered BAL cells were enumerated in the presence of trypan blue using a hemacytometer (Hausser Scientific) and centrifuged onto glass slides for differential staining (Diff-Quick). Differential counts were performed by microscopic examination of the slides. Multi-parameter flow cytometric analyses were also performed in BAL samples. Anti-CD4-Fluorescein (clone RM4-4) and anti-CD8a-Peridinin Chlorophyll-α Protein (clone 53-6.7), were purchased from BD Biosciences (San Diego, CA). The anti-CD4 clone RM4-4 was used to confirm CD4⁺ cell depletion *in vivo* in spleen samples because it is not blocked by the CD4-depleting antibody (clone GK1.5). Cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA)

Quantification of *Pneumocystis* burden

Real-time quantitative PCR method was utilized to quantify lung *Pneumocystis* burden in lung homogenate preparations. Right lung lobes were homogenized, subjected to three

freeze/thaw cycles, boiled for 20 min, and then centrifuged at max speeding a μ fuge. The lung homogenate supernatants were utilized for quantification of *Pneumocystis* burden. Quantitative PCR using TaqMan primer/fluorogenic probe chemistry was performed with a primer/probe set specific for the single copy mouse *Pneumocystis* kexin gene as previously described (22). The Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) was used to quantify *Pneumocystis* kexin gene copies.

Measurement of *P. carinii*-specific IgG and IgM

Pneumocystis-specific immunoglobulin G (24) and immunoglobulin M (IgM) in mouse serum was determined by enzyme-linked immunosorbent assay as described previously (25, 26). Flat-bottom microtitration plates (Flow Laboratories, McLean, Va.) were coated with mouse *Pneumocystis* soluble total protein from the lungs of infected SCID mice (10 mg of protein per ml), or protein from uninfected mouse lung. After blocking using 5% nonfat dry milk in phosphate-buffered saline, test sera were diluted 1:25 in phosphate-buffered saline–0.05% Tween 20. After washing the plates, goat anti-mouse IgG+IgM (H+L) alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:5,000 was used as secondary antibody. The plates were developed with *p*-nitrophenylphosphate as a substrate, and optical density (20) was measured at 405 nm. The background OD from uninfected lung protein coated plates was subtracted from *Pneumocystis*-infected mice lung protein coated plates.

Histological examination of lung sections

Left lung lobes were inflated with 15 cm gravity flow-pressure of 10% formalin (Sigma, St. Louis, MO). The lungs were fixed *in situ* for 10 minutes, removed from the mouse and placed in fixative solution for 16 h at 4°C. Lung tissue was embedded in paraffin and 4 μ M sections were cut. Lung sections were stained with hematoxylin and eosin to visualize tissue.

Cytokine and chemokine enzyme linked immunosorbent assay (ELISA)

BAL fluid was collected and centrifuged at 12,000 \times g for 10 min to remove cell debris. BAL fluids were stored at –80°C. Chemokine levels of CCL2 and CXCL2 and cytokine levels of TNF- α , IL-5, IL-17, IFN- γ , IL-1 α and IL-1 β were determined by commercially available ELISA kits (R&D Systems), and utilized according to manufacturer's instructions.

Generation of chimeras by bone marrow transplantation (BMT)

Bone marrow transplant (BMT) chimeras were generated as previously described (27). Briefly, (donor \rightarrow recipient) WT \rightarrow WT, KO \rightarrow WT, WT \rightarrow KO and KO \rightarrow KO (WT=C57BL/6; KO= C57BL/6 MyD88^{-/-}) BMT chimeras were prepared by radioablation by total body irradiation of female recipients (6 Gy \times 2 doses), followed by reconstitution with male donor bone marrow (Shepherd irradiator, 6000 Ci 137Cs source). To extract bone marrow from donors of the respective mouse strains, femurs and tibias were flushed into HBSS with 1% FCS. Cells were dispersed through a 21-gauge needle, and pooled by strain. Erythrocytes were removed by hypotonic lysis. The cells were counted, resuspended, and delivered to recipient mice by tail vein injection of 1×10^7 cells per mouse in a volume of 100 μ L. After BMT, animals were allowed to reconstitute for 8–10 weeks under

microisolator conditions, supplied with high efficiency particulate filtered air, sterilized food, acid water, and bedding. Fluorescent in situ hybridization (FISH) to detect the Y chromosome, was used on BAL cells from some mice to confirm the male origin of cells transplanted to female recipients, as previously described (28).

Statistical Analysis

Data are presented as mean \pm 1 standard error measurement (SE). In some experiments differences between strains at different time points were analyzed using two-way ANOVA and Bonferroni's Multiple Comparison Test as a post test. Differences between infected animals and control uninfected were analyzed using one-way analysis of variance (ANOVA) using Tukey's Multiple Comparison Test as a post test. Differences were considered significant at $P < 0.05$. All data were analyzed using GraphPad Prism (version 5.00) software (GraphPad Software, San Diego, CA).

Results

MyD88 deficient mice maintain effective host defense against *Pneumocystis* infection

The MyD88 adaptor molecule functions in the generation and maintenance of innate and adaptive immune responses. However, the specific contribution of MyD88 to host defense against respiratory infection with *Pneumocystis* is unknown. To address this question, non-immunosuppressed C57BL/6 WT and MyD88 deficient mice were infected with freshly isolated *Pneumocystis* organisms, and organism burden over time was determined. As expected, immunocompetent WT mice harbored *Pneumocystis* organisms in the lung for approximately 3 weeks before an adaptive immune response cleared the infection (Fig. 1A, B). However, MyD88 deficient mice had significantly higher *Pneumocystis* burden than WT mice on day 14 post-infection (Fig. 1A). At later time points there was no difference between WT and MyD88 deficient mice with respect to lung burden, and the MyD88 deficient mice were competent to mount an effective immune response, which included the production of anti-*Pneumocystis* antibody (Fig. 1B). These results demonstrated that MyD88 plays a role in the control of *Pneumocystis* growth early after infection, but that MyD88 is not essential for effective adaptive immunity, the production of anti-*Pneumocystis* antibody, or the clearance of *Pneumocystis* from the lung.

In addition to monitoring fungal growth and clearance, pulmonary function was also measured to determine whether loss of MyD88 affected PcP-related lung disease independent of changes in *Pneumocystis* burden. As expected, immunocompetent WT mice showed little evidence of respiratory impairment during *Pneumocystis* growth and immune-mediated clearance. Furthermore, lung compliance and lung resistance measurements were very similar in WT and MyD88 deficient mice at all time points examined, and were not statistically different from uninfected control mice (Fig. 2A and B). In addition, neither group lost weight during *Pneumocystis* infection, which is another indicator of PcP-related respiratory disease (data not shown). These findings confirm that MyD88 is not required for effective host defense against *Pneumocystis*, and that loss of MyD88 does not render mice more susceptible to lung injury associated with clearance of *Pneumocystis* by the normal immune system.

MyD88-dependent signals regulate the normal immune response to *Pneumocystis*

Although there were not obvious respiratory consequences of *Pneumocystis* infection in MyD88 deficient mice, it remained possible that the nature or magnitude of the immune response to *Pneumocystis* was altered by the absence of MyD88. Total cells recovered in the BAL fluid of WT and MyD88 deficient mice were determined on days 14, 18, 21 and 25 post-infection. *Pneumocystis*-infected WT mice had a significantly greater number of total BAL cells than uninfected mice at day 14 post-infection, and this number decreased over time coincident with *Pneumocystis* clearance (Fig. 3A). Interestingly, *Pneumocystis*-infected MyD88 mice showed a similar increase in the number of total BAL cells following infection. However, in contrast to WT mice, the number of BAL cells recovered from MyD88 mice did not diminish over the course of the study (Fig. 3A). *Pneumocystis*-infected MyD88 mice had more lymphocytes, macrophages, eosinophils and PMNs than *Pneumocystis*-infected WT mice at all time points. These differences reached statistical significance on day 21 post-infection for all cell types, and additionally on day 25 for PMNs (Fig. 3B–E).

To determine whether the altered immune response in *Pneumocystis*-infected MyD88 deficient mice was associated with an altered pattern of cytokine and chemokine production, protein levels were measured in BAL fluid of experimental mice. MyD88 deficient mice had statistically higher lung levels of the chemokine CCL2 than WT mice on days 14, 18 and 21 post-infection, although on day 25 both strains have similar baseline levels of the chemokine (Fig. 4A). These results correlated with increased numbers of lymphocytes and macrophages in lungs of *Pneumocystis*-infected MyD88 deficient mice (Fig. 3B, C). IL-4 levels in the BAL of MyD88 deficient mice were also significantly elevated as compared to WT mice on days 14, 18 and 21 post-infection, but decreased to basal levels on day 25 (Fig. 4B), suggesting a Th2-associated immune response. IL-5 levels were also elevated relative to WT mice in the lungs of MyD88 deficient mice and peaked at day 21 (Fig. 4C), coincident with the peak number of eosinophils in the lungs (Fig. 3E). Furthermore, IL-17 levels were also elevated in the lungs of *Pneumocystis*-infected MyD88 deficient mice compared to WT (Fig. 4D), and was associated with increased numbers of neutrophils (Fig. 3D). There was no significant difference in TNF- α levels, although there was a trend towards higher TNF- α levels in MyD88 deficient mice on days 14, 18 and 21 post-infection (Fig. 4E). IFN- γ levels in the BAL fluid were very low and nearly at the limit of detection in both strains, and there was no difference at any time point (Fig. 4F). These results further confirm that loss of MyD88 signaling alters the normal immune response to *Pneumocystis*, including more CCL2, more Th2-associated IL-4 and IL-5, and Th17-associated IL-17. These results suggest that MyD88 helps to regulate the degree of inflammation during a normal immune response to *Pneumocystis* infection, and that MyD88 deficiency results in exacerbated Th2 and Th17 responses following *Pneumocystis* infection.

MyD88 signaling contributes to PcP-related immunopathogenesis during early stages of infection, but is protective during later stages of disease

MyD88 plays a role in the early inflammatory response to *Pneumocystis* (16). To investigate the role of MyD88 in the immunopathogenesis of PcP, a model of AIDS-related immunosuppression was utilized to induce susceptibility to PcP. For this, WT and MyD88

deficient mice were depleted of CD4⁺ T cells and infected with freshly isolated *Pneumocystis* organisms. During the progression of the infection, body weights and respiratory rates were measured non-invasively to assess overall health and disease progression. WT and MyD88 deficient mice exhibited no weight loss over the first 4 weeks of infection, but both began to show weight loss after 4 weeks of infection. However, MyD88 deficient mice displayed a much more rapid weight loss than WT mice at this time (Fig. 5A). There were also differences in respiratory rates. WT mice displayed increased respiratory rates compared to MyD88 deficient mice during the first 3 weeks of infection (Fig. 5B). However, between 3 and 5 weeks post-infection, the MyD88 deficient mice displayed a much more dramatic elevation in respiratory rate than WT mice. The respiratory rates of MyD88 deficient mice rose approximately 100%, compared to a 12% increase observed in WT mice over this period. By day 31 post-infection MyD88 deficient mice had higher respiratory rates than WT mice, with 514 ± 31 and 465 ± 46 breaths per minute, respectively.

To directly assess the contribution of MyD88 to PcP-related respiratory impairment, pulmonary function was measured on experimental mice at 4 and 5 weeks post-infection. We chose two time points that reflected the distinct stages of disease in the MyD88 mice; prior to weight loss and respiratory rate increase (Week 4), and when MyD88 deficient mice displayed signs of exacerbated disease compared to WT (Week 5). At weeks 4 and 5 post-infection WT mice showed significantly lower dynamic lung compliance and higher lung resistance relative to uninfected mice, which is characteristic of PcP (Fig. 6A and B). In contrast, at 4 weeks post-infection MyD88 deficient mice were protected from the PcP-related lung function deficits observed in WT mice. The *Pneumocystis*-infected MyD88 deficient mice had significantly higher dynamic lung compliance than WT infected mice, which was similar to lung compliance measurements in uninfected control mice (Fig. 6A). Similarly, MyD88 deficient mice also showed comparable lung resistance measurements to control uninfected mice at week 4 post-infection (Fig. 6B). However, between 4 and 5 weeks post-infection, MyD88 deficient mice displayed a 50% drop in dynamic lung compliance and a 25% increase in lung resistance, signifying a dramatic acceleration in the severity of PcP in these mice over this period. These values were significantly different from uninfected mice, and worse than infected WT mice (Fig. 6A and B). Together these data demonstrate that MyD88 signaling contributes to PcP-associated respiratory impairment early during infection, but protects at later stages of disease. Thus, MyD88 signaling likely plays distinct roles during different stages of PcP.

MyD88-dependent mechanisms limit fungal burden during active PcP

To determine whether MyD88 plays a role in controlling *Pneumocystis* growth in a CD4⁺ T cell-depleted model of PcP, lungs of WT and MyD88 deficient mice were tested for *Pneumocystis* burden using quantitative real-time PCR as we have described (22). There was no difference in *Pneumocystis* burden between WT and MyD88 deficient mice at 4 weeks post-infection (Fig. 7). However, by 5 weeks post-infection the lungs of MyD88 deficient mice contained nearly 2-fold more *Pneumocystis* organisms than WT mice (Fig. 7). These data show that MyD88-dependent mechanisms help to control fungal burden in CD4⁺ T cell-depleted mice.

MyD88 contributes to lung inflammation early during *Pneumocystis* infection

To determine whether the differences observed in overall health and lung function between *Pneumocystis*-infected MyD88 deficient and WT mice correlated with the degree of pulmonary inflammation, total cell counts in BAL fluid were performed. *Pneumocystis*-infected MyD88 deficient mice had significantly fewer BAL cells than infected WT mice at week 4 (Table I). Significantly fewer numbers of macrophages and CD8⁺ T cells were present in the lung of MyD88 deficient mice at this time. Both of these cell types are effectors of PcP-related immunopathogenesis, and reduced numbers in the lungs of MyD88 deficient mice is likely a factor in the preserved lung function observed in these mice. At week 5 post-infection, there was not a significant difference in total cells or any specific cell type recruited to the lungs of *Pneumocystis*-infected WT or MyD88 deficient mice, suggesting that mice can compensate for the loss of MyD88 at least with respect to cell recruitment to the lung.

Histological examination of lung sections from experimental mice demonstrated increased cellular infiltration and inflammation in the alveolar region of *Pneumocystis*-infected WT mice at 4 weeks post-infection, as compared with uninfected lungs (Fig. 8A and B). In contrast, the lungs of *Pneumocystis*-infected MyD88 deficient mice displayed fewer alveolar infiltrates and less inflammation than WT mice at this time. Furthermore, while the cell infiltrates appeared dispersed throughout the alveoli in WT mice, they were localized mainly to peribronchial regions of the MyD88 deficient lungs, with less involvement of the alveolar regions. At week 5 post-infection, the lungs of *Pneumocystis*-infected MyD88 deficient mice showed more signs of inflammation and cell recruitment throughout the lung, which was similar to that observed in WT mice (Fig. 8C). Interestingly, the lungs of MyD88 deficient mice displayed more foamy exudates filling the alveolar spaces than were evident in WT mice (Fig. 8C, D). These results demonstrate that MyD88 regulates cell recruitment and inflammation in the lung during PcP.

MyD88 regulates chemokine and cytokine production during PcP

PcP-related immunopathogenesis is characterized by an increase in cytokine and chemokine production in the lungs. To determine whether the reduced cell-mediated pulmonary inflammation and preserved pulmonary physiology in the *Pneumocystis*-infected MyD88 deficient mice early during infection is associated with reduced chemokine and/or cytokine production in the lung, ELISA was used to evaluate BAL fluid from experimental mice. As expected, CCL2 levels were increased in the lungs of *Pneumocystis*-infected WT mice compared to uninfected controls. Also consistent with our earlier work (16), CCL2 levels were significantly reduced in the lungs of MyD88 deficient mice compared to WT mice at 4 weeks post-infection (Fig. 8A), which likely explains our finding of fewer macrophages and CD8⁺ T cells in the lungs of MyD88 deficient mice at this time (Table I). However, by 5 weeks post-infection CCL2 levels were similar in the lungs of MyD88 deficient and WT mice (Fig. 9A), indicating that CCL2 production can proceed via a MyD88-independent mechanism at later time points. Surprisingly, CXCL2, TNF- α , and IFN- γ levels were higher in the lungs of *Pneumocystis*-infected MyD88 deficient mice compared to WT lungs (Fig. 9B, C, and D), and elevated TNF- α and IFN- γ in the lungs at 5 weeks post-infection likely contributes to the MyD88-independent inflammation observed at this time. IL-1 α and IL-1 β

levels were also higher in infected MyD88 deficient mice compared to WT mice (Fig. 9E and F), which is likely related to lack of negative feedback regulation due to impaired IL-1 receptor signaling. These data demonstrate that deletion of MyD88 causes dysregulated cytokine and chemokine responses in the lung during PcP.

MyD88-dependent hematopoietic cell responses control fungal burden during active PcP

To determine whether the MyD88-dependent mechanisms required for the control of *Pneumocystis* burden rely on lung resident cells or hematopoietic cells, we generated bone-marrow chimera mice. WT and MyD88^{-/-} mice were lethally irradiated and reconstituted with WT or MyD88^{-/-} bone marrow cells. After 8 to 10 weeks post-reconstitution, mice were CD4-depleted and infected with *Pneumocystis*. At week 4 post-infection, there were not statistically significant differences in *Pneumocystis* lung burden between any of the groups, although WT mice reconstituted with MyD88^{-/-} cells (KO→WT) showed higher organism burden (Fig. 10). On week 5 post-infection, irradiated WT mice that were reconstituted with WT bone-marrow derived cells (WT→WT) presented with similar *Pneumocystis* counts as MyD88^{-/-} mice reconstituted with WT cells (WT→KO). However, WT mice that received MyD88^{-/-} bone marrow-derived cells (KO→WT) were unable to control *Pneumocystis* growth, as demonstrated by significant increase in organism burden in the lungs of these mice (Fig. 10). MyD88^{-/-} mice reconstituted with MyD88^{-/-} (KO→KO) bone marrow cells showed slightly higher *Pneumocystis* burden than WT→WT mice, but significantly lower burden than KO→WT (Fig. 10), suggesting that MyD88 signaling in hematopoietic and parenchymal cells may have opposing effects on control of *Pneumocystis* infection. These data suggest that the MyD88 signaling pathway in hematopoietic derived cells is important for control of organism burden during *Pneumocystis* infection in immunocompromised host.

Discussion

The MyD88 signaling pathway plays an important role in the generation of effective innate and adaptive immune responses to a variety of pathogens. In vitro studies have determined that MyD88 is required for alveolar epithelial cell and alveolar macrophage chemokine and cytokine responses to *Pneumocystis* (15), (16). However, the role of MyD88-dependent signaling during *Pneumocystis* infection and immunity *in vivo* has not been determined. In this study we expanded upon our previous work by; 1) identifying a regulatory role for MyD88 during the normal adaptive immune response against *Pneumocystis*; and 2) demonstrating that MyD88 signaling participates in PcP-related immunopathogenesis. While MyD88 signaling is not required for resistance to *Pneumocystis* infection in immunocompetent mice, it does help control fungal burden early during infection and limits the subsequent protective immune response. In the CD4-depleted AIDS-related model of active PcP, MyD88 contributes to inflammation, cell recruitment, and impaired pulmonary function during the early stages of PcP. However, at later stages of disease, MyD88 signaling limits inflammation and lung injury, and helps control *Pneumocystis* burden. These results suggest that MyD88 regulates the intensity of the immune response to *Pneumocystis* in immunocompetent hosts. However, MyD88 plays a dual role during active

PcP by contributing to immunopathogenesis at early stages, but protecting against lung injury and uncontrolled growth of the organism at later stages.

Our results suggest that MyD88-dependent mechanisms control *Pneumocystis* burden early during the CD4⁺ T cell-mediated immune response. Even though there were slightly higher numbers of macrophages in the lungs of MyD88^{-/-} mice early at day 14 post-infection, it seems that the MyD88 deficient macrophages were less efficient in controlling the fungal burden. MyD88 deficiency may prevent effective killing of *Pneumocystis* by AMs, either by defects in phagocytosis, or through impaired production of antimicrobial agents such as reactive oxygen or nitrogen species. Other studies have shown similar increased susceptibility of *Pneumocystis* infection in dectin-1 or TLR2 deficient mice, and have suggested a role for reactive oxygen and nitrogen species in clearance of *Pneumocystis* (29, 30). Interestingly, a similar pattern of increased fungal burden early during infection followed by effective resolution has been observed in MyD88^{-/-} mice infected with *Aspergillus* (12). Importantly, MyD88^{-/-} mice were able to clear *Pneumocystis* infection similar to WT mice, suggesting that MyD88 is not required for host defense against this infection. The ability of MyD88^{-/-} mice to clear *Pneumocystis* is probably due to an effective adaptive response and production of *Pneumocystis*-specific antibody that was comparable to WT mice. Production of *Pneumocystis* specific IgG and IgM antibody has been shown to correlate with clearance of *Pneumocystis* organisms (25, 31, 32).

In addition, we found that MyD88 regulates the intensity and resolution of the *Pneumocystis*-specific immune response in non-immunosuppressed mice. Although MyD88^{-/-} mice were able to clear the infection, the mice displayed an exacerbated immune response characterized by elevated CCL2 which correlated with higher numbers of lymphocytes and macrophages recruited to the lung, and an increase in the Th2-related cytokines IL-4 and IL-5 which may explain increased lung eosinophilia. Interestingly, MyD88^{-/-} mice that were infected with the pathogens *Legionella pneumophila*, *Salmonella typhimurium*, *Chlamydia muridarum* or *Leishmania major* all presented polarized Th2 responses that were associated with enhanced disease pathology (33–36). In addition, levels of the Th17 cytokine, IL-17, were also elevated in MyD88^{-/-} mice, which correlated with higher numbers of neutrophils recruited to the lungs. Increased inflammation and lung pathology in *Pneumocystis* infected nude IFN γ ^{-/-} mice correlated with higher levels of IL-17 in the lung (37). It should be noted that despite displaying an exacerbated immune response against *Pneumocystis*, non-immunosuppressed MyD88^{-/-} mice did not exhibit reduced pulmonary function relative to WT mice. We speculate that the recruited MyD88 deficient inflammatory cells are less activated than WT cells, and thus do not have profound effects on lung function.

Recent studies have suggested a role for TLR2 and TLR4 in the *in vivo* responses to *Pneumocystis* in a CD4-depleted, AIDS-related model of PcP (18, 29). However, both studies suggest that either TLR2 or TLR4 are not the sole pathways responsible for the immunopathogenesis during *Pneumocystis* infection. Our results using infected MyD88^{-/-} mice show an increase in lung TNF- α and CXCL2 compared to WT mice, which is consistent with results reported using a model of pulmonary *Aspergillus* infection (12). Conversely, our results show a decrease in CCL2 in the BAL of CD4-depleted MyD88^{-/-}

mice with PcP. CCL2 (or MCP-1), is a member of the CC chemokine family which recruits macrophages and T cells to sites of infection (23, 38–40). Macrophages and CD8⁺ T cells contribute to PcP-related immunopathogenesis, and reduced CCL2 in MyD88^{-/-} mice correlated with fewer macrophages and CD8⁺ T cells recruited to the lungs and preserved lung function. (9, 41, 42). These results suggest that MyD88 mediates the inflammatory response and lung injury early during infection through CCL2-dependent recruitment of CD8⁺ T cells and macrophages. MyD88 also regulates other chemokines and cytokines such as CXCL2, TNF- α , IFN- γ , IL-1 α and IL-1 β (Fig. 8). Our data also suggests that additional MyD88-dependent receptors other than TLR2 or TLR4 may be involved in the response to *Pneumocystis*. For example, IL-1R was found to mediate the chemokine response to *Pneumocystis* in primary AECs, and may play a role in the immunopathogenesis of PcP (16). Interestingly, IL-1 β is required for effective resolution of important and perhaps somewhat redundant roles in the immunopathogenesis of PcP. For this reason, our study attempted to determine the role during PcP of the adaptor molecule MyD88, which participates in the TLR/IL-1 pathways.

MyD88 is required for optimal alveolar macrophage TNF- α responses to *Pneumocystis* β -glucan (15), and the MyD88-dependent pattern recognition receptors TLR2 and TLR4 also contribute to AM chemokine and cytokine responses to *Pneumocystis* (18, 29). In vivo studies found that CD4-depleted TLR2^{-/-} mice harbored higher *Pneumocystis* burdens than WT mice, similar to our findings in MyD88^{-/-} mice (29). AECs also respond to *Pneumocystis*, and we recently reported that the alveolar epithelial cell chemokine response is dependent on MyD88 and IL-1 receptor, but independent of TLR2 or TLR4 (16). It is interesting that our chimeric studies determined that loss of AEC MyD88 signaling did not affect fungal burden in CD4-depleted mice, whereas loss of hematopoietic cell MyD88 caused increased burden. These findings indicate that AEC responses may not directly contribute to control of *Pneumocystis* burden during active PcP in immunosuppressed mice. However, it remains possible that AEC responses contribute to inflammatory cell recruitment and PcP-related immunopathogenesis through MyD88-dependent mechanisms. Further studies are required to explore this possibility.

MyD88-independent pathways may also limit the inflammatory response during active PcP in CD4-depleted mice, especially at later stages of disease. At 5 weeks post-infection, when MyD88^{-/-} mice developed severe inflammation and exhibited signs of impaired lung function, BAL TNF- α levels were significantly higher than in WT mice. Excessive TNF- α is associated with inflammatory cell recruitment, uncontrolled activation of pro-inflammatory pathways, and endothelial leakiness. A previous study from our group showed that TNF receptor signaling drives pulmonary inflammation and respiratory impairment in *Pneumocystis*-infected CD4-depleted mice (22). While this study found that the presence of CD8⁺ T cells is required for maximal TNF production during PcP in CD4-depleted mice (22), CD8⁺ T cell-independent mechanisms of TNF production may also contribute to elevated lung levels in MyD88^{-/-} mice. For example, excessive lung injury in MyD88^{-/-} mice due to higher organism burdens at 5 weeks post-infection could cause elevated TNF- α release. A similar outcome was observed in a study in which lymphocyte deficient SCID mice were infected with *Pneumocystis*. These mice produced very little lung TNF- α until

the very late stages of disease when lungs were injured and the *Pneumocystis* burden was very high (43). It is possible that various redundant pathways work in cooperation to detect and respond to *Pneumocystis*. For example, dectin-1 has been found to cooperate with TLR2 to stimulate TNF- α production in macrophages exposed to mycobacteria infection (44). In addition, macrophage mannose receptor (MR) deficient mice infected with *Pneumocystis* showed increased total protein, albumin and inflammatory cells in lungs compared to WT mice (45). Recently, a role for intracellular osteopontin in the control of *Pneumocystis* burden in immunocompromised mice was reported. The authors suggested that osteopontin clusters TLR2, dectin-1 and MR in macrophages exposed to *Pneumocystis* (46). It is possible that MyD88-independent pathways cooperate with the MyD88 pathway during the host response to *Pneumocystis*, although more studies are required to confirm these interactions.

Our data demonstrate that MyD88 signaling in hematopoietic cells is required to control *Pneumocystis* infection during active PcP in CD4-depleted mice. These results agree with recent findings showing that MyD88 signaling in the hematopoietic compartment was required for control of *Legionella pneumophila* (47). This appears to be dependent on the specific pathogen, since MyD88 expression in both resident and hematopoietic cells contribute to control of *Klebsiella pneumoniae* infection. In contrast, resident non-hematopoietic cells were important for control of *Pseudomonas aeruginosa* growth (24, 48). While we have clearly shown that MyD88 signaling modulates pulmonary immunity and contributes to PcP-related immunopathogenesis, many questions remain. For example, we have neither identified the exact cell types that utilize MyD88 signaling during *Pneumocystis* infection, nor defined the specific MyD88-dependent mechanisms that regulate host defense and immunopathogenesis. MyD88-dependent responses are known to regulate both innate and adaptive immunity, and many cell types, including epithelial cells, macrophages, neutrophils, lymphocytes, and DCs utilize the MyD88 signaling pathway during the course of an immune response. More in-depth follow-up studies are needed to define the cell type-specific role of MyD88 in the complicated network of immune interactions that occur in response to *Pneumocystis in vivo*.

The blockade of MyD88 signaling is being explored as a possible therapeutic strategy to attenuate inflammatory disease (49, 50). Our preliminary findings suggest that MyD88 may represent a potential target for immunotherapy in *Pneumocystis* infected patients. Since lack of MyD88 early during PcP limits inflammation and promotes better lung function, it is conceivable that blocking the MyD88 pathway in combination with effective antibiotic treatment may improve disease outcome. MyD88 represents a more specific pathway that might be blocked temporarily while antibiotic treatment to *Pneumocystis* is administered. This is in contrast to corticosteroids that sometimes cause undesired systemic side effects. However, we also found that loss of MyD88 signaling could have negative effects on the severity of PcP at later stages of disease by increasing fungal burden, which raises concerns regarding the therapeutic blockade of MyD88 during PcP. In addition, the MyD88 and TLR pathways have been found to protect against lung injury through recognition of extracellular matrix components such as hyaluronan. Hyaluronan degradation products present during lung injury promote lung repair and protect the lung epithelium through interactions with

TLRs (51, 52). Thus, we speculate that the later deterioration in CD4-depleted MyD88 deficient mice with active PcP may be partly related to loss of MyD88-dependent protective mechanisms that function during lung injury. Further studies utilizing MyD88 blocking agents *in vivo* are required to thoroughly evaluate the potential of targeting MyD88 for anti-inflammatory therapy during PcP.

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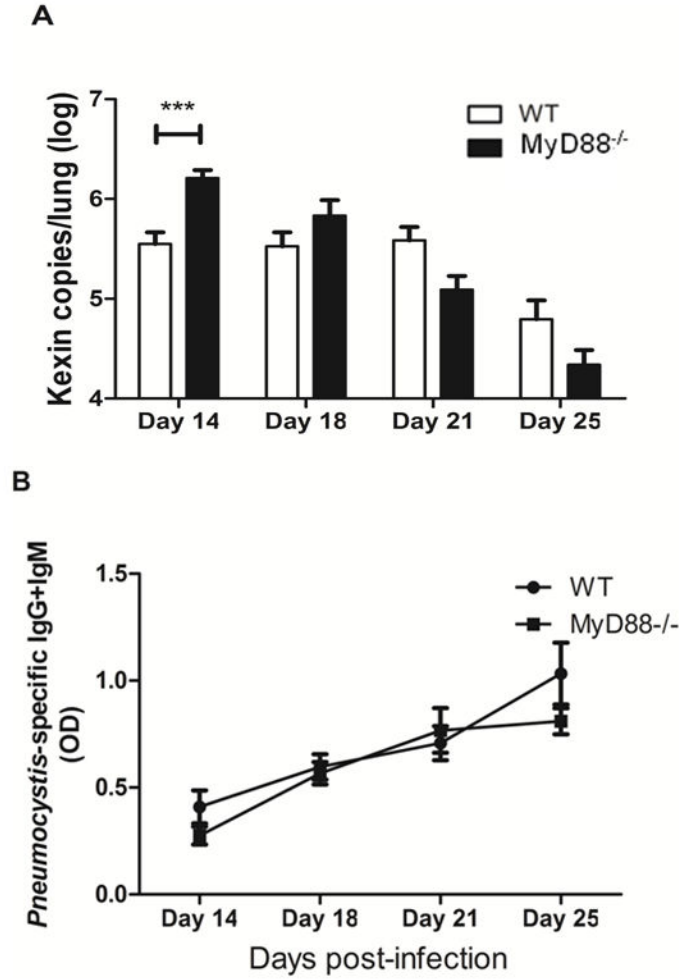


Figure 1. MyD88 deficient mice maintain effective host defense against *Pneumocystis* infection
Pneumocystis burden was determined by quantitative real-time PCR for *Pc kexin* gene copies in WT and MyD88^{-/-} mice on days 14, 18, 21 and 25 post-*Pneumocystis* infection (A). *Pneumocystis* specific IgG and IgM in the serum of mice were measured by ELISA using soluble *Pneumocystis* antigens from infected mouse lung homogenates as target (B). Values are mean ± 1 standard error measurement (n=4 to 8/timepoint/group). ***, $P < 0.001$ in WT compared to MyD88^{-/-}. Mean represents combined data from two independent experiments.

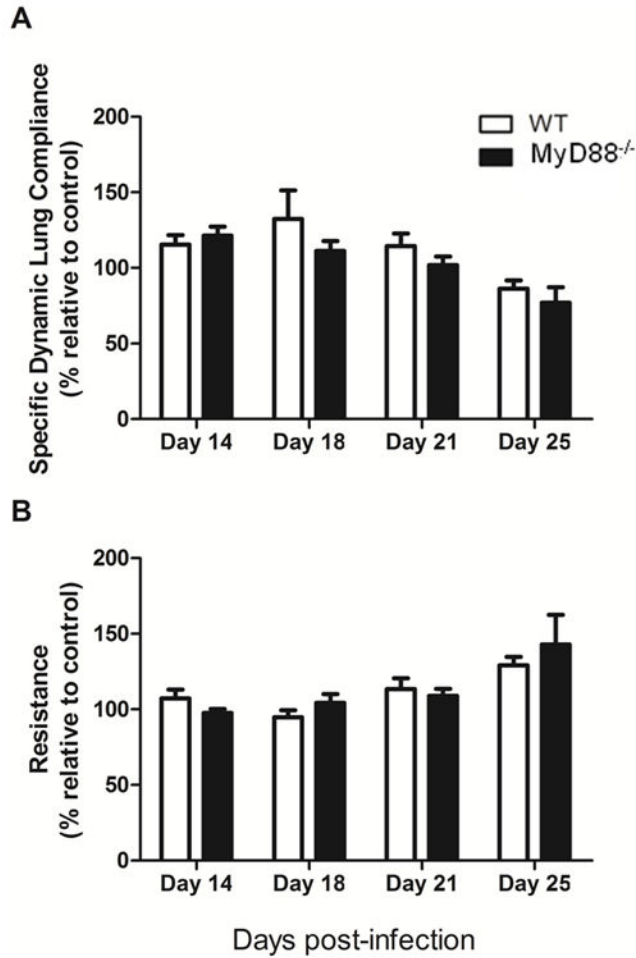


Figure 2. MyD88 deficiency does not alter lung function during *Pneumocystis* infection in the immunocompetent host

Dynamic lung compliance and lung resistance were measured in C57BL/6 (WT) and MyD88^{-/-} mice on days 14, 18, 21 and 25 after *Pneumocystis* infection. Uninfected mice were used as a control. Values are mean \pm 1 standard error measurement (n=4 to 8/ timepoint/group) of combined data from two independent experiments ($P>0.05$).

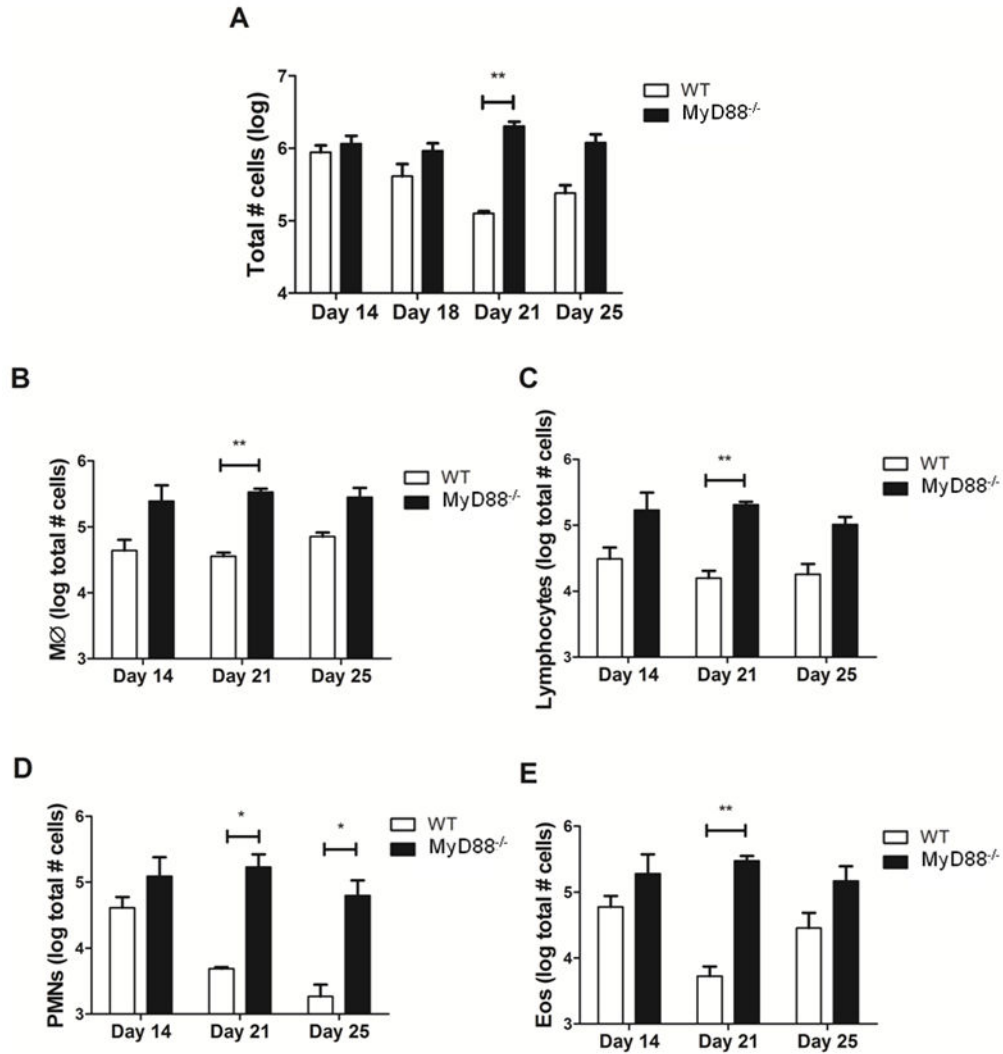


Figure 3. MyD88-dependent signals regulate cell recruitment during normal immune response to *Pneumocystis*

C57BL/6 (WT) and MyD88^{-/-} mice were infected with *Pneumocystis*. On days 14, 18, 21 and 25 post-infection total cells, macrophages (MØ), lymphocytes, neutrophils (PMNs) and eosinophils (Eos) were enumerated from BAL. Values are mean ± 1 standard error measurement (n=4 to 8/timepoint/group) of combined data from two independent experiments (*, $P < 0.05$; **, $P < 0.01$).

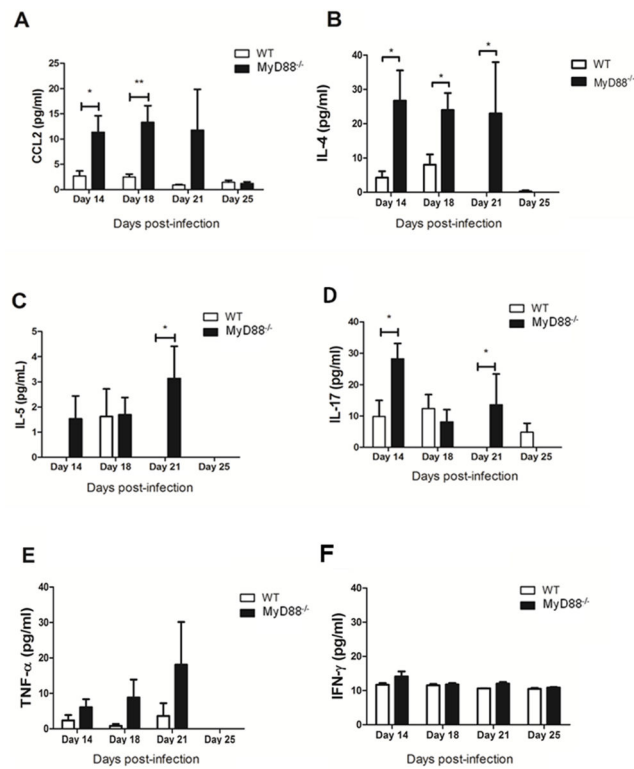


Figure 4. MyD88-deficiency alters chemokine and cytokine responses to *Pneumocystis*
 C57BL/6 (WT) and MyD88^{-/-} mice were infected with 5×10^5 freshly isolated *Pneumocystis* cysts. On days 14, 18, 21 and 25 post-infection CCL2 (A), IL-4 (B), IL-5 (C), IL-17 (D), TNF-α (E) and IFN γ (F) were measured by ELISA from BAL. Values are mean \pm 1 standard error measurement (n=4 to 8/timepoint/group) of combined data from two independent experiments (*, $P < 0.05$; **, $P < 0.01$).

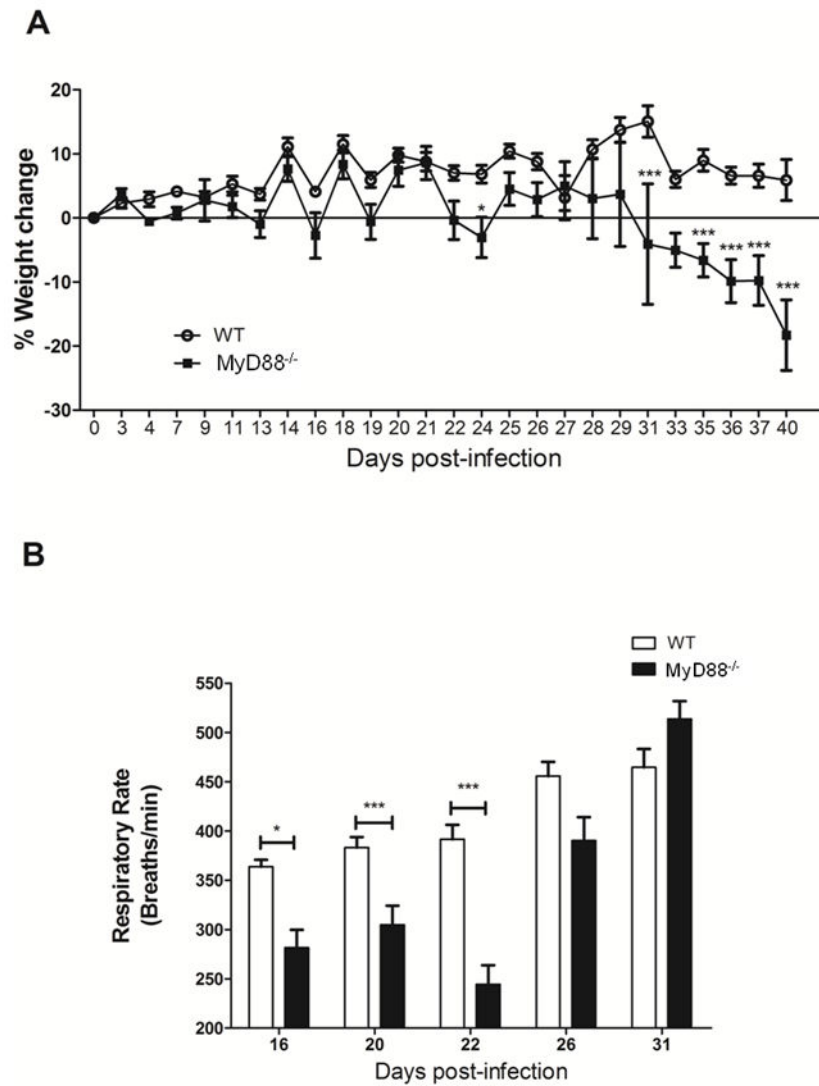


Figure 5. MyD88 signaling contributes to severity of PcP and PcP-associated respiratory impairment during early stage of infection, but is protective during later stage of disease
 CD4⁺ T cell depleted C57BL/6 (WT) and MyD88^{-/-} mice were infected with 5×10^5 freshly isolated *Pneumocystis* cysts. Mice were weighed from two to three times a week, and changes in body weight (% weight change) were calculated. Respiratory rates were measured non-invasively on days 16, 20, 22, 26 and 31 post-infection. Values are mean \pm 1 standard error measurement (n=6 to 11/timepoint/group) of combined data from two independent experiments (*, $P < 0.05$; ***, $P < 0.001$).

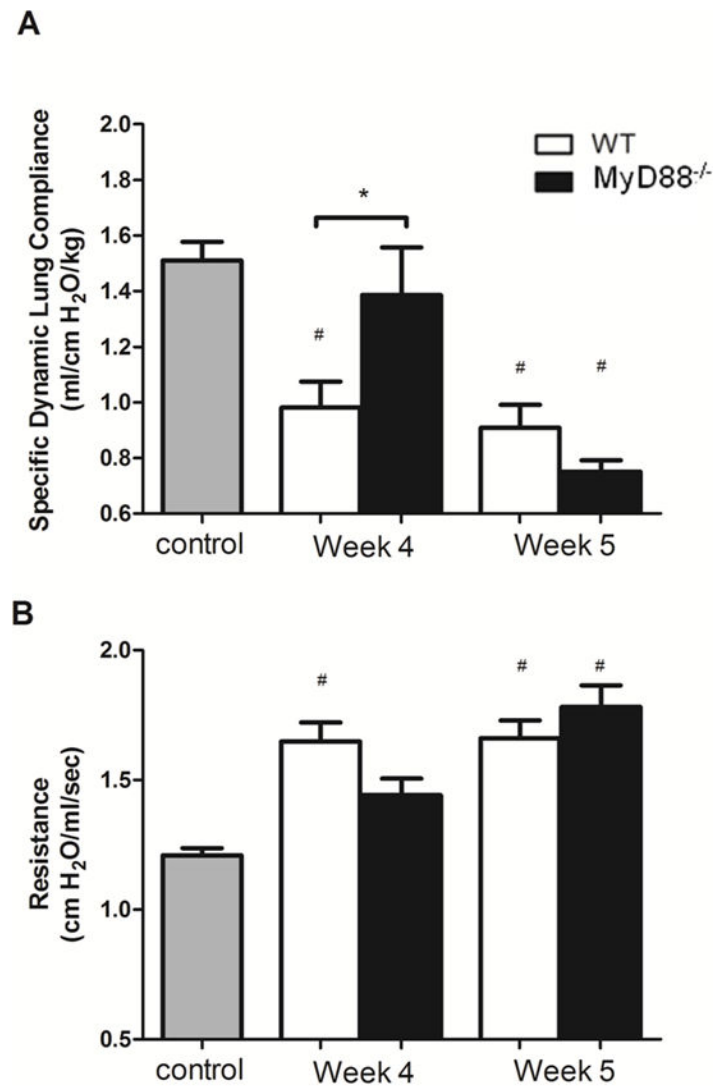


Figure 6. MyD88 signaling contributes to PcP-related lung function deficits during the early stage of infection, but is protective during the later stage of disease

CD4⁺ T cell depleted C57BL/6 (WT) and MyD88^{-/-} mice were infected with 5×10^5 freshly isolated *Pneumocystis* cysts. Dynamic lung compliance and lung resistance were measured on week 4 and week 5 post-infection. Uninfected mice were used as a control. Values are mean \pm 1 standard error measurement (n=6 to 11/timepoint/group) of combined data from two independent experiments (*, $P < 0.05$; #, $P < 0.05$ compared to control).

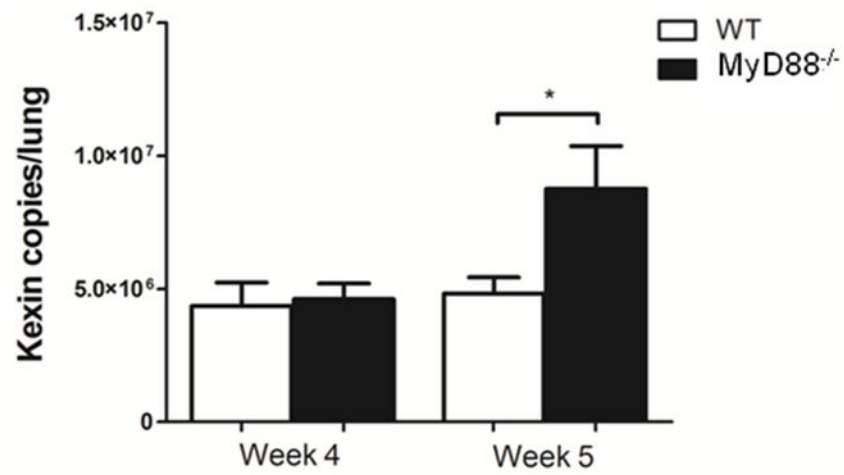


Figure 7. MyD88-dependent mechanisms limit fungal burden during active PcP
Pneumocystis burden was determined by quantitative real-time PCR of *kex1* gene copies in CD4-depleted WT and MyD88^{-/-} mice on week 4 and week 5 post-*Pneumocystis* infection. Values are mean ± 1 standard error measurement (n=6 to 11/timepoint/group) *, $P < 0.05$. Mean represents combined data from two independent experiments.

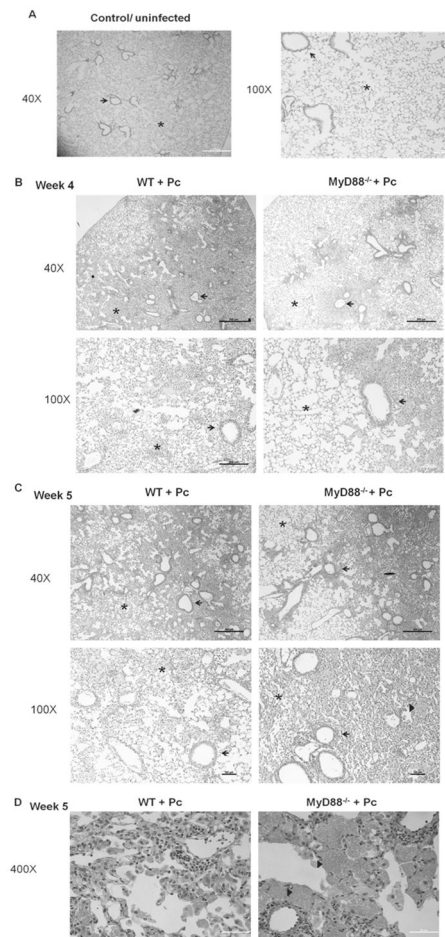


Figure 8. MyD88 contributes to lung inflammation early during *Pneumocystis* infection
 CD4⁺ T cell depleted C57BL/6 (WT) and MyD88^{-/-} mice were infected with 5e5 freshly isolated *Pneumocystis* cysts. Lung sections from uninfected control mice (A), or *Pneumocystis*-infected mice at week 4 (B), or week 5 post-infection (C, D) were stained with Hematoxylin and Eosin. Representative pictures were taken by microscopy under 40×, 100× (A–C) or 400× (D) magnification as indicated. Arrows denote peribronchiolar regions in each section, while asterisks denote alveolar regions. In C and D arrow heads indicate foamy exudates in alveolar spaces.

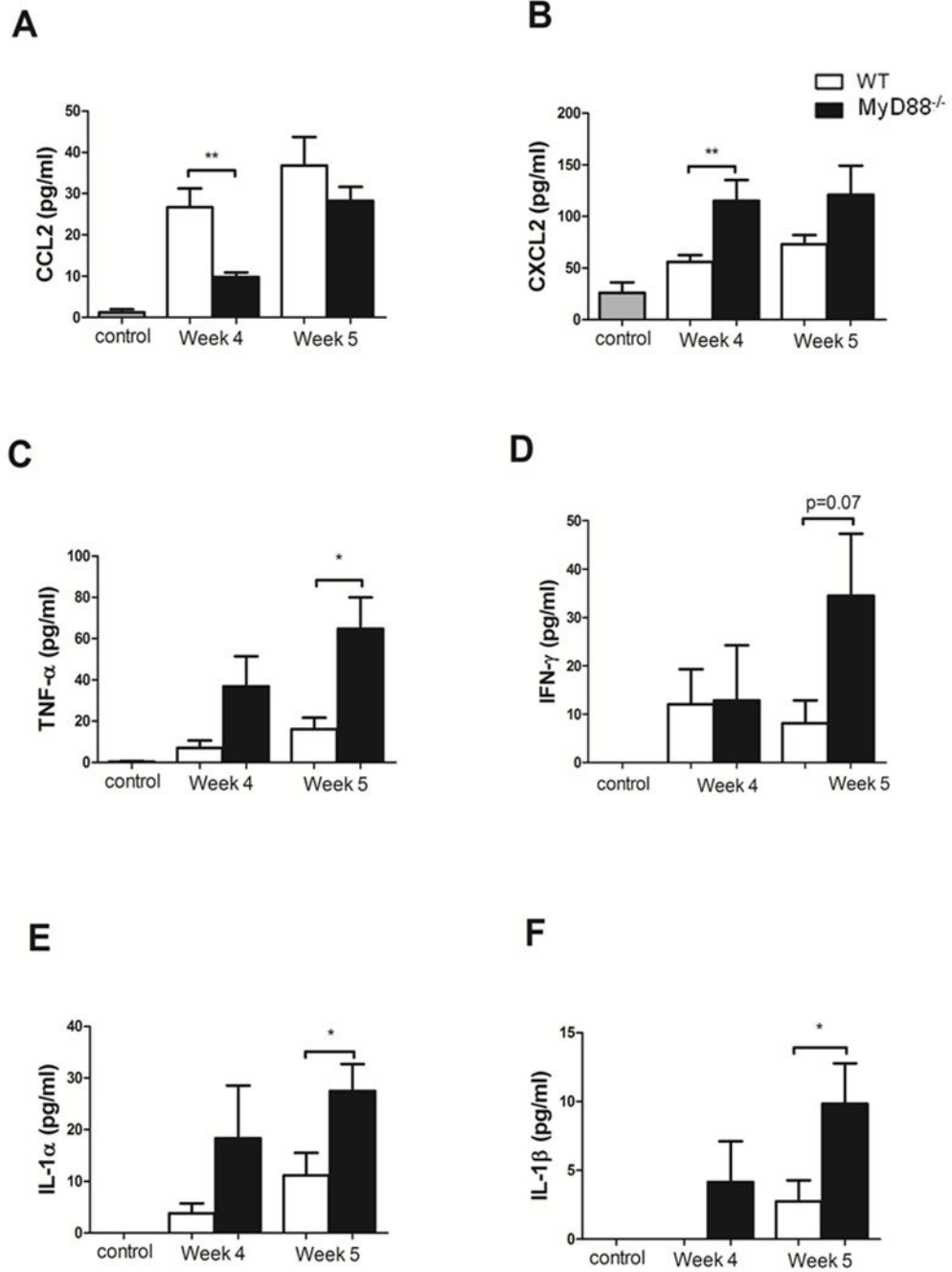


Figure 9. MyD88 regulates chemokine and cytokine production during PcP

CD4⁺ T cell depleted C57BL/6 (WT) and MyD88^{-/-} mice were infected with 5×10^5 freshly isolated *Pneumocystis* cysts. CCL2 (A), CXCL2 (B), TNF-α (C), IFN-γ (D), IL-1α (E) and IL-1β (E) levels were measured in the BAL fluid from *Pneumocystis*-infected mice and control uninfected mice. Values are mean ± 1 standard error measurement (n=6 to 11/ timepoint/group) of combined data from two independent experiments (*, $P < 0.05$; **, $P < 0.01$).

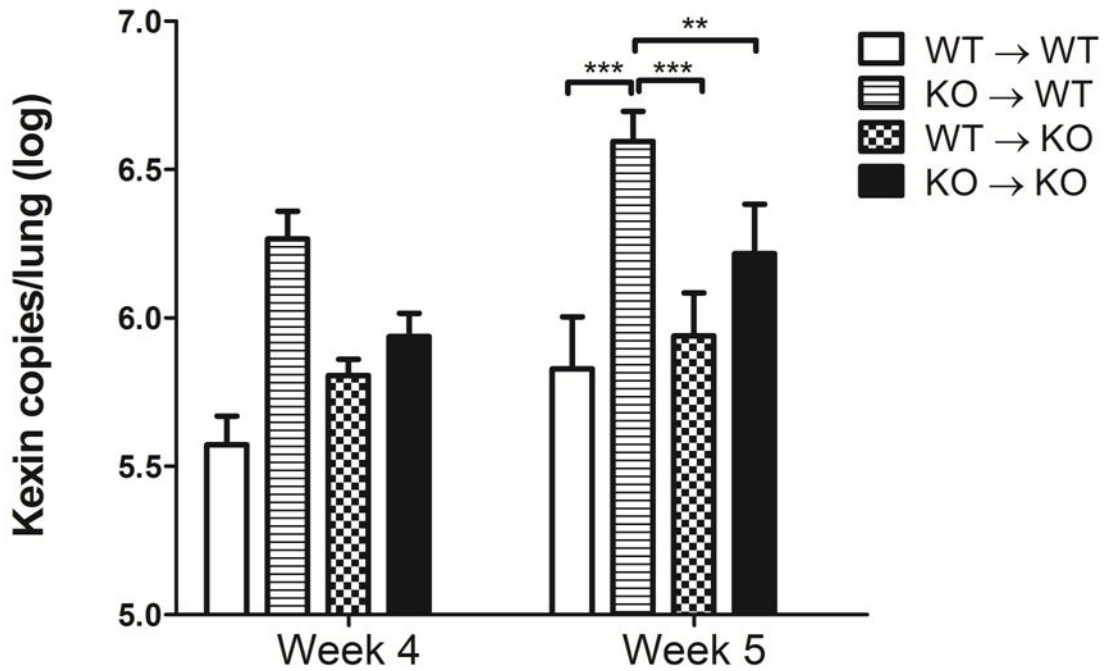


Figure 10. MyD88-dependent signals in hematopoietic cells control fungal burden during active PcP

Pneumocystis burden was determined by quantitative real-time PCR for *kex1* gene copies in CD4-depleted WT and MyD88^{-/-}(KO) bone marrow chimeras (BMC) at weeks 4 and week 5 post-*Pneumocystis* infection. BMC mice were prepared by lethal irradiation of either WT or MyD88^{-/-} mice (KO) as described in Materials and Methods. Irradiated WT mice reconstituted with WT bone marrow cells (WT→WT), irradiated WT mice reconstituted with MyD88^{-/-} bone marrow cells (KO→WT), irradiated MyD88^{-/-} mice reconstituted with WT bone marrow cells (WT→KO), irradiated MyD88^{-/-} mice reconstituted with MyD88^{-/-} bone marrow cells (KO→KO). Values are mean ± 1 standard error measurement (n=6 to 11/timepoint/group) **, $P < 0.01$; and ***, $P < 0.001$. Mean represents combined data from two independent experiments.

Table 1

Cellular composition of BAL fluid from *Pneumocystis*-infected, CD4-depleted WT and MyD88^{-/-} mice

Cell Type (10 ⁵)	Week 4		Week 5	
	WT	MyD88 ^{-/-}	WT	MyD88 ^{-/-}
Total cells	4.87 ± 0.51	2.85 ± 0.59*	3.34 ± 0.37	4.08 ± 0.54
Macrophages	1.46 ± 0.12	0.97 ± 0.19*	1.01 ± 0.25	0.92 ± 0.28
PMNs	1.78 ± 0.39	0.84 ± 0.29	1.27 ± 0.22	1.49 ± 0.28
Lymphocytes	1.59 ± 0.20	0.96 ± 0.24	1.05 ± 0.16	1.63 ± 0.27
CD8⁺ T cells	1.30 ± 0.33	0.23 ± 0.14*	0.81 ± 0.20	0.49 ± 0.17

BAL fluid was isolated from one lung and cell populations were distinguished by differential counts or flow cytometry.

* , p<0.05 as compared with *Pneumocystis*-infected WT mice at the same time point. Values are mean ± 1 standard error measurement (n=6 to 11/time point/group). Data is pooled from two independent experiments.