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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- κ B 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 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

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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- κ B.[10] NF- κ B 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. 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 wk 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 exsanguinations. 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 to 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 μ m nylon mesh (BD Biosciences, Franklin Lakes, NJ), followed by a 40 μ m 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 μ l 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 minutes 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 Mix Reagent Kit (Applied Biosystems). Ct values of the samples were converted to rRNA copy number using the standard curve.

2.8. Quantification of TNF- α in bronchoalveolar lavage fluids (BALF) by ELISA

Lungs were lavaged with 1 ml sterile saline through an intratracheal catheter as described previously[11]. BALF was centrifuged at 10,000 $\times$ g for 5 min to remove cellular debris. TNF- α concentrations in BALF were determined by sandwich ELISA as described previously.[11]

2.9. Determination of NF- κ B Activation by electrophoretic mobility shift assays (EMSA)

A biotin-labeled consensus double-stranded NF- κ B oligonucleotide probe (5'-AGTTGAGGGGACTTTCCAGGC-3') was used for EMSA. Nuclear extract was obtained from lung tissue homogenates using the nuclear extraction kit (Active Motif, Carlsbad, CA). Two micrograms of nuclear protein was used to detect NF- κ B activation using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). Cold competition was carried out using

specific unlabeled probe. Unlabeled oligonucleotide that does not bind NF- κ B was used as nonspecific competitor.

2.10. Quantification of nitrate and nitrite in BALF

Since nitric oxide is quickly oxidized to nitrite and nitrate, nitrite levels were determined. Briefly, Freshly obtained BALF was incubated with cadmium granules to reduce nitrate to nitrite, followed by adding Griess reagent of the QuantiChrom™ Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA), and absorbance at 540 nm was measured using a microplate reader (Molecular Dynamics, Sunnyvale, CA). Total nitrite concentration was calculated from the standard curve formed by serial dilution of nitrite standards.

2.11. Statistics

Data were presented as mean $\pm$ standard deviation (S.D.) of the indicated number of experiments. Differences between groups were determined using the two-tail Student's t-test and were considered statistically significant if p was < 0.05 .

3. Results

3.1 Decreased inflammation in the lungs of TLR2 $^{-/-}$ mice with PcP

To examine the role of TLR2 in the inflammatory response to *Pneumocystis* infection, wild type and TLR2 $^{-/-}$ mice were sacrificed eight weeks after infection with *P. murina*. Inflammation in the lungs was evaluated by histopathology examination. As shown in Fig. 1, lungs of wild type mice with PcP displayed severe inflammation, characterized by increased numbers of monocytes, lymphocytes, neutrophils, and eosinophils and destruction of alveolar parenchyma and small airways. In contrast, there were fewer mononuclear cells and less neutrophil infiltration in the lungs of TLR2 $^{-/-}$ mice with PcP. Although there was more foamy exudate in the alveoli, the alveolar structure was better preserved in the lungs of TLR2 $^{-/-}$ mice with PcP (Fig. 1). The pulmonary inflammation score of TLR2 $^{-/-}$ PcP mice was significantly lower than that of the wild type mice with PcP (3.6 ± 0.4 vs. 4.5 ± 0.7 , $p = 0.012$). Flow cytometry analysis of single cell preparations showed that the percentage of neutrophil in total lung cells was decreased by 86% ($0.71 \pm 0.38\%$ vs. $5.13 \pm 0.93\%$, $p = 0.007$), and that of monocyte/macrophage was decreased by 67% ($3.99 \pm 1.02\%$ vs. $12.10 \pm 1.15\%$, $p = 0.02$) in the lungs of TLR2 $^{-/-}$ mice with PcP compared to those of wild type mice with PcP. The number of CD8 $^{+}$ lymphocytes in the lungs of TLR2 $^{-/-}$ mice with PcP was not significantly different from that of wild type mice with PcP ($0.90 \pm 0.37\%$ vs. $1.14 \pm 0.46\%$, $p = 0.165$).

3.2 Decreased production of TNF- α in TLR2 $^{-/-}$ mice with PcP

To investigate whether the decreased inflammation in the lungs of TLR2 $^{-/-}$ mice with PcP was due to decreased TNF- α production, TNF- α mRNA expression levels of lung tissue and TNF- α protein levels in the BALF were determined. As shown in Fig. 2, TNF- α mRNA abundance (Fig. 2a) and TNF- α production (Fig. 2b) were both increased in wild type mice and TLR2 $^{-/-}$ mice during *P. murina* infection. However, compared to wild type mice with PcP, TLR2 $^{-/-}$ mice with PcP showed significantly lower levels of TNF- α mRNA expression (39.5 ± 16.8 fold increase vs. 90.5 ± 18.7 fold increase, $p = 0.009$) and TNF- α production (90.0 ± 12.2 pg/ml vs. 172.8 ± 18.4 pg/ml, $p = 0.043$). This result suggests that TLR2 is partially involved in TNF- α production in response to *Pneumocystis*. Similar pattern was found in the mRNA expression level of another pro-inflammatory cytokine MIP-2. TLR2 $^{-/-}$ mice with PcP showed significantly lower levels of MIP-2 mRNA expression (41.8 ± 11.3 fold increase vs. 91.7 ± 31.6 fold increase, $p = 0.02$).

3.3 Decreased NF- κ B activation in the lungs of TLR2^{-/-} mice with PcP

Since the expression of TNF- α is regulated by NF- κ B,[10] the role of TLR2 in the activation of NF- κ B during PcP was examined. Lung homogenates were used to isolate nuclear extracts for determination of NF- κ B activation by EMSA. As shown in Fig. 3, *P. murina*-induced NF- κ B activation was seen in both wild type mice and TLR2^{-/-} mice, but TLR2^{-/-} mice with PcP showed a markedly reduced level of NF- κ B activation in the lungs. Densitometry analysis of the autoradiograph shown in Fig. 3 indicated that *Pneumocystis* infection induced 1.3 fold and 3.48 fold increases of NF- κ B activation in TLR2^{-/-} mice and wild type mice, respectively. These data are consistent with the less robust TNF- α response in TLR2^{-/-} PcP mice as compared to wild type PcP mice shown in Fig. 2, suggesting that TLR2 deficiency results in less NF- κ B activation, TNF- α production, and inflammation in the lungs during *P. murina* infection.

3.4 More severe PcP in TLR2^{-/-} mice

To investigate the progression of PcP, wild type and TLR2^{-/-} mice with PcP were examined for several parameters of disease. As shown in Fig. 4, both wild-type and TLR2^{-/-} mice started to lose weight 6 wk after initiation of infection, but the loss was greater in TLR2^{-/-} mice than in wild type mice at 7 and 8 wk of infection. TLR2^{-/-} mice with PcP displayed more severe symptoms of disease including reduced grooming, dyspnea, and hypokinesia after 8 wk of infection. The survival rates of TLR2^{-/-} mice and wild type mice at 8 wk of infection were 78.6% and 96.4%, respectively (n = 28). Arterial blood oxygen tension (PaO₂) of TLR2^{-/-} mice with PcP was significantly lower than that of wild type mice with PcP (67.8 $\pm$ 0.5 mm Hg vs. 80.1 $\pm$ 3.5 mm Hg, p = 0.03, n = 8).

3.5 Increased organism burden in the lungs of TLR2^{-/-} mice with PcP

Silver staining of the lung tissue sections from TLR2^{-/-} mice with PcP showed a significantly higher level of *P. murina* cyst burden than those from wild type mice with PcP (Fig. 5a). The increased organism burden was confirmed by real-time RT-PCR for *Pneumocystis* rRNA with an average of 6.71 $\times$ 10⁸ copies in the lungs of TLR2^{-/-} mice and 2.99 $\times$ 10⁸ copies in those of wild type mice with PcP (p = 0.04) (Fig. 5b). These data suggest that TLR2 knockout leads to a reduced clearance of *P. murina* organisms.

3.6 Impaired inducible nitric oxide synthase (iNOS) induction and nitric oxide production in TLR2^{-/-} mice with PcP

To explore a possible reason why TLR2^{-/-} mice were less able to clear *P. murina* organisms, iNOS mRNA levels in the lungs and nitric oxide levels in the BALF were determined. As shown in Fig. 6a, iNOS mRNA expression was significantly increased in wild type mice and TLR2^{-/-} mice during *P. murina* infection. However, compared to wild type mice with PcP, TLR2^{-/-} mice with PcP showed significantly lower levels of iNOS mRNA expression (949.1 $\pm$ 193.8 fold increase vs. 1818.2 $\pm$ 216.0 fold increase, p = 0.022). Determination of nitric oxide levels in BALF showed that TLR2^{-/-} mice with PcP contained significantly less nitric oxide than wild type mice with PcP (20.1 $\pm$ 2.6 μ M vs. 27.1 $\pm$ 4.5 μ M, p = 0.012) (Fig. 6b).

3.7 Impaired NADPH oxidase induction in TLR2^{-/-} mice with PcP

NADPH oxidase is the key enzyme in the production of reactive oxygen species (ROS). To investigate the effect of TLR2 knockout on ROS production during PcP, the mRNA level of NADPH oxidase p47phox subunit in the lungs was determined. As shown in Fig. 6c, p47phox mRNA abundance was significantly increased in wild type mice and TLR2^{-/-} mice during *P. murina* infection. However, compared to wild type mice with PcP, TLR2^{-/-} mice with PcP showed a significantly smaller increase in p47phox mRNA (17.7 $\pm$ 6.5 fold increase vs. 59.6 $\pm$ 5.7 fold increase, p = 0.03).

4. Discussion

As a pattern recognition receptor that recognizes fungal cell wall components, TLR2 was shown in our previous study to mediate alveolar macrophage inflammatory response to *P. murina* in vitro,[11] but the role of TLR2 in the host response during *Pneumocystis* pneumonia had not been determined. In this study, we used TLR2^{-/-} mice to investigate the roles of TLR2 in the host response against *Pneumocystis* infection and found that TLR2 knockout mice with PcP had less severe pulmonary inflammation than wild type mice with PcP (Fig. 1). We also found that TLR2^{-/-} mice with PcP displayed less TNF- α in BALF (Fig. 2) and lower level of NF- κ B activation than the wild type mice with PcP (Fig. 3). Since NF- κ B regulates the expression of TNF- α ,[10] a reduction in NF- κ B activation would certainly result in a decreased production of TNF- α . TLR2 is one of many receptors that can trigger NF- κ B activation. It is conceivable that TLR2 knockout leads to diminished NF- κ B activation, reduced TNF- α production, and subsequently less pulmonary inflammation.

Although the levels of NF- κ B activation and TNF- α production were significantly lower in TLR2^{-/-} mice with PcP than in wild type mice with PcP, they were still significantly higher than those in uninfected control mice. TLR2^{-/-} mice with PcP still showed some degree of pulmonary inflammation (Fig. 1), suggesting that TLR2 is not the only receptor that mediates inflammatory response to *Pneumocystis* in vivo. This is consistent with previous studies showing that other receptors, such as dectin-1 and mannose receptors, also play important roles in the host response to *Pneumocystis* infection.[9,14] Other studies have shown that TLR2 collaborates with dectin-1 and mannose receptors to mediate inflammatory response to *Pneumocystis*. [15,16] A recent review by Dennehy and Brown described that dectin-1 is critical in the control of fungal infection since it can mediate its own signaling, as well as synergize with TLR to initiate responses to fungal pathogens.[17] Study by Ding *et al.* showed that there was a reduced level of MIP-2 in bronchoalveolar lavage fluid of TLR4 mutant mice with PcP, suggesting that TLR4 also mediates the inflammatory response to *Pneumocystis*. [18]

Host defense against infection is critically dependent upon recruitment of immune cells into infected tissue. These cells may include lymphocytes, neutrophils, monocytes and macrophages, and serve as the effector cells for the clearance of microorganisms. However, over-exuberant inflammation may cause tissue damage. In fact, inflammation is considered the major cause of lung damage in PcP. Wright *et al.* have found that immune-mediated inflammation directly impairs pulmonary function during PcP.[19] A recent study showed that sensitized CD8⁺ T cells fail to clear organisms but accelerate the onset of lung injury during PcP.[20] In this study, we found that the numbers of neutrophil and monocyte/macrophage were decreased in TLR2^{-/-} mice with PcP and the number of CD8⁺ T cells in the lungs of TLR2^{-/-} mice with PcP was found to be similar to that of wild type mice with PcP. These results suggest that neutrophil and monocyte/macrophage play a role in TLR2-mediated inflammatory response during PcP. Whether this inflammation leads to lung damage remains to be investigated since it has been shown that neutrophils do not contribute to lung damage during PcP.[21] The observation that TLR2^{-/-} mice developed more severe PcP symptoms with increased organism burden as compared to wild type mice (Fig. 3, 4) indicates that TLR2-mediated response contributes to the elimination of *Pneumocystis* organisms.

Clearance of the organisms by the host is mediated by direct phagocytosis, and the production of toxic antimicrobial metabolites such as nitric oxide and ROS by macrophages. Nitric oxide has been shown to be an important antimicrobial intermediate on organisms. *Pneumocystis* organisms can stimulate alveolar macrophages to produce nitric oxide,[22] and nitric oxide is toxic to *Pneumocystis* organisms.[23] iNOS is the key enzyme in the production of nitric oxide. The promoter region of the mouse iNOS gene contains several binding sites for transcription factors including NF- κ B.[24] Therefore, it is possible that a reduction in NF- κ B leads to a

decreased production of nitric oxide. This is indeed the case in the TLR2^{-/-} mice with PcP, as iNOS mRNA levels in lung homogenates and nitric oxide concentration in BALF from these mice were significantly decreased as compared to wild type mice with PcP (Fig. 6a, 6b). This may account for the defect in the clearance of the organism in TLR2^{-/-} mice. However, the roles of nitric oxide and iNOS in the clearance of *Pneumocystis* organisms require to be more carefully investigated as both molecules have been shown to be inadequate in the control of *Pneumocystis* infection.[25]

ROS, such as super oxide and hydrogen peroxide, are also important antimicrobial intermediates. *Pneumocystis* can induce ROS production in alveolar macrophages.[26] A recent study shows that ROS are important in the protection against *Pneumocystis* infection because defective production of ROS correlated with increased susceptibility to *Pneumocystis* infection in dectin-1 knockout mice.[27] Superoxide production is regulated by the multisubunit enzyme NADPH oxidase. In our study, the mRNA level of the p47phox component of NADPH oxidase was found to be much lower in TLR2^{-/-} mice with PcP (Fig. 6c). The impaired production of ROS in TLR2^{-/-} mice may be another reason for the decreased clearance of *Pneumocystis* organisms.

Since TLR2^{-/-} mice with PcP exhibited more severe symptoms despite decreased inflammation in the lungs, there must be factors other than inflammation that cause the impaired lung function. The increased organism burden may directly impair lung function by disrupting oxygen diffusion inside the alveoli. Previous studies suggest that *Pneumocystis* organisms require attachment or close apposition to the alveolar epithelium in order to proliferate.[28] An ultrastructural study shows that cysts and trophozoites are held together by slender membranotubular extensions to form the foamy alveolar cast on the surface of the pulmonary epithelium, which may impair the gas exchange function of the alveoli.[29]

In this study, wild type mice with PcP had more severe inflammation, but had lower organism burden, less body weight loss, and most importantly a higher survival rate than TLR2^{-/-} mice with PcP. This result suggests that inflammation is one of the host defense mechanisms against *Pneumocystis* infection and that anti-inflammatory therapy for PcP needs to be used with caution. A complete inhibition of inflammation may have adverse effects. There have been reports of clinical benefit from the use of adjunctive corticosteroids in addition to the specific anti-*Pneumocystis* therapy. Some of the benefits are related to the decreased hypersensitive reactions to antibiotic therapy. However, a small, but significant, increased rate of infection in steroid-treated patients is noted.[30] The anti-inflammatory therapy for PcP is beneficial only in conjunction with effective anti-microbial treatments.[30]

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Fig. 1.

Decreased inflammation in the lungs of TLR2^{-/-} mice with PcP. Immunosuppressed wild-type and TLR2^{-/-} mice were infected with *P. murina* for eight weeks. Lungs were fixed, embedded, and sectioned. Lung sections were stained with H&E, and examined with light microscopy at magnifications of 100X and 400X. Data presented are representative for 6–8 mice per group. Similar results were obtained in three independent experiments. Scale bar = 100 μ m.

Fig. 2.

Decreased production of proinflammatory cytokine TNF- α in TLR2^{-/-} mice with PcP. (a) Total RNA was isolated from lung cells of uninfected mice and those that had been infected for eight weeks. TNF- α mRNA expression levels were determined by real-time RT-PCR. The mRNA of the ribosomal protein S8 (RPS8) gene was co-amplified with that of TNF- α to serve as the internal PCR control in each assay. Fold increase in TNF- α mRNA levels in the lungs is relative to that of the lungs from uninfected wild-type control mice, which was set to a value of one. (b) BALF of the same infected or uninfected mice was analyzed for TNF- α protein level (pg/ml) by ELISA. Bars represent means $\pm$ S.D. for 6–8 mice per group. Similar results were obtained in three independent experiments.

Fig. 3. Decreased NF-κB activation in the lungs of TLR2^{-/-} mice with PcP. Nuclear protein was isolated from lung cells of uninfected mice and those that had been infected for eight weeks. NF-κB activation level in the nuclear extract was determined by EMSA. The blank lane is the control reaction mixture containing biotin-labeled DNA probe without adding nuclear extract. Data presented are representative for 6–8 mice per group. Similar results were obtained in three independent experiments.

Fig. 4. Body weight change in *Pneumocystis*-infected wild type and TLR2^{-/-} mice. The mice were weighed every week, and changes in body weight (g) were calculated. Bars represent means $\pm$ S.D. for 6–8 mice per group. Similar results were obtained in three independent experiments.

Fig. 5. Increased organism burden in TLR2^{-/-} mice with PcP. Immunosuppressed wild-type and TLR2^{-/-} mice were infected with *P. murina* for eight weeks. (a) Lungs were fixed, embedded, and sectioned. Lung sections were stained with the GMS silver staining kit, and examined with light microscopy at magnifications of 100X and 200X. Results shown are representative of 6–8 mice per group. (b) Total RNA was isolated from the lungs and subjected to real-time RT-PCR for Pc rRNA. Pc rRNA copy numbers in the lungs of wild type and TLR2^{-/-} mice with PcP were calculated from a standard curve generated with known copy numbers of Pc rRNA. Bars represent means $\pm$ S.D. for 6 mice per group.

Fig. 6.

Impaired iNOS induction, nitric oxide production, and NADPH oxidase induction in TLR2-/- mice with PCP. (a) Total RNA was isolated from lung cells of uninfected mice and those that had been infected for eight weeks, and iNOS mRNA expression levels were determined by real-time RT-PCR. The mRNA of the RPS8 gene was co-amplified with that of iNOS to serve as the internal PCR control in each assay. Fold increase in iNOS mRNA levels in the lungs is relative to that of the lungs from uninfected wild-type control mice, which was set to a value of one. (b) Nitrate and nitrite levels (μM) in BALF were determined by using the Griess reagent of the QuantiChrom™ Nitric Oxide Assay Kit. (c) Total RNA was isolated from cells of infected and uninfected lungs, and p47phox mRNA levels were determined by real-time RT-PCR. Fold

increase in p47phox mRNA levels in the lungs is relative to that of the lungs from uninfected wild-type control mice, which was set to a value of one. Bars represent means $\pm$ S.D. for 6–8 mice per group. Similar results were obtained in three independent experiments.