A Critical Role for CARD9 in Pneumocystis Pneumonia Host Defense

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Abstract

Caspase recruitment domain-containing protein 9 (CARD9) is an adaptor molecule critical for key signaling pathways initiated through C-type lectin receptors (CLRs). Previous studies demonstrated that Pneumocystis organisms are recognized through a variety of CLRs. However, the role of the downstream CARD9 adaptor signaling protein in host defense against Pneumocystis infection remains to be elucidated. Herein, we analyzed the role of CARD9 in host defense against Pneumocystis both in CD4-depleted CARD9⁻/⁻ and immunocompetent hosts. Card9 gene-disrupted (CARD9⁻/⁻) mice were more susceptible to Pneumocystis, as evidenced by reduced fungal clearance in infected lungs compared to wild-type infected mice. Our data suggests that this defect was due to impaired proinflammatory responses. Furthermore, CARD9⁻/⁻ macrophages were severely compromised in their ability to differentiate and express M1 and M2 macrophage polarization markers, to enhanced mRNA expression for Dectin-1 and Mincle, and most importantly, to kill Pneumocystis in vitro. Remarkably, compared to wild-type mice, and despite markedly increased organism burdens, CARD9⁻/⁻ animals did not exhibit worsened survival during PCP, perhaps related to decreased lung injury due to altered influx of inflammatory cells and decreased levels of proinflammatory cytokines in response to the organism. Lastly, although innate phase cytokines were impaired in the CARD9⁻/⁻ animals during PCP, T-helper cell cytokines were normal in immunocompetent CARD9⁻/⁻ animals infected with Pneumocystis. Taken together, our data demonstrate that CARD9 has a critical function in innate immune responses against Pneumocystis.

Keywords

CARD9; Pneumocystis; macrophage; host defense; inflammation

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CONFLICT OF INTEREST

The authors have no conflict of interest.
INTRODUCTION

In advanced HIV infections, fungi are major contributors to opportunistic infections. *Pneumocystis jirovecii* is the most common pathogen in this cohort causing Pneumocystis Pneumonia (PCP) in AIDS patients across the world. The use of highly active antiretroviral therapy (HAART) has decreased the overall incidence of PCP, but those requiring hospitalization still remains quite high (Thomas Jr et al., 2007). However, *Pneumocystis* pneumonia continues to represent a major concern in patients who are unaware that they are infected with HIV, particularly in resource limited settings, and in those patients who refuse to take or stop taking HAART, and in those with failure of antiretroviral drugs (Limper et al., 2017).

Myeloid cells such as neutrophils, monocytes, macrophages, and dendritic cells utilize pattern recognition receptors (PRRs) including the C-type lectin receptors (CLRs), Toll-like receptors (TLRs), and the intracellular nucleotide-binding oligomerization domain-like receptors (NLRs) expressed on their surface to recognize and initiate innate immune response against fungi (Hardison et al., 2012). CLRs such as Dectin-1, Dectin-2, and Mincle converge at a common point in the innate signaling pathway which involves SYK and an adaptor protein known as Caspase recruitment domain-containing protein 9 (CARD9) (Gross et al., 2006, Drummond et al., 2016). CARD9 encompasses a coiled-coil region at the C-terminus and a CARD domain in the N-terminus but lacks a PDZ domain, which is commonly shared by other CARD-containing membrane-associated guanylate kinase (CARMA) family proteins needed for their association with the plasma membrane (Gringhuis et al., 2009). CARD9 is expressed in a variety of tissues including spleen, liver, placenta, lung, peripheral blood, brain, bone marrow, and fetal liver with high expression in macrophages and dendritic cells, indicating a prominent role in inflammation and immune cell activation (Nakamura et al., 2005, Ruland, 2008).

The signaling events leading to the linkage of SYK and CARD9 are still being elucidated, but subsequent to SYK activation, CARD9 forms a trimolecular complex with BCL-10 and MALT1. Binding of these complexes together activates downstream signaling events required for production of a proinflammatory response against microbial pathogens. Studies have shown important roles of CARD9 in Dectin-1-mediated NF-kB activation and in the activation of mitogen-activated protein kinases (MAPK) such as p38 and JNK, which have crucial roles in immune responses against fungal infections (Dong et al., 2002, Wang et al., 2002, Han et al., 2005, Ashwell, 2006). CARD9 has been shown to be critical for host defenses against *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. Furthermore, humans carrying *CARD9* mutations possess increased susceptibility to lethal infections from *C. albicans* (Glocker et al., 2009, Saijo et al., 2010, Yamamoto et al., 2014, Drummond et al., 2016). More than 15 *CARD9* mutations associated with various mucosal and/or systemic fungal infections have been reported so far (Drewniak et al., 2013, Lanternier et al., 2013, Gavino et al., 2014, Wang et al., 2014, Drummond et al., 2015, Grumach et al., 2015, Herbst et al., 2015, Jachiet et al., 2015, Lanternier et al., 2015a, Lanternier et al., 2015b, Liang et al., 2015, Celmeli et al., 2016, Yan et al., 2016). Although CARD9 is clearly implicated in the innate immune response against a variety of fungal infections, the tropism of CARD9 deficiency varies across different fungal infections.
Glocker et al., 2009, Drummond et al., 2015). The mechanisms by which CARD9 provides organ-specific immune defense is not fully known and requires characterizing different fungal infections individually in the setting of CARD9 deficiency.

Accordingly, we implemented a CARD9-deficient murine model of PCP to investigate the possible function of CARD9 in host defense against *Pneumocystis*. We demonstrate that CARD9-mediated signaling is necessary for innate immune activation and for the clearance of *Pneumocystis* organisms in an immunosuppressed mouse model of PCP. In addition, we observed delays in the clearance of *Pneumocystis* from healthy wild-type (WT) animals, though the animals did not display any ill effects and survived normally. Furthermore, we present *in vitro* data supporting critical roles for CARD9 deficiency in innate lung immunity against *Pneumocystis*. Taken together, these data may provide insight into the important activity of CARD9 directly and/or indirectly in host defense during PCP.

**METHODS:**

**Ethical statement.**

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011). The Mayo Clinic Institutional Animal Care and Use Committee approved this research (A00002653).

**Animals.**

Mice with a targeted mutation in CARD9 (CARD9−/−) on the C57BL/6 background generated as described previously (PMID: 17187069) and were a kind gift from Dr. Marcel Wüthrich (University of Wisconsin). Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Approximately equal numbers of both male and female mice aged 6–10 weeks were used in all experiments. For studies of genetically modified CLR responses (i.e. CARD9−/−), mice were required. Mice have been essential for studying various components of immune response to *Pneumocystis murina* since there are many parallels to human disease (Elsegeiny et al., 2018). Rats derived *Pneumocystis carinii* were required for some studies that required large amounts of organisms for *Pneumocystis* component isolation, generated as we previously reported (Kottom et al., 2018). Of importance, our prior studies indicate the *Pneumocystis*-host interactions are highly similar in rat and mouse derived organisms and cells (Kottom et al., 2018).

**Isolation of *Pneumocystis*.**

*P. murina* and *P. carinii* organisms were derived from the American Type Culture Collection and propagated through the *Rag2tm1FwaIl2rgtm1Wjl* mice (purchased from Taconic Biosciences) and rats, respectively (Krajicek et al., 2010). All *Pneumocystis* organisms were quantified and used from frozen aliquots. Animals were infected with *P. murina* or *P. carinii* and after 8 weeks of infection, organisms harvested as previously described (Krajicek et al., 2010, Kottom et al., 2018).
Murine models of infection.

Mice from CARD9\(^{-/-}\) and wild-type strains were inoculated with \(1\times10^5\) P. murina nuclei intratracheally. The *Pneumocystis* inoculum used was purified by differential filtration as we reported to remove host related debris (Kottom et al., 2017a). We utilized intratracheal instillation to insure that each mouse received comparable organism inoculation load, and such intratracheal instillation has been used to study host responses to *Pneumocystis* (Rapaka et al., 2010, Evans et al., 2016, Hoy et al., 2020). For the immunocompetent infection model, mice were sacrificed at 20 days after infection (Kottom et al., 2018). To establish the PCP immunosuppressed animal model, wild-type and CARD9\(^{-/-}\) mice received weekly intraperitoneal injection of GK1.5 (0.3 mg) antibody to deplete CD4 T-cells (Shellito et al., 1990). After 14 days, mice were anesthetized (ketamine) and inoculated with \(1\times10^5\) *P. murina* intratracheally. Finally, mice were sacrificed 8 weeks after infection. More than 10 animals per group were used in each experiment and *P. murina* burdens and inflammatory markers were assayed on lung tissue by ELISA and qPCR. Survival analyses were also performed with identical groups of mice in separate experiments.

BMDM and alveolar macrophage isolation and culture.

For Bone Marrow-Derived Macrophages (BMDM) generation, the tibia and femur bones were excised from rats, and bone marrow cells were flushed and dispersed into culture medium. BMDM were derived in culture using RPMI 1640 medium (Life Technologies, Gaithersburg, MD) containing 20% fetal bovine serum, L-glutamine/L-alanyl L-glutamine supplementation (GlutaMAX; Thermo Fisher Scientific), MEM-nonessential amino acids, sodium pyruvate, penicillin, and streptomycin (all purchased from Life Technologies) and recombinant rat macrophage colony-stimulating factor (M-CSF; 50 ng/ml; Peprotech, Rocky Hill, NJ) for 6 days in a non-tissue culture dish. An additional volume of the medium described above was added once on the fourth day of culture. BMDM cells were removed from differentiation dishes using cold sterile pyrogen-free PBS containing 5 mM EDTA and used for the subsequent infection and stimulation experiments. Alveolar macrophages were isolated as previously described (Nakamura et al., 2015).

Cell infection experiments.

For cell infection with *P. carinii*, BMDMs were primed with Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). GM-CSF (10 ng/ml) and interferon gamma (IFN\(\gamma\)) (25 U/ml), (PeproTech, Rocky Hill, NJ) for 18 hours and were further plated into fresh RPMI 1640 medium. For all stimulation experiments, the BMDMs were challenged at a ratio of 10 *Pneumocystis* organisms per cell based on total *Pneumocystis* nuclei. The infecting inoculum is a mixture of whole *Pneumocystis* trophic forms and cysts typically in a ratio of about 10 trophic forms per cyst, as per our prior published studies (Kottom et al., 2018). Supernatants were collected for ELISA and total RNA used for real-time qPCR analysis. For alveolar macrophage infections, Similar ratio of *P. carinii* organisms were added immediately after isolation as noted above and supernatants collected for ELISA.
ELISA determination of cytokine release.

Cytokines were analyzed from the cell culture supernatants or from total lung homogenates. ELISA kits to measure mouse TNF-α, IL-12P40, IL-6, IL-1β, IFN-γ, IL-17 and IL-4 were purchased from R&D Systems, Minneapolis, MN.

qPCR.

Macrophages or lung tissues (10 mg) were lysed using QIAshredders and TissueLyser LT respectively, both purchased from Qiagen, (Germantown, MD). Total RNA was purified with the RNAasy™ mini kit (Qiagen). An iScript™ Select cDNA synthesis kit (Bio-Rad, Hercules, CA) was used for reverse transcription using oligo (dT) primers and random hexamer primer mix. A SYBR green PCR kit (Bio-Rad) was used for quantitative real-time PCR and was performed and analyzed on an CFX Connect™ PCR machine (Bio-Rad). The sequences of the primer pairs are listed in Table S1 in the supplementary section.

Determination of Pneumocystis burden.

After sacrifice, the right lung was flash frozen for quantification of Pneumocystis burden using qPCR. DNA was isolated with the DNeasy Blood and tissue kit (Qiagen). A qPCR assay was performed to enumerate relative P. murina organism burden with primers to P. murina 16S mitochondrial ribosomal RNA DNA. Amplifications of unknown samples were compared to a standard curve and the resulting measure of DNA copies were proportioned to the relative P. murina burden. To quantify live P. carinii organisms in cell cultures, total RNA was isolated from the entire contents of the well, converted to cDNA using iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA) and similar procedures were followed using primers to the 16S mitochondrial rRNA DNA.

Immunohistochemistry.

Paraffin embedding and staining were performed at the Mayo Clinic Histology Core, Scottsdale, AZ. The left lungs were inflation fixed with 10% buffered formalin overnight and embedded in paraffin. Sections (5 μm) were stained with H&E and GMS and the pixel intensities were scored using the Aperio ImageScope algorithm.

Flow cytometry analysis.

Lung cell suspensions were prepared by collagenase digestion of whole lung tissue (Miltenyi Biotec, Bergisch Gladbach, Germany) from healthy and infected animals. RBCs were lysed with lysis buffer (Thermo Fisher). Gating strategy was adopted from as described previous after exclusions of doublets and debris (Misharin et al., 2013). Briefly, the following expression patterns were used to identify alveolar macrophages (Siglec F+ CD11b−CD11c+ CD64+), CD103+ dendritic cells (CD11c+ CD64+), neutrophils (CD11b+ Ly6G+), eosinophils (Siglec F+ CD11b+ CD11c−), Ly6C+ monocytes (CD11b+ MHCII− CD64−/−Ly6C+), and CD11b+ dendritic cells (CD11b+ MHCII+ CD11c+ CD24− CD64+). Cells were washed and re-suspended in Fluorescent Activated Cell Sorting (FACS) buffer. Data was collected on the Attune NXT flow cytometer and analyzed with FlowJo software (Tree Star, Inc., Ashland OR).

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Cell signaling analysis by Western blotting.

To study the activation of MAP kinases and NF-κB, their phosphorylation kinetics were compared to the levels of the respective total proteins using Western blotting following infection of wild-type and CARD9−/− BMDMs with P. carinii for 30 min. Total lysates were prepared from cell cultures using RIPA buffer (BD Biosciences, San Jose, CA) or as the manufacturer’s instructions in the Active Motif kit (Carlsbad, CA) for phospho-IκB-α detection, and samples were further separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose membranes, immunoblotting analyses were performed as previously described (Kottom et al., 2000). Antibodies recognizing the respective phosphorylated and total proteins of ERK, JNK, p38, p-IκB-α, and GAPDH (loading control) were purchased from Cell Signaling Technology (CST), (Danvers, MA) (Kottom et al., 2017b). Phosphorylation levels of respective signaling proteins was quantified using Image Studio Lite (version 5.2.5) normalized against the total protein amount or GAPDH levels.

Overexpression of CARD9 in RAW macrophages. enhances the TNF-α secretion in response to Pneumocystis.

RAW264.7 macrophages stably expressing full length CARD9, CARD domain, or coiled-coil domain (kindly provided and characterized by David Underhill, Cedars-Sinai Medical Center, Los Angeles, CA) (Goodridge et al., 2009) were stimulated with P. carinii (10 P. carinii:1 cell) for 24 h and supernatants were harvested and analyzed by ELISA for TNF-α protein.

Statistical analysis.

Statistical differences between various experimental conditions were first assessed using ANOVA and then by Student’s t tests as indicated. Nonparametric statistics were used when data were distributed in a non-Gaussian manner. For survival analyses, comparisons were done using the Mantel-Cox (log rank) test. Statistical testing was performed using GraphPad Prism version 8.2.1 software, and statistical differences were considered significant when p < 0.05 or greater.

RESULTS

CD4 Depleted CARD9−/− mice display marked increased lung burdens in the PCP model.

To determine the overall effects of CARD9 deficiency during PCP, CARD9−/− and WT mice were immunosuppressed by depletion of their CD4 T-cells and were infected with Pneumocystis murina. Relative organism numbers (burden) in the lungs was quantified 8 weeks after infection. As shown (Fig 1A), the relative lung burdens of P. murina evaluated by 16S mitochondrial ribosomal DNA copy number were significantly higher in CD4 depleted CARD9−/− P. murina-infected mice compared to CD4 depleted WT P. murina infected mice. Silver staining on lung sections of CARD9−/− P. murina-infected mice revealed the presence of larger and more dense clusters of cysts (Fig 1B, subpanel 4) in comparison to their WT infected counterparts (Fig 1B, subpanel 3). Higher magnification (Fig 1B, subpanel 5) of the CARD9−/− mouse lung illustrates the copious amounts of cysts
present. Lastly, pixel intensity determination of the cyst life forms using the Aperio positive pixel count algorithm showed significantly more intensity corresponding to greater cyst numbers in the CARD9−/− P. murina-infected mice than WT P. murina-infected animals (Fig 1C). These data indicate the striking role of CARD9 in mediating the clearance of Pneumocystis during PCP in CD4 depleted immune suppressed animals.

**Role of CARD9 in host defense against Pneumocystis during PCP.**

The production of inflammatory cytokines is a key event in host defense against Pneumocystis. Accordingly, we investigated the effect of CARD9 deficiency on lung cytokine production during PCP. In addition to the defective Pneumocystis clearance observed, the production of inflammatory cytokines was also severely altered in the CARD9−/− lungs affected with PCP (Fig 2). This included marked suppression of the production of TNF-α, IL-6, and IL-1β in the CD4 depleted CARD9−/− compared to wild type controls. These results indicate that CARD9 mediates the production of inflammatory responses during Pneumocystis infection.

**CARD9−/− mice survive despite their significantly increased relative Pneumocystis burden.**

We further sought to determine whether there was enhanced mortality in the CD4 T-cell depleted mice lacking CARD9 in comparison to the WT CD4-depleted mice with PCP. Surprisingly, despite the markedly enhanced relative organism burdens, the CARD9−/− mice exhibited similar mortality rates compared to their WT PCP infected counterparts with a median survival of 58 and 62 days for the WT and CARD9−/− animals infected with P. murina, respectively (Fig 3A). No significant survival differences were noted for up to 9 weeks of PCP. At this time, the mice were all excessively moribund, and further time points were deemed unethical, as independently determined by our veterinarian medicine staff.

It has been established that lung inflammation strongly contributes to respiratory impairment during PCP and that death during PCP often times occurs due to excessive inflammation and not directly from the actual fungal burden (Thomas et al., 2004, Thomas Jr et al., 2007). Therefore, we next assessed the levels of Myeloperoxidase (MPO), whose concentrations are generally elevated in neutrophilic degranulation and lung injury (McCabe et al., 2001). Interestingly, we noticed a significant reduction in the levels of MPO in the CARD9−/− lungs affected with PCP compared to the wild type infected animals (Fig 3B). Furthermore, granulocyte colony stimulating factor (G-CSF), an important cytokine implicated in neutrophil differentiation and recruitment (Furze et al., 2008) was also markedly decreased in the CARD9−/− P. murina-infected lungs compared to the wild type infected controls (Fig 3B). These data support a significant reduction in neutrophilic inflammation and lung injury in CARD9−/− P. murina-infected lungs.

Next, we evaluated inflammatory cell influx in the WT vs CARD9−/− P. murina-infected lungs using lung cell suspensions prepared from whole lung tissue. Consistent with a decrease in inflammation, cells in the myeloid compartment such as alveolar macrophages, neutrophils, conventional CD11b+ DC’s, and inflammatory Ly6C+ monocytes were significantly decreased. Furthermore, eosinophils and CD103+ cells were also markedly reduced in the CARD9−/− P. murina-infected lungs compared to the wild type P. murina-
infected lungs, although not to a significant degree (Fig 3C). Furthermore, H&E staining suggested that overall lung inflammation was lower in the lungs of CARD9−/− animals (Fig 3D, subpanel 4) infected with Pneumocystis compared to wild type P. murina-infected animals (Fig 3D, subpanel 3). These observations indicate less inflammation and injury in the lungs of CARD9−/− mice and no significant impact on their survival despite, fascinatingly, the presence of substantially more Pneumocystis organisms in the lungs of the CARD9−/− mice compared to that of the wild type P. murina-infected controls.

**CARD9 participates in organism clearance in immune competent animals, but is not required for T-helper responses during Pneumocystis infection.**

Next, we wanted to determine if CARD9 deficiency affects the clearance of and immune responses to Pneumocystis in immunocompetent hosts. To test this, we infected WT and CARD9−/− animals with P. murina for 20 days and analyzed the Pneumocystis burdens and T-helper cell cytokine profile in the lungs of these animals (Kottom et al., 2018). Interestingly, there was an approximate threefold increase in organism burden in the CARD9−/− lungs isolated from the infected animals compared to that of the WT infected animals (Fig 4A), indicating reduced clearance of Pneumocystis in the absence of CARD9 in the immunocompetent host.

However, we did not observe any defects in any of the T-helper cell derived cytokines such as IL-17 (Th-17), IL4 (Th-2) and IFN-γ at 20 days post-infection (Fig 4B). In fact, the IFN-γ levels were increased about twofold in the lungs of the CARD9−/− animals infected with P. murina in comparison to that of the infected WT controls, although these numbers did not reach significance (Fig 4B). Consistent with an increase in the IFN-γ secretion, the levels of IL-12, a Th-1 stimulating cytokine were increased to a significant degree in the lungs of the CARD9−/− P. murina-infected animals compared to wild type infected counterparts (Fig 4C). While this increase in the IFN-γ levels may be attributed to the delayed clearance of Pneumocystis in the CARD9−/− lungs at 20 days, the actual defect in clearance might be due to a dysregulation of early inflammatory response against the organism. Nevertheless, these data demonstrate an important role of CARD9 in the clearance of Pneumocystis during natural elimination of the organism in healthy hosts in a similar manner to animals that were immunosuppressed and infected with PCP.

**CARD9 is required for the inflammatory response from macrophages in response to Pneumocystis.**

Although Pneumocystis clearance is largely mediated by adaptive CD4 T-cell-driven immunity, macrophages are the major effector cells for Pneumocystis host defense in the immunosuppressed hosts that lack T-cells. Therefore, we evaluated the potential of bone marrow-derived macrophages (BMDMs) to release cytokines upon stimulation with Pneumocystis in vitro. Wild type BMDMs secreted significant levels of IL-6, and IL-12 in response to Pneumocystis but we observed a severe defect in the production of these inflammatory cytokines from the CARD9-deficient macrophages in the presence of Pneumocystis (Fig 5). We also noted significant defects in the release of these two cytokines using freshly isolated alveolar macrophages stimulated with P. murina as well.
These results suggest that CARD9 is an central signaling factor for the inflammatory responses of macrophages to *Pneumocystis*.

**CARD9 is required for macrophage differentiation and induction of Dectin-1 and Mincle in response to *Pneumocystis***.

We next investigated whether the differentiation potential and the phenotypic characteristics of the CARD9-deficient bone marrow-derived macrophages would be altered when challenged with *Pneumocystis*, perhaps through an indirect feedback mechanism as a result of a defective cytokine release (O’Riordan et al., 1995, Limper et al., 1997, Koziel et al., 1998). Interestingly, qPCR analyses of CARD9−/− macrophages stimulated with *Pneumocystis* for 24 hours show a marked decrease in the expression levels of iNOS and Arg-1, which are M1 and M2 macrophage markers respectively, compared to wild type stimulated cells (Fig 6A). In addition, we observed a severe reduction in the expression levels of Dectin-1 and Mincle, two critical CLRs implicated for *Pneumocystis* host defense in the CARD9−/− macrophages compared to the wild type macrophages (Fig 6B) (Saijo et al., 2007, Kottom et al., 2017a). To assess the functional impacts of these alterations, we also tested the killing activity of the CARD9-deficient macrophages and determined they were severely compromised in their ability to kill *Pneumocystis* compared to wild type macrophages (Fig 6C). These observations suggest that CARD9 regulates the expression of Dectin-1 and Mincle and that this is required for the effective killing of *Pneumocystis* by macrophages.

**CARD9 triggers MAPK activation in macrophages following challenge with *Pneumocystis***.

MAPK activation is involved in macrophage response to *Pneumocystis* infection (Wang et al., 2011). The CARD9 adaptor protein through its interaction with the BCL-10-MALT1 complex is implicated in playing a significant role in MAPK activation downstream of the ITAM-coupled receptors in myeloid cells (Hara et al., 2009). We sought to determine whether CARD9 was indeed required for the activation of these signaling molecules in response to *Pneumocystis* stimulation in macrophages. To test this, we checked the activation of different MAPK signaling events through Western blotting and by probing these proteins with antibodies against their phosphorylated states, signifying the activation of the associated signaling events. Interestingly, in the absence of CARD9, there was a severe impairment in the activation of MAPK (p38, p-ERK, p-JNK) and NFκB-associated signaling events in response to *Pneumocystis* while the wild type macrophages, showed prompt activation of these signaling processes following organism challenge (Figure 7A-B). Thus, the CARD9 adaptor protein is required for macrophage signaling events through MAPK and NFκB pathways in response to *Pneumocystis*.

**Overexpression of CARD9 enhances macrophage TNF-α production in response to *Pneumocystis***.

Our data suggest a critical role for CARD9 in mediating an inflammatory response for the elimination of *Pneumocystis*. Lack of CARD9 leads to a blunted immune response from macrophages and an increased accumulation of the organism in the lungs of immunosuppressed *Pneumocystis* infected animals. Based on these observations, we postulated that enhancing CARD9 levels in macrophages during PCP infection, may boost...
inflammatory responses. To test this hypothesis, we used the RAW macrophage stable cell line expressing Dectin-1 and full-length CARD9 or CARD9 mutants (containing only the CARD9 domain or coiled-coil domain) and checked for the production of TNF-α after overnight stimulation with *Pneumocystis*. As expected, we noted an increase in the production of TNF-α in the CARD9 WT overexpressing cells challenged with *P. carinii in vitro* (Fig 8). Interestingly, cells expressing CARD9 mutants and overexpressing Dectin-1 did not show enhanced TNF-α secretion despite their increased concurrent overexpression of Dectin-1 (Fig 8). These data indicate the importance of CARD9 in macrophage inflammation to the organism and the requirement for the full-length protein in order for this response to occur.

**DISCUSSION**

*Pneumocystis jirovecii* causes fatal pneumonia in immunocompromised individuals, especially those with AIDS (Thomas et al., 2004). Previous studies evaluating the roles CLRs such as Dectin-1, Dectin-2, and Mincle in *Pneumocystis* infection demonstrate their importance in mediating innate immune signaling during PCP (Hoving, 2018). After ligand binding, the above mentioned CLRs initiate the intracellular signaling processes by activating SYK kinase. Downstream of SYK, CARD9 is an adaptor molecule that forms a complex with BCL-10 and MALT1, which acts as a scaffold for further signaling events resulting in MAPK and NF-κB activation and inflammatory cytokine responses (Gross et al., 2006) CARD9 deficiency is associated with a wide range of bacterial and fungal diseases and the outcome of the disease depends largely on the site of infection and the organism involved (Jhingran et al., 2015, Drummond et al., 2016). In that light, the effects of CARD9 deficiency during PCP have previously been unknown. In the current study, we demonstrate the following important points about CARD9-dependent cellular or host responses in *Pneumocystis* host defense; (i) CARD9 appears to represent a key component for host clearance of organisms during infection with *Pneumocystis*, both in immune compromised and immune competent hosts; (ii) CARD9 deficiency leads to an abrogation in the production of inflammatory cytokines during PCP; (iii) the lungs of the CARD9<sup>−/−</sup> PCP animals demonstrate less lung inflammation and markedly similar survival despite significantly greater organism burdens than CARD9 replete animals; (iv) although CARD9 was required for proper immune signaling from innate immune cells, the T-helper cell responses were normal in the CARD9-deficient *P. murina*-infected immunocompetent animals; (v) CARD9 was also required for the functional differentiation and expression of Dectin-1 and Mincle in macrophages upon its interactions with *Pneumocystis*, which in turn appears to influence macrophages ability to kill *Pneumocystis* organisms; and finally (vi) CARD9 was required for activating MAPK and NF-κB signaling events in response to *Pneumocystis* organisms. While macrophages participate in host inflammatory responses, it is noteworthy that CD4 cells remain essential for effective and lasting elimination of *Pneumocystis* infection.

Adaptor signaling molecules such as CARD9, BCL-10, and MALT1 retain important functions linking C-type lectin receptors to downstream events that are required for the activation of various inflammatory signaling pathways (Gross et al., 2006). In this study, we have characterized the critical role of the adaptor protein, CARD9 in innate immune...
responses to *Pneumocystis* through the use of a CARD9-deficient murine model of *Pneumocystis* pneumonia. Our results indicate that CARD9 is involved in the activation of MAPK such as ERK, JNK, and p38 and in the activation of NF-κB signaling events following interactions of host cells with *Pneumocystis*. It is well documented that the activation of MAPK and NF-κB in response to *Pneumocystis* are essential events for the production of proinflammatory cytokines in both macrophages and epithelial cells (Dong et al., 2002, Ashwell, 2006, Hsu et al., 2006). Consistent with this, CARD9-deficient macrophages were severely impaired in *Pneumocystis*-induced cytokine production. Although CARD9 is involved in the activation of both MAPK and NF-κB signaling events in response to *Pneumocystis*, it is interesting to note that CARD9 was selective in the activation of only specific signaling pathways in other infections. For example, in response to intracellular bacterial and viral infections, CARD9 was responsible for activating p38 and JNK but there was only a modest activation of NF-κB (Hsu et al., 2006). In contrast, CARD9 can facilitate a Dectin-1 mediated NFκB activation for antifungal immunity against the β-1,3 and β-1,6-linked β-glucan carbohydrates that are enriched in the fungal cell wall preparations of fungal derived zymosan (Gross et al., 2006). Furthermore, murine CARD9-deficient monocytes were severely compromised in *C. albicans* and zymosan-induced cytokine production (Roth et al., 2013). Another study demonstrated the involvement of CARD9 selectively in Dectin-2 induced NF-κB activation in response to *C. albicans* hyphae challenge but not to that of its heat killed form (Bi et al., 2010). These investigations emphasize the selective roles exhibited by CARD9, depending on organism and site of infection. Although the exact molecular mechanisms of these events are unknown, it’s possible that CARD9 adopts different molecular partners for selective activation of its downstream events in different cell types and with different stimulus. In support of this, one study found that Dectin-1 induced CARD9 signaling failed to activate NF-κB and drive inflammatory responses in bone marrow-derived macrophages unless they were primed with GM-CSF as opposed to the peritoneal and alveolar macrophages that readily produced an inflammatory response to the external stimulus received (Goodridge et al., 2009). Our data further support this concept, since our in vitro analyses using bone marrow-derived macrophages, required additional priming with GM-CSF and IFN-γ in order to induce the cytokine production when challenged with *Pneumocystis*.

The ability of CARD9-deficient macrophages to induce the production of proinflammatory cytokines when challenged with *Pneumocystis in vitro* was severely diminished compared to wild type cells. These events, however, were not completely abrogated and may indicate the involvement of additional CARD9-independent pathways during innate activation of macrophages to *Pneumocystis* infection (Gringhuis et al., 2009, Drummond et al., 2011, Strasser et al., 2012). Furthermore, these immune responses were insufficient to mediate the complete clearance of the organisms, as shown by a severe impairment in the in vitro elimination of *Pneumocystis* by the CARD9-deficient macrophages compared to wild type cells.

In contrast, impairment of the innate immune responses in vivo from the lungs of PCP mice that were CARD9-deficient was noted with a complete absence of proinflammatory cytokine production in comparison to wild type *P. marina*-infected mice. This may be due to defective host cytokine responses in the absence of CARD9, which could result in a milieu that does
not appropriately support the further differentiation and functional responses of various host
cells to *Pneumocystis*. In support of this, we observed defective M1 and M2 macrophage
differentiation potential, as well as suppression in the expression of CLRs Dectin-1 and
Mincle in the CARD9-deficient cells challenged with *Pneumocystis*. Other studies have
similarly highlighted the importance of CARD9 in downstream signaling events required for
the production of a proinflammatory response upon pathogen interactions (Roth et al.,
2013).

Although CARD9 functions downstream of multiple CLRs, it is interesting to note the
varying outcomes of CARD9 deficiency in different settings and compartments of infection
(Jhingran et al., 2015, Drummond et al., 2016). One reason for this may come from differing
CLR-triggered CARD9-dependent events following ligation of different microbial
components. Numerous studies have highlighted the critical role of Dectin-1, Dectin-2, and
Mincle in recognizing the distinct cell wall components of *C. albicans*, *A. fumigatus*, and *P.
carinii* and inducing effector responses against the organisms (Drummond et al., 2011, Roth
et al., 2013). Although no direct comparisons have been performed, its hypothesized that
CARD9 deficiency leads to more severe outcomes than mice lacking individual CLRs given
its non-redundant function downstream of these receptors (Drummond et al., 2011). Mice
harboring targeted CARD9 deletions are extremely susceptible to several fungal infections
from *C. albicans*, *C. tropicalis*, *A. fumigatus*, and *C. neoformans* and to certain bacterial
infections including *Listeria monocytogenes* and *Mycobacteria tuberculosis* (Dorhoi et al.,
2010, Gavino et al., 2014, Drummond et al., 2015, Jhingran et al., 2015, Drummond et al.,
2016, Rieber et al., 2016). In humans, CARD9 defect due to missense and non-sense
mutations have been associated with fungal infections such as chronic mucocutaneous
candidiasis, cutaneous/subcutaneous skin infections, or systemic fungal diseases such as
fungal meningoencephalitis caused by *Candida* species (Drewniak et al., 2013, Lanternier et
al., 2013, Gavino et al., 2014, Wang et al., 2014, Drummond et al., 2015, Grumach et al.,
2015, Herbst et al., 2015, Jachiet et al., 2015, Lanternier et al., 2015a, Lanternier et al.,
2015b, Liang et al., 2015, Celmeli et al., 2016, Yan et al., 2016).

Besides microbial-specific innate immune effects, other reasons potentially exist for the
varying outcomes of CARD9 deficiency that may stem from requirements found in differing
specific anatomical site of infection, that mediate specific modes of immune protection
(Jhingran et al., 2015). Thus, it remains essential to study each infection individually in the
CARD9-deficient setting. For instance, during PCP, CARD9-deficient immune suppressed
animals were severely impaired in their ability to clear *Pneumocystis* from their lungs.
However, despite such excessive organism burden, the animals survived to a similar manner
compared to their wild type immune suppressed counterparts with *Pneumocystis* infection.
We posit that this may be due to the absence of exuberant injurious lung inflammatory
responses that are abrogated in the CARD9-deficient animals. Lethality from PCP more is
correlated with deleterious inflammation and lung damage, rather than from direct toxic
effects of the organism itself (Thomas et al., 2004). Supporting this, we have shown that
CARD9-deficient *Pneumocystis* infected animals exhibit reduced inflammatory cell influx,
reduced G-CSF production and neutrophil-secreted products such as MPO, which are
correlated with lung injury.
In stark contrast to our observations, CARD9 deficiency during *M. tuberculosis* infection actually led to an accumulation of neutrophils and their secreted products, resulting in excessive inflammation and lung injury, accounting for the excess mortality of these animals (Dorhoi et al., 2010). However, there are other reports consistent with our observation, documenting the role of CARD9 in the recruitment of neutrophils and other inflammatory cells during infections from *Candida* and *Aspergillus* (Gavino et al., 2014, Drummond et al., 2015, Jhingran et al., 2015, Rieber et al., 2016). Unlike what has been reported for *Pneumocystis* killing, neutrophils are major effector cells for *Aspergillus* and *Candida* and animal mortality during these infections in the absence of CARD9 had been attributed to the lack of neutrophils in the sites of infection. Nevertheless, one cannot rule out the possibility that timely clearance of the pathogen through a CLR/CARD9-mediated pro-inflammatory response and subsequent activation of the anti-inflammatory feedback loop could hinder development of lethal inflammation and tissue damage, such as during *Pneumocystis* infection.

CARD9 is highly expressed in antigen presenting cells such as dendritic cells and macrophages and its activity along with CLRs and SYK leads to the production of stimulatory factors for the differentiation of naïve lymphocytes into optimal T-helper cell lineage desired for various antifungal responses (Hardison et al., 2012). Several studies have demonstrated the development of Th-1 and Th-17 responses via CARD9 following Dectin1, Dectin-2, and Mincle ligation. It had been shown that the CD4 T-cell responses to *P. carinii* involve Th-1 and Th-17, and predominantly Th-2 cell subsets (Hu et al., 2009). Although there were severe defects in the innate immune responses to *Pneumocystis* in the immunosuppressed PCP animals with CARD9 deficiency, we observed normal or increased T helper cell response in the lungs of CARD9-deficient immunocompetent animals challenged with *Pneumocystis*. The increased amounts of cytokines in the lungs of CARD9−/− mice compared to that of wild-type animals at 20 days after infection may be due to the delay in the effective clearance of *Pneumocystis*. Clearly, other CARD9-independent mechanisms are also involved in the orchestration of adaptive immune responses against *Pneumocystis*. Nevertheless, CARD9 deficient CD4 replete animals were significantly defective in their ability to clear *Pneumocystis* in spite of normal T-helper cell responses, most likely due to a defect in their innate phase immune responses.

Lastly, our data also shows a striking increase in the secretion of TNF-α via ectopic overexpression of CARD9 in *Pneumocystis* challenge in vitro. Manipulation of the CARD9 pathway in the control of cytokine production and subsequent immune responses is an exciting possibility for PCP therapy. Furthermore, future efforts in trying to understand individual CLR-induced CARD9 signaling through the use of mice lacking both CARD9 and the individual CLRs and conditional deletion of CARD9 in specific lineages to comprehend the cell specific role of CARD9 during *Pneumocystis* infection may open up new therapeutic targets along the CARD9 pathway to not only kill the pathogen but also to mitigate the deleterious proinflammatory pathogenesis associated with PCP. Our study sets the stage for such future work, by outlining the general contribution of CARD9 signaling in the control of the host response against *Pneumocystis*.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
CARD9−/− mice exhibit significantly increased organism burden during PCP. CARD9−/− and wildtype (WT) mice were immunosuppressed by depletion of CD4 cells and subsequently inoculated with P. murina organisms. (A) After 8 weeks, the P. murina organism burden was determined by P. murina 16S mitochondrial ribosomal DNA copy number and assessed using a standard curve. (Data are derived from 10 to 12 mice per group + SEM; *Denotes p < 0.05, comparing P. murina burden in the CARD9−/− and (WT) mice and are representative of 2 separate experiments.) (B) After 8 weeks of infection, the lungs were fixed in 10% phosphate-buffered formalin and 5-μm sections were obtained. Gomori Methenamine Silver stains (GMS) for P. murina organisms were performed, demonstrating clusters of stained organisms (subpanel 3, 4, and 5) whereas no organisms were noted in uninfected and CARD9−/− mice, respectively (subpanel 1 and 2). As indicated, for original magnification ×100, scale bars are 200 μm and for original magnification ×400, scale bars are 20 μm (C) GMS-stained slides were scanned using the Aperio ScanScope System at 100× magnification, typically scanning from 5 sections per slides and about 10 slides per condition. Pixel intensity was determined using the Aperio positive pixel count algorithm. The algorithm counts the number of positive (brown) and negative stains, the number of pixels, and the average intensity of staining using a hot spot approach. Data shown is the average pixel intensity + SEM from 50 sections per mouse lung and are representative of one experimental run with 12 total mice. ** Denotes p < 0.01 comparing silver stain pixel intensity in in the CARD9−/− and (WT) mice with P. murina pneumonia.
Figure 2. 
Levels of inflammatory cytokines are reduced in the lungs of PCP-induced mice in absence of CARD9. WT or CARD9−/− mice were infected with P. murina. After 8 weeks of infection, the lungs were harvested and protein lysates obtained. Levels of the indicated inflammatory cytokines were measured by ELISA. ****p < 0.0001, comparing P. murina-infected CARD9−/− mice to P. murina infected WT mice and the data shown are derived from n>10 animals + SEM per group and are representative of 2 separate animal runs.
Figure 3.
CD4 depleted CARD9−/− PCP-infected mice had significantly reduced inflammation and lung injury but similar survival rates compared to CD4 depleted controls with PCP. (A) Data shown are Kaplan-Meier survival curves of WT and CARD9−/− mice that were immunosuppressed by depletion of CD4 cells and subsequently inoculated with P. murina organisms for a course of 65 days. Despite the enhanced organism burden in the CARD9−/− mice with PCP, we did not observe any worsened mortality compared with that of the WT mice with PCP. ns=not significant, p =0.7884, by Log-rank (Mantel-Cox) test with n = 9 mice total inoculated in each group. After 8 weeks of infection WT and CARD9−/− mice that were immunosuppressed by depletion of CD4 cells, the lungs were harvested and (B) levels of the lung injury marker, MPO, and the neutrophil recruitment cytokine, G-CSF, were measured by ELISA using protein lysates and (C) inflammatory cell influx was assessed by flow cytometry and shown are the average percentages of different leukocytes in the lungs. Data in (B-C) are derived from n>10 animals per group + SEM and are
representative of 2-3 separate experimental runs; *p < 0.05; ****p < 0.0001 comparing P. murina-infected CARD9−/− mice to P. murina infected WT mice. (D) H&E staining of CARD9−/− animals infected with Pneumocystis compared to that of WT animals shows that overall lung inflammation was lower in the lungs of CARD9−/− animals infected with Pneumocystis compared to that of WT animals.
Figure 4.
CARD9 is not required for T-helper responses during Pneumocystis infection. WT or CARD9−/− mice were infected with P. murina. After 20 days of infection, the lungs were harvested and, (A) Organism burden was determined by 16S mitochondrial ribosomal DNA–targeted qPCR and copy number assessed using a standard curve. Protein lysates prepared and levels of (B) T-helper cell secreted-cytokines as measured by ELISA and, (C) IL-12, Th-1 stimulating cytokine were measured by ELISA. Data in (A-C) are derived from n=13-14 animals per group + SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 comparing P. murina–infected CARD9−/− mice to P. murina–infected WT mice.
Figure 5.
CARD9 is a critical factor in macrophages for the production of inflammatory response during Pneumocystis infection. BMDM were stimulated with P. carinii (10 P. carinii:1 cell) for 24 h after overnight priming with 10 ng/ml of GM-CSF or 25 U/ml IFNγ. After stimulation, supernatants were harvested and analyzed by ELISA for protein levels of different cytokines as indicated. The data shown are integrated means ± the SEM of 4-5 independent experiments; *p < 0.05, **p < 0.01.
Figure 6.
Macrophages require CARD9 for differentiation and induction of Dectin-1 and Mincle in response to Pneumocystis. BMDM were stimulated with P. carinii (10 P. carinii:1 cell) for 24 h. After stimulation, macrophage mRNA samples were harvested and analyzed by qPCR for mRNA expression levels of (A) M1 (iNOS) and M2 (Arg-1) markers and (B) Dectin-1 and Mincle, respectively. The mRNA expression levels were normalized to GAPDH and data shown are integrated means ± the SEM of at least three independent experiments; *p < 0.05, p** < 0.01. (C) BMDMs were stimulated with P. carinii for 24 hours and total DNA was extracted from the contents of each well and qPCR for P. carinii 16S mitochondrial ribosomal DNA copy number was performed. The data are integrated means ± the SEM from five independent experiments; *p < 0.05.
Figure 7. CARD9 triggers MAPK and NF-κB activation in macrophages following challenge with Pneumocystis.

(A). Macrophage lysates from BMDM stimulated with P. carinii were prepared at the indicated times and expression levels of total and phospho-proteins as indicated were analyzed by Western blot to assess the activation of the different signaling events in the presence and absence of CARD9. GAPDH is used as a loading control for p-IκB-α, since the total IκB-α degrades with activation. Data are representative of three independent experiments. (B). Quantification of phosphorylation of signaling proteins was quantified with Image Studio Lite software and normalized against the total protein amount or GAPDH levels as denoted. (* Denotes p<0.05 compared to control).
Overexpression of CARD9 in macrophages enhances the TNF-α secretion in response to Pneumocystis. RAW264.7 macrophages stably expressing full length CARD9, CARD domain, or coiled-coil domain were stimulated with P. carinii (10 P. carinii:1 cell) for 24 h. After stimulation, supernatants were harvested and analyzed by (A) ELISA for TNF-α protein. Data shown are integrated means ± the SEM of three independent experiments; *p < 0.05, **p < 0.01 compared with the cells expressing the empty control vector or comparing full length CARD9 to the respective CARD9 mutants.