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Critical role of intracellular osteopontin in anti-fungal innate immune responses

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Abstract

We found that absence of osteopontin (OPN) in immunocompromised *Rag2*$^{-/-}$ mice, which lack T and B cells, made the mice extremely susceptible to an opportunistic fungus *Pneumocystis*, although immunocompetent OPN-deficient mice could clear *Pneumocystis* as well as wild-type mice. OPN has been studied as an extracellular protein, and the role of an intracellular isoform of osteopontin (iOPN) is still largely unknown. Here, we elucidated the mechanism by which iOPN was involved in anti-fungal innate immunity. First, iOPN was essential for cluster formation of fungal receptors that detect *Pneumocystis*; including dectin-1, TLR2, and mannose receptor. Second, iOPN played a role as an adaptor molecule in TLR2 and dectin-1 signaling pathways, and mediated ERK activation and cytokine production by zymosan, which simultaneously activates TLR2 and dectin-1 pathways. Third, iOPN enhanced phagocytosis and clearance of *Pneumocystis.* Our study here suggests the critical involvement of iOPN in anti-fungal innate immunity.

Introduction

Pneumocystis is an opportunistic fungal pathogen that cause of serious morbidity and mortality in immunocompromised individuals. Host cells recognize *Pneumocystis* via pattern recognition receptors (PRRs), such as dectin-1, Toll-like receptor 2 (TLR2), and mannose receptor (MR), followed by phagocytosis, cytokine production, and killing the pathogen (1–3). These PRRs are recruited in proximity on the cell surface and cooperatively integrate their signals (4–7). However, the molecular mechanisms by which these PRRs collaborate are largely unknown.

Osteopontin (OPN) is a protein, which is expressed by a variety of tissues and cell types, including macrophages, dendritic cells (DCs), NK cells, neutrophils, and T and B lymphocytes. The role of OPN in innate immunity is reflected by its protective role in infectious diseases, but only limited numbers of reports are available on the involvement of OPN in host responses against fungi (8, 9). A recent microarray study, however, showed that

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expression of *Opn* mRNA had the highest upregulation 8 weeks after *Pneumocystis* infection in alveolar macrophages from immunosuppressed rats (10). OPN has two types of isoforms; secreted OPN (sOPN) and intracellular OPN (iOPN) that was recently characterized (11–17). $Opn^{-/-}$ mice showed attenuated resistance to a low dose *Candida albicans* infection (9), but depletion of OPN by antibody (Ab) in wild-type (WT) mice did not attenuate the resistance to *C. albicans* (9). The finding suggested that the host resistance to *C. albicans* is attributed to iOPN, but not sOPN, because OPN Ab only depletes sOPN.

OPN has been studied as extracellular protein, *i.e.,* as sOPN, and the role of iOPN is much less characterized compared to that of sOPN. Thus, current understanding on iOPN is limited, but it is known that innate immune phagocytes, such as macrophages and DCs, constitutively express high levels of iOPN (and sOPN), but T cells prefer to express sOPN (12, 14, 18). Currently, iOPN is known to participate in TLR9 signaling, by interacting with MyD88, and enhances IFNα production by plasmacytoid DCs (14). These results suggested that iOPN plays a role as an adaptor molecule in innate immune signaling pathways.

Adaptive immunity is considered to be the ultimate mechanism to resolve opportunistic fungal infection. However, our study here demonstrated that iOPN in $Rag2^{-/-}$ mice, which lack adaptive immunity, make a significant difference in host resistance against opportunistic *Pneumocystis* fungal infection. In contrast, OPN-deficient immunocompetent mice can clear *Pneumocystis*, suggesting that OPN is not essential in the presence of adaptive immunity. However, OPN still matters in immunocompromised hosts -- patients who contract opportunistic fungal infections are immunocompromised. In this study, we also demonstrated that iOPN does not only play a critical role in formation of fungal PRR clusters on the cell surface, but iOPN is also involved in TLR2 and in dectin-1 signaling transduction pathways as an adaptor molecule.

Materials and Methods

Animals and Pneumocystis

C57BL/6 background mice were used in this study. $Opn^{-/-}$ mice were a gift from D. Denhardt and S. Rittling (Rutgers University), and backcrossed to C57BL/6 mice for 15 generations. $Rag2^{-/-}$ mice (Jackson Laboratory) were crossed to $Opn^{-/-}$ mice to generate $Opn^{-/-}$ $Rag2^{-/-}$ mice. All the mice were kept in a barrier facility. This study was approved by the Duke University Institutional Animal Care and Use Committee. *Pneumocystis* was obtained from ATCC (*Pneumocystis murina,* PRA-111), propagated *in vivo* by infecting $Rag2^{-/-}$ mice, and was harvested from lungs as previously described (19).

qPCR conditions and primer sequences

mRNA expression levels were determined by using the $-\Delta\Delta Ct$ method of real-time PCR. qPCR primers/TaqMan probe for *Pneumocystis rRNA* (20) and *PCMei2* (19) are previously described. *Actb* mRNA (encoding β-actin) was used as an internal control (14).

Confocal microscopy

To evaluate colocalization of *Pneumocystis* recognition receptors and iOPN, we stained cells with antibodies against OPN (2A1, Santa Cruz), MR (BioLegend), dectin-1 (BioLegend), and TLR2 (Molecular Probe) with fluorophore-conjugated secondary antibodies. The frequency (%) of cells that show clustering of iOPN and PRRs was calculated by enumerating cells that formed the cluster from at least 50 cells, which were either attached to *Pneumocystis* or had phagocytosed *Pneumocystis*.

Immunoprecipitation, immunoblotting, and ELISA

Immunoprecipitation was carried out with OPN antibody (2A1), followed by immunoblotting with IRAK1 antibody (Assay Designs) or Syk antibody (BioLengend) with Pam_2CSK_4 (TLR2 ligand) and curdlan (dectin-1 ligand), respectively. To evaluateERK activation by zymosan, peritoneal macrophages from WT and *Opn*$^{-/-}$ mice were used. To neutralize secreted OPN, we treated cells with a neutralizing antibody, AF808 (R&D Systems) at 10 μg/ml to the cells 10 min before *Pneumocystis* treatment. *Opn*$^{-/-}$ macrophages were infected using lentiviral vectors to express GFP and iOPN, as previously described (14). ERK and phospho-ERK were detected by immunoblotting. Levels of IL-1β and IL-10 in the culture supernatants of zymosan-stimulated macrophages were determined by ELISA (BD Pharmingen).

Analyzing phagocytosis, ROS production, and Pneumocystis clearance

Pneumocystis was labeled as previously reported (21), except for using *Pneumocystis* (1×10^7 cysts) with 2 μl of 10 mg/ml Alexa fluor 647 (Molecular Probe). Macrophages were stained with CD11b, and phagocytosis was evaluated by detecting CD11b and Alexa-647 double positive cells. To evaluate reactive oxygen species (ROS) generation by macrophage stimulation with *Pneumocystis*, dihydroethidium (DHE) staining was detected in CD11b-positive cells. Detection of *Pneumocystis* clearance was described previously (22).

Statistical analysis

Statistical analysis was evaluated using Student's *t* tests. The criterion of significance was set as $p<0.05$. All results are expressed as mean ± SEM.

Results and Discussion

OPN-deficient Rag2$^{-/-}$ mice are susceptible to spontaneous Pneumocystis infection

Immunocompromised *Rag2*$^{-/-}$ mice, which lack T and B cells, are susceptible to opportunistic fungal infection by *Pneumocystis*. *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice became sick after 12–13 weeks of age, under the environment that *Opn*$^{+/+}$*Rag2*$^{-/-}$ mice were healthy and showing no signs of disease. *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice were sick and clearly smaller in size compared to the *Opn*$^{+/+}$*Rag2*$^{-/-}$ mice at 13-week-old (Supplemental Fig. 1A), and started dying from 12–13 weeks of age (Fig. 1A). We performed pathology analyses on 14-week-old mice. Although the lungs from *Opn*$^{+/+}$*Rag2*$^{-/-}$ mice appeared normal (Fig. 1B, left), those from the *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice indicated typical *Pneumocystis* histopathology, *i.e.*, foamy exudates, stained in pink with H&E, which were filling alveolar spaces (Fig. 1B. center and right). Lung sections of *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice also showed extensive inflammatory cell infiltration, and lack of airway spaces (Fig. 1B). *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice could recruit cells into lungs (Fig. 1B), but the ability of cell recruitment did not appear to be a decisive factor to clear Pneumocystis, because of the high loads of *Pneumocystis* in *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice.

Pneumocystis cannot be cultured; therefore, it is not possible to assess the fungal loads by CFU (colony forming unit). Therefore, we confirmed *Pneumocystis* infection by detecting the expression of the mitochondrial large subunit *rRNA* of *Pneumocystis* (22) and *Pneumocystis*-specific gene *PCMei2* (19). Four-week old *Opn*$^{-/-}$*Rag2*$^{-/-}$ (double knockout; DKO) mice looked healthy, and the levels of the large subunit *Pneumocystis rRNA* and *PCMei2* mRNA were around the detection limit in their lungs (Fig. 1C, supplemental Fig. 1B). However, 14-week-old *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice showed much higher expression levels of both transcripts than the 4-week-old *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice. This suggested that the mice developed intensive *Pneumocystis* infection. In addition to lungs, the presence of *Pneumocystis* was also detected in mediastinal (lung draining) lymph nodes (LNs) from the 14-week-old *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice. Lungs from single gene deficient mice, *i.e.,*

Opn$^{+/+}$*Rag2*$^{-/-}$ and *Opn*$^{-/-}$*Rag2*$^{+/+}$ mice, at the age of 14 weeks did not have detectable levels of *Pneumocystis rRNA* and *PCMei2* transcripts (Fig. 1C, supplemental Fig. 1B), suggesting that single knockouts remained highly resistant to opportunistic *Pneumocystis* infection. The data strongly suggested that OPN is not essential to protect hosts against opportunistic *Pneumocystis* when adaptive immunity is equipped. In summary, OPN plays a role to protect hosts against opportunistic *Pneumocystis* infection in innate immunity, but OPN is dispensable in the presence of adaptive immunity.

Co-localization of iOPN and fungal PRRs in macrophages stimulated by Pneumocystis

Macrophages contribute to the host immune responses to *Pneumocystis* by phagocytosis and subsequent killing of the fungus. TLR2, dectin-1, and MR are known to play a critical role in detection and clearance of *Pneumocystis* (1–3, 6, 23). Previous studies showed co-localization of the PRRs, and such physical proximity of the PRRs makes their signals integrated and synergized to enhance host responses (4–7). To evaluate involvement of iOPN in the association of fungal PRRs, we detected iOPN, TLR2, and dectin-1 or MR in wild-type (WT) macrophages stimulated with *Pneumocystis*. Thirty min after *Pneumocystis* treatment, cells showed co-localization of iOPN, TLR2, dectin-1, and MR upon contact with *Pneumocystis* (Fig. 2A, B; Supplemental Fig. 2A). On the other hand, *Opn*$^{-/-}$ macrophages failed to co-localize the PRR cluster (Fig. 2C, D; Supplemental Fig. 2B), suggesting that iOPN is essential for PRR clustering.

To elucidate a role of iOPN in downstream signaling pathways from TLR2, we first tested whether iOPN associates with IRAK1, which is one of downstream kinases of the TLR2 signaling pathway. IRAK1 was selected, because of possible interdependence between iOPN and IRAK1 from previous studies, demonstrating that iOPN and IRAK1 are required for TLR9-mediated IRF7 activation (14, 24). TLR2 stimulation with Pam_2CSK_4, a TLR2 ligand,induced OPN-IRAK1 association (Fig. 2E). This finding suggested the involvement of iOPN in TLR2 signaling. We also found that iOPN is involved in a dectin-1 signaling pathway by associating with Syk after stimulation with curdlan, a dectin-1 ligand (Fig. 2F). It should be noted that whole cell lysates include both iOPN and sOPN. Yet, OPN that associates with IRAK1 and Syk is iOPN, because iOPN, IRAK1 and Syk are cytosolic proteins but sOPN is segregated in secretory vesicles. In summary, we found that iOPN is essential for fungal PRR cluster formation and involved in signaling downstream of TLR2 and dectin-1 pathways by associating with IRAK1 and Syk, respectively.

iOPN facilitates phagocytosis and clearance of Pneumocystis

We next tested the requirement of iOPN in the phagocytosis of *Pneumocystis. Pneumocystis* labeled with Alexa-647 was cultured with WT or *Opn*$^{-/-}$ macrophages, and phagocytosis of *Pneumocystis* by macrophages was detected by flow cytometry. Phagocytosis was significantly reduced in *Opn*$^{-/-}$ macrophages, compared in WT macrophages (Fig. 3A; Supplemental Fig. 3A). We next evaluated which OPN, sOPN or iOPN, is involved in phagocytosis of *Pneumocystis,* by antibody-mediated sOPN depletion in WT macrophages and lentivirus-mediated iOPN expression in *Opn*$^{-/-}$macrophages. sOPN depletion with OPN antibody (AF808; 10 μg/ml) in WT cell culture treatment did not reduce phagocytic activity (Fig. 3B, left panel), but expression of iOPN in *Opn*$^{-/-}$ macrophages rescued the OPN-deficient phenotype of decreased phagocytic ability (Fig. 3B, right panel). These findings strongly suggested that iOPN, but not sOPN, was critical for the phagocytosis of *Pneumocystis* by macrophages.

Once pathogen is phagocytosed, ROS production is critical for its killing (25, 26). It was reported that ROS generation is impaired in dectin-1-deficient macrophages upon stimulation with *Pneumocystis* (2). We found that *Opn*$^{-/-}$ macrophages had significantly

reduced ROS generation (Fig. 3C; Supplemental Fig. 3B). In addition, ROS activity was not affected by treatment with OPN antibody (Fig. 3D, left panel), but lentiviral reconstitution of iOPN expression in *Opn*$^{-/-}$ macrophages induced ROS generation (Fig. 3D, right panel). The result suggests that iOPN, not sOPN, is involved in ROS generation in macrophages that phagocytosed *Pneumocystis*. It is of note that deficiency of p91^{phox} (Nox2), a component of the Nox2 NADPH oxidase that produces ROS, did not affect tissue damages in *Pneumocystis*-infected CD4 T cell-depleted immunocompetent mice (27). Since Sasaki et al. (28) demonstrated that Nox1 could compensate ROS generation in Nox2-deficient bone marrow macrophages/monocytes, the Nox1 NADPH oxidase may compensate resistance against *Pneumocystis* in Nox2-deficient mice. We also found that *Pneumocystis* was not effectively killed in *Opn*$^{-/-}$ macrophages as in WT macrophages. Higher levels of *Pneumocystis rRNA* and *PCMei2* expression were detected in *Opn*$^{-/-}$ macrophages than wild-type macrophages, which phagocytosed *Pneumocystis* (Fig. 3E; Supplementary Fig. 3C). Collectively, our findings strongly suggested that iOPN in macrophages played a critical role to clear *Pneumocystis* by enhancing phagocytosis, ROS generation, and fungal killing.

iOPN enhances zymosan-induced cytokine production in macrophage

We next tested the requirement of iOPN in the cytokine production by macrophages stimulated with *Pneumocystis*. Although *Opn*$^{-/-}$ macrophages appeared to produce less IL-1β and IL-10 than WT macrophages when stimulated with *Pneumocystis* (Supplementary Fig. 3D), the levels of cytokines were not high enough by ELISA detection. To better assess the consequence of simultaneous stimulation of multiple receptors that detect *Pneumocystis*, we therefore used zymosan, a glucan from fungal cell wall. Zymosan, simultaneously ligates dectin-1 and TLR2, which are essential for the detection of *Pneumocystis* by host cells (5, 29–31). *Opn*$^{-/-}$ macrophages had significantly reduced production of both IL-1β and IL-10 (Fig. 4A), but not TNFα and IFNγ (Supplemental Fig. 3D), upon zymosan stimulation. Because iOPN is essential for clustering of dectin-1 and TLR2, and involved in PRR signaling as an adaptor molecule (Fig. 2), we asked whether iOPN is involved in the production of IL-1β and IL-10 by zymosan stimulation. iOPN expression successfully restored the production of IL-1β and IL-10 in *Opn*$^{-/-}$ macrophages (Fig. 4B), suggesting the involvement of iOPN in signal transduction pathways activated by zymosan. It is known that zymosan induces IL-10 production through ERK activation (30). *Opn*$^{-/-}$ macrophages had reduced ERK activation compared to WT macrophages, and iOPN expression in *Opn*$^{-/-}$ macrophages restored ERK activation (Fig. 4C). Collectively, our findings demonstrated that iOPN mediated signal transduction of dectin-1 and TLR2, by interacting with IRAK1 and Syk, leading to ERK-dependent cytokine production.

In conclusion, we have shown that *Opn*$^{-/-}$*Rag*$^{-/-}$ mice are extremely susceptible to spontaneous *Pneumocystis* infection. iOPN is essential for generating anti-fungal innate immune responses in PRR recognition, signal transduction, phagocytosis, and clearance of *Pneumocystis,* and cytokine production. It appears that iOPN not only clusters fungal PRRs together, but iOPN also functions as an adaptor molecule that transduces signals from PRRs (Supplemental Fig. 4). This study demonstrated a new mechanism by which a novel OPN isotype, iOPN, plays a critical role. Lack of iOPN in the innate immune system may explain the extremely susceptible phenotype of *Opn*$^{-/-}$*Rag*$^{-/-}$ mice to opportunistic *Pneumocystis* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ezekowitz RA, Williams DJ, Koziel H, Armstrong MY, Warner A, Richards FF, Rose RM. Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. Nature. 1991; 351:155–158. [PubMed: 1903183]
2. Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, Kinjo T, Nakamura K, Kawakami K, Iwakura Y. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat Immunol. 2007; 8:39–46. [PubMed: 17159982]
3. Wang SH, Zhang C, Lasbury ME, Liao CP, Durant PJ, Tschang D, Lee CH. Decreased inflammatory response in Toll-like receptor 2 knockout mice is associated with exacerbated Pneumocystis pneumonia. Microbes Infect. 2008; 10:334–341. [PubMed: 18400546]
4. Ip WK, Takahashi K, Moore KJ, Stuart LM, Ezekowitz RA. Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome. J Exp Med. 2008; 205:169–181. [PubMed: 18180310]
5. Dennehy KM, Ferwerda G, Faro-Trindade I, Pyz E, Willment JA, Taylor PR, Kerrigan A, Tsoni SV, Gordon S, Meyer-Wentrup F, Adema GJ, Kullberg BJ, Schweighoffer E, Tybulewicz V, Mora-Montes HM, Gow NA, Williams DL, Netea MG, Brown GD. Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Eur J Immunol. 2008; 38:500–506. [PubMed: 18200499]
6. Tachado SD, Zhang J, Zhu J, Patel N, Cushion M, Koziel H. Pneumocystis-mediated IL-8 release by macrophages requires coexpression of mannose receptors and TLR2. J Leukoc Biol. 2007; 81:205–211. [PubMed: 17020928]
7. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol. 2007; 7:179–190. [PubMed: 17318230]
8. Nishikaku AS, Scavone R, Molina RF, Albe BP, da Cunha SC, Burger E. Osteopontin involvement in granuloma formation and in the severity of Paracoccidioides brasiliensis infection. Med Mycol. 2009; 47:495–507. [PubMed: 19048431]
9. Sato I, Yamamoto N, Rittling SR, Denhardt DT, Hino M, Morimoro J, Sakai F, Fujie A, Uede T. Osteopontin is dispensable for protection against high load systemic fungal infection. Int Immunopharmacol. 2008; 8:1441–1448. [PubMed: 18590834]
10. Cheng BH, Liu Y, Xuei X, Liao CP, Lu D, Lasbury ME, Durant PJ, Lee CH. Microarray studies on effects of Pneumocystis carinii infection on global gene expression in alveolar macrophages. BMC Microbiol. 2010; 10:103. [PubMed: 20377877]
11. Cantor H, Shinohara ML. Regulation of T-helper-cell lineage development by osteopontin: the inside story. Nat Rev Immunol. 2009; 9:137–141. [PubMed: 19096390]
12. Shinohara ML, Kim HJ, Kim JH, Garcia VA, Cantor H. Alternative translation of osteopontin generates intracellular and secreted isoforms that mediate distinct biological activities in dendritic cells. Proc Natl Acad Sci U S A. 2008; 105:7235–7239. [PubMed: 18480255]
13. Shinohara ML, Kim JH, Garcia VA, Cantor H. Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. Immunity. 2008; 29:68–78. [PubMed: 18619869]
14. Shinohara ML, Lu L, Bu J, Werneck MB, Kobayashi KS, Glimcher LH, Cantor H. Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. Nat Immunol. 2006; 7:498–506. [PubMed: 16604075]
15. Suzuki K, Zhu B, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CA, Sodek J. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. J Bone Miner Res. 2002; 17:1486–1497. [PubMed: 12162503]

16. Zhu B, Suzuki K, Goldberg HA, Rittling SR, Denhardt DT, McCulloch CA, Sodek J. Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: evidence of a role for an intracellular form of osteopontin. J Cell Physiol. 2004; 198:155–167. [PubMed: 14584055]
17. Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA, Sodek J. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol. 2000; 184:118–130. [PubMed: 10825241]
18. Shinohara ML, Jansson M, Hwang ES, Werneck MB, Glimcher LH, Cantor H. T-bet-dependent expression of osteopontin contributes to T cell polarization. Proc Natl Acad Sci U S A. 2005; 102:17101–17106. [PubMed: 16286640]
19. Burgess JW, Kottom TJ, Limper AH. Pneumocystis carinii exhibits a conserved meiotic control pathway. Infect Immun. 2008; 76:417–425. [PubMed: 17984205]
20. Motz GT, Eppert BL, Sun G, Wesselkamper SC, Linke MJ, Deka R, Borchers MT. Persistence of lung CD8 T cell oligoclonal expansions upon smoking cessation in a mouse model of cigarette smoke-induced emphysema. J Immunol. 2008; 181:8036–8043. [PubMed: 19017996]
21. Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, morphotype-specific Candida albicans beta-glucan exposure during infection and drug treatment. PLoS Pathog. 2008; 4:e1000227. [PubMed: 19057660]
22. Nelson MP, Metz AE, Li S, Lowell CA, Steele C. The absence of Hck, Fgr, and Lyn tyrosine kinases augments lung innate immune responses to Pneumocystis murina. Infect Immun. 2009; 77:1790–1797. [PubMed: 19255189]
23. Zhang C, Wang SH, Liao CP, Lasbury ME, Durant PJ, Tschang D, Lee CH. Toll-like receptor 2 knockout reduces lung inflammation during Pneumocystis pneumonia but has no effect on phagocytosis of Pneumocystis organisms by alveolar macrophages. J Eukaryot Microbiol. 2006; 53(Suppl 1):S132–133. [PubMed: 17169030]
24. Uematsu S, Sato S, Yamamoto M, Hirotani T, Kato H, Takeshita F, Matsuda M, Coban C, Ishii KJ, Kawai T, Takeuchi O, Akira S. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. J Exp Med. 2005; 201:915–923. [PubMed: 15767370]
25. Forman HJ, Torres M. Signaling by the respiratory burst in macrophages. IUBMB Life. 2001; 51:365–371. [PubMed: 11758804]
26. Henricks PA, Nijkamp FP. Reactive oxygen species as mediators in asthma. Pulm Pharmacol Ther. 2001; 14:409–420. [PubMed: 11782121]
27. Swain SD, Wright TW, Degel PM, Gigliotti F, Harmsen AG. Neither neutrophils nor reactive oxygen species contribute to tissue damage during Pneumocystis pneumonia in mice. Infect Immun. 2004; 72:5722–5732. [PubMed: 15385471]
28. Sasaki H, Yamamoto H, Tominaga K, Masuda K, Kawai T, Teshima-Kondo S, Matsuno K, Yabe-Nishimura C, Rokutan K. Receptor activator of nuclear factor-kappaB ligand-induced mouse osteoclast differentiation is associated with switching between NADPH oxidase homologues. Free Radic Biol Med. 2009; 47:189–199. [PubMed: 19409483]
29. Lamkanfi M, Malireddi RK, Kanneganti TD. Fungal zymosan and mannan activate the cryopyrin inflammasome. J Biol Chem. 2009; 284:20574–20581. [PubMed: 19509280]
30. Goodridge HS, Simmons RM, Underhill DM. Dectin-1 stimulation by Candida albicans yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. J Immunol. 2007; 178:3107–3115. [PubMed: 17312158]
31. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med. 2003; 197:1119–1124. [PubMed: 12719478]

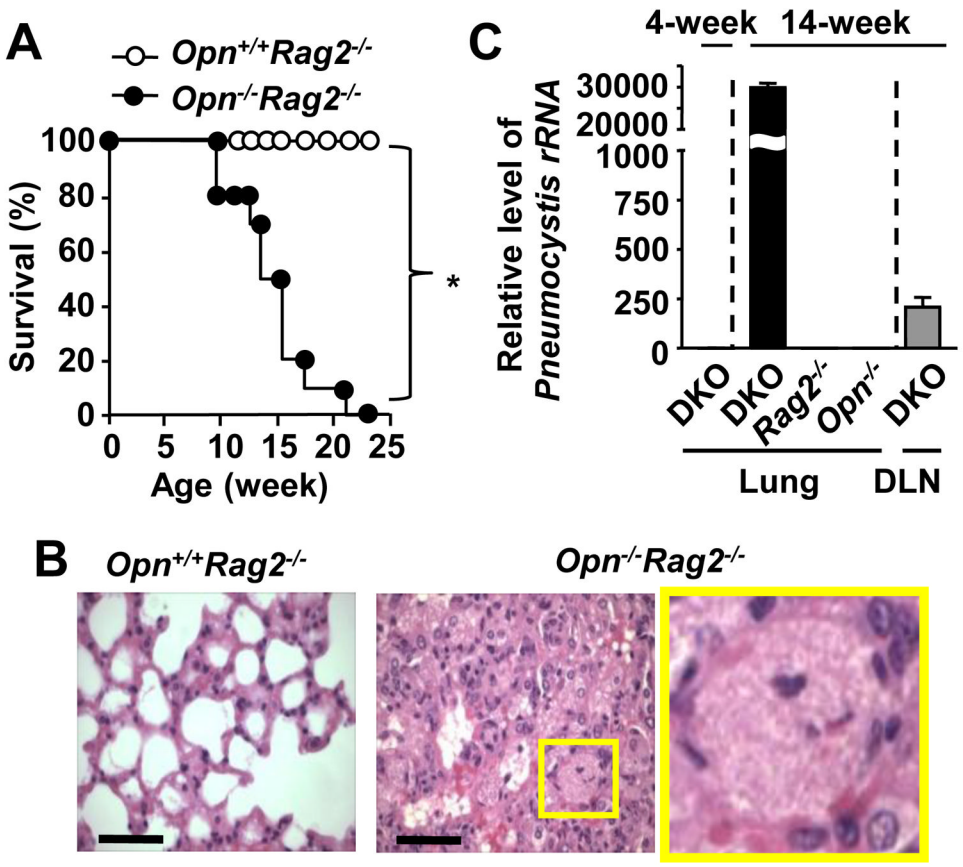

Figure 1. OPN-deficient *Rag2*$^{-/-}$ mice are extremely susceptible to spontaneous *Pneumocystis*

(A) Survival ratio of *Opn*$^{+/+}$*Rag2*$^{-/-}$ mice and *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice. n=10. **(B)** Representative H&E staining in the lungs of 13-week-old *Opn*$^{+/+}$*Rag2*$^{-/-}$ mice and *Opn*$^{-/-}$*Rag2*$^{-/-}$ mice. The image in a yellow rectangle in the middle panel is enlarged in the right panel. Scale bars = 50 μm. **(C)** *PCMei2* mRNA was detected by qPCR, normalized with β-actin mRNA. First column denotes *PCMei2* levels in lungs of 4-week-old *Opn*$^{-/-}$*Rag2*$^{-/-}$ (DKO) mice, otherwise all the samples are from 14-week-old mice of DKO, and single KO of *Rag2*$^{-/-}$ or *Opn*$^{-/-}$. The last column denotes values from lung draining LNs from 14-week-old DKO mice.

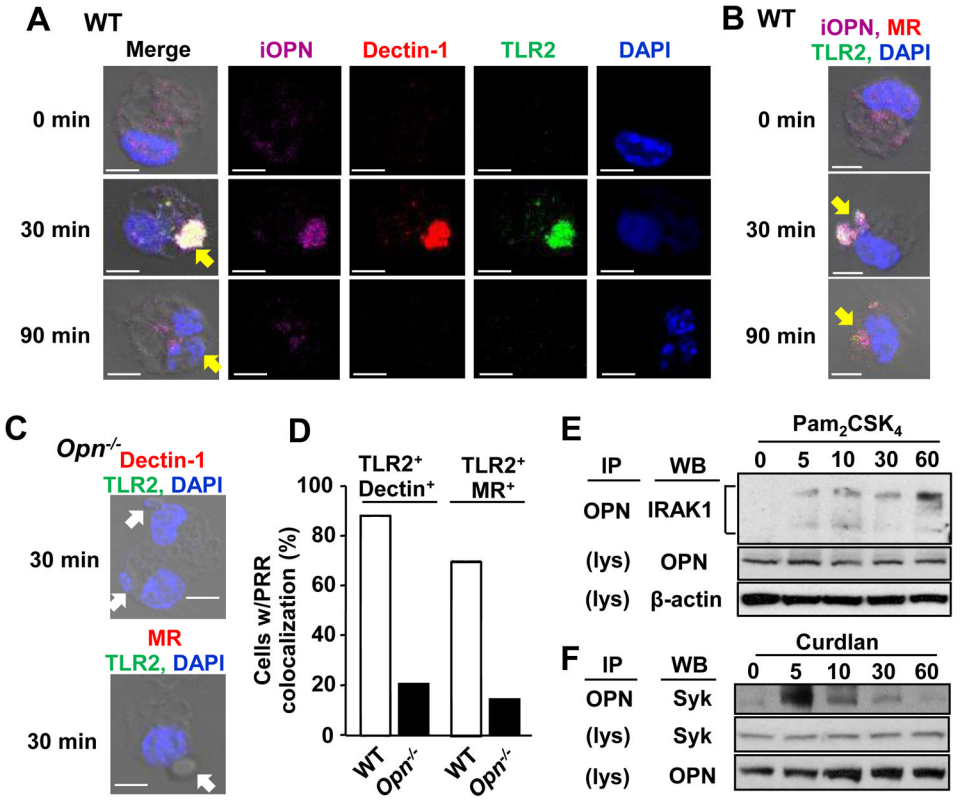

Figure 2. Interaction of iOPN and fungal PRRs by *Pneumocystis* treatment

(A) Representative confocal microscopic images of OPN (magenta), dectin-1 (red), TLR2 (green) and DAPI (blue) staining in WT macrophages at indicated time points after co-culture with *Pneumocystis*. **(B)** Same as (A), except that MR is indicated with red. **(C)** No distinct cluster formation of PRRs in $Opn^{-/-}$ macrophages 30 min after *Pneumocystis* co-culture. (A–C) Yellow and white arrows represent phagocytosed *Pneumocystis* and *Pneumocystis* contacting to macrophages, respectively. Scale bars = 5 μm. See Supplemental Fig. 2A and 2B for single color staining images for (B) and (C), respectively. **(D)** Calculated frequency of macrophages with PRR colocalization (TLR2 and dectin-1 or MR) at 30 min after *Pneumocystis* co-culture. **(E)** Immunoprecipitation with OPN in RAW 264.7 cells stimulated with Pam_2CSK_4 (100 ng/ml) for 0, 5, 10, 30, and 60 min. Immunoblot were performed with anti-IRAK-1. OPN and β-actin were detected in non-immunoprecipitated cell lysates (indicated as “lys”). **(F)** Co-immunoprecipitation of iOPN and Syk in peritoneal macrophages stimulated with curdlan (100 μg/ml).

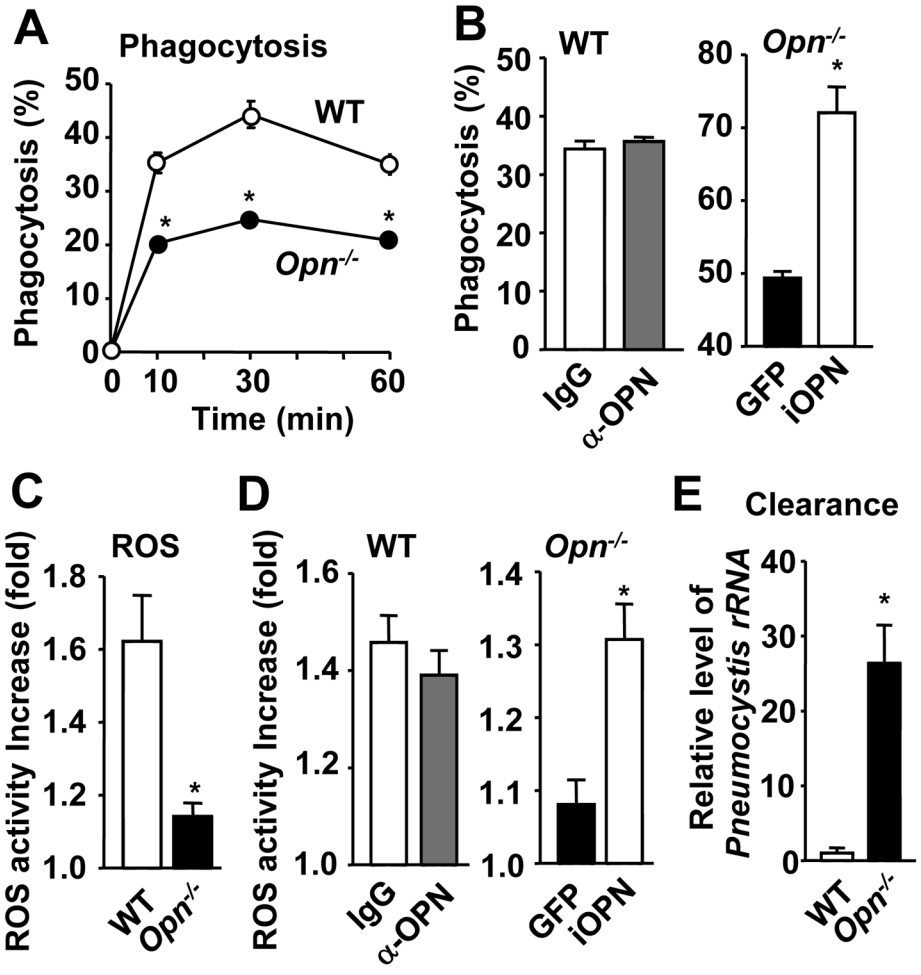

Figure 3. Involvement of iOPN in phagocytosis, ROS generation, and fungal clearance by macrophage

(A) Phagocytosis of *Pneumocystis* by macrophages. Percentages of Alexa-647/CD11b double-positive cells over total CD11b positive cells were calculated (Supplemental Fig. 3A). **(B)** WT macrophages were cultured with *Pneumocystis* with OPN antibody (AF808, 10 μg/ml) or control IgG (left panel). iOPN was lentivirally expressed in $Opn^{-/-}$ macrophages (right panel). Phagocytic activity at 60 min. **(C)** ROS activity increase is calculated as MFI value increase (%) of DHE staining in the initial 1 h after *Pneumocystis* stimulation (Supplemental Fig. 3B). **(D)** Comparison of ROS generation by sOPN depletion in WT cells and by iOPN expression in $Opn^{-/-}$ cells, as described in (B). **(E)** *Pneumocystis* clearance was detected with *Pneumocystis rRNA* in macrophages.

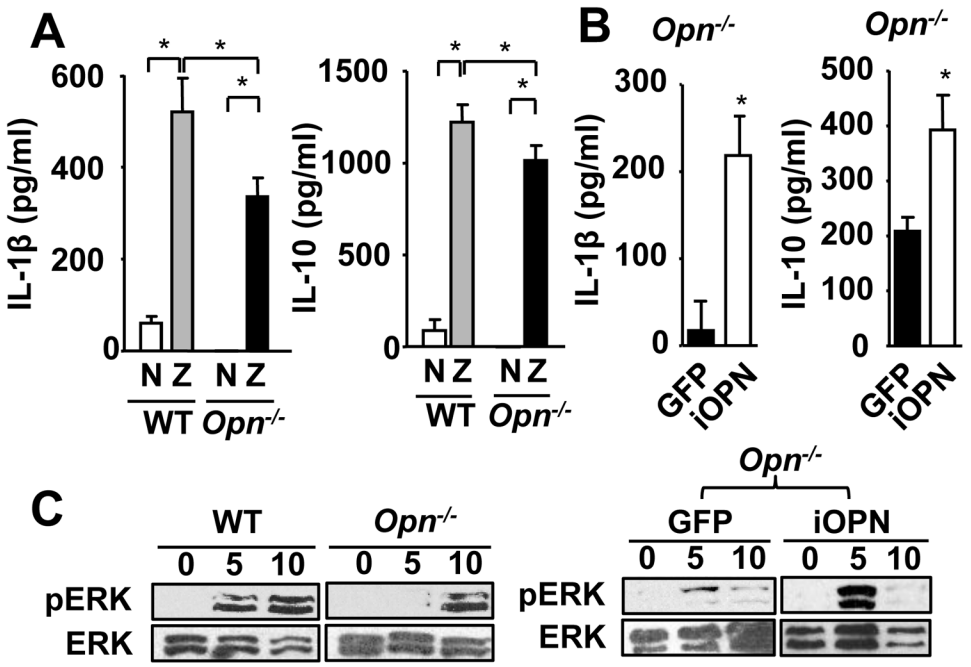

Figure 4. Involvement of iOPN in activating signal transduction triggered by zymosan

(A) Expression of IL-1β and IL-10 by WTand *Opn*$^{-/-}$ macrophages stimulated with (indicated as "Z") or without (indicated as "N") 100 μg/ml zymosan for 24 hr. Macrophage culture supernatants were analyzed by ELISA. **(B)** Induction of IL-1β and IL-10 production by lentiviral iOPN expression by *Opn*$^{-/-}$ macrophages with zymosan stimulation. **(C)** Reduction of phospho-ERK (pERK) in zymosan-stimulated *Opn*$^{-/-}$macrophages. **(D)** Induction of zymosan–induced ERK activation in *Opn*$^{-/-}$ macrophages by lentiviral iOPN expression.