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Cutting Edge: Critical Role of Intracellular Osteopontin in Antifungal Innate Immune Responses

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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 OPN (iOPN) is still largely unknown. In this study, we elucidated the mechanism by which iOPN was involved in antifungal 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 suggests the critical involvement of iOPN in antifungal innate immunity. The Journal of Immunology, 2011, 186: 000–000.

Pneumocystis is an opportunistic fungal pathogen that causes serious morbidity and mortality in immunocompromised individuals. Host cells recognize Pneumocystis via pattern recognition receptors (PRRs), such as dectin-1, 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 that 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 Opn mRNA was one of the most highly expressed transcripts 8 wk after Pneumocystis infection in alveolar macrophages from immunosuppressed rats (10). OPN has two types of isoform; 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 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 an extracellular protein (i.e., as sOPN), and the role of iOPN is much less characterized compared with 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 IFN-α production by plasmacytoid DCs (14). These results suggested that iOPN plays a role as an adaptor molecule in innate immune signaling pathways.

Our study demonstrated that OPN in Rag2−/− mice, which lack adaptive immunity, makes 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. Yet, OPN still matters in immunocompromised hosts: Patients who contract opportunistic fungal infections are immunocompromised. In this study, we demonstrated that iOPN plays a critical role in formation of fungal PRR clusters on the cell surface, in addition to iOPN’s involvement 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, Piscataway, NJ), and

The online version of this article contains supplemental material.

Abbreviations used in this paper: DC, dendritic cell; DKO, double knockout; iOPN, intracellular osteopontin; IRAKI, IL-1R–associated kinase 1; MR, mannose receptor; OPN, osteopontin; PRR, pattern recognition receptor; ROS, reactive oxygen species; sOPN, secreted osteopontin; WT, wild-type.

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backcrossed to C57BL/6 mice for 15 generations. Rag2−/− mice (The Jackson Laboratory, Bar Harbor, ME) were crossed to Opn−/− mice to generate Opn−/− Rag2−/− mice. All of the mice were kept in a barrier facility. This study was approved by the Duke University Institutional Animal Care and Use Committee (Durham, NC). Pneumocystis was obtained from American Type Culture Collection (Pneumocystis murina, PRA-111; Manassas, VA), propagated in vivo by infecting Rag2−/− mice, and harvested from lungs as previously described (19).

Quantitative PCR conditions and primer sequences
mRNA expression levels were determined by using the −ΔΔ Ct method of real-time PCR. Quantitative PCR 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 Abs against OPN (2A1, Santa Cruz Biotechnology, Santa Cruz, CA), MR (Biolegend, San Diego, CA), dectin-1 (Biolegend), and TLR2 (Molecular Probes, Eugene, OR) with fluorophore-conjugated secondary Abs. The frequency (percentage) of cells that show clustering of iOPN was determined by ELISA (BD Pharmingen, San Diego, CA).

Immunoprecipitation, immunoblotting, and ELISA
Immunoprecipitation was carried out with OPN Ab (2A1), followed by immunoblotting with IL-1R−/−-associated kinase 1 (IRAK1) Ab (Assay Designs, Plymouth Meeting, PA) or Syk Ab (Biolegend) with Pam3CSK4, TLR2 ligand, and curdian (dectin-1 ligand), respectively. To evaluate ERK activation by zymosan, peritoneal macrophages from WT and Opn−/− mice were used. To neutralize secreted OPN, we treated cells with a neutralizing Ab, AF808 (R&D Systems, Minneapolis, MN) 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, San Diego, CA).

Analyzing phagocytosis, reactive oxygen species production, and Pneumocystis clearance
Pneumocystis was labeled as previously reported (21), except for using Pneumocystis (1 × 105 cysts) with 2 μl 10 μg/ml Alexa Fluor 647 (Molecular Probes). Macrophages were stained with CD11b Ab, and phagocytosis was evaluated by detecting CD11b and Alexa 647 double-positive cells. To evaluate reactive oxygen species (ROS) generation by macrophage stimulated with Pneumocystis, dihydroethidium staining was evaluated in CD11b-positive cells. Detection of Pneumocystis clearance was described previously (22).

Statistical analysis
Statistical analysis was evaluated using Student 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, under the environment that Opn−/− Rag2−/− mice were healthy and showing no signs of disease. Opn−/− Rag2−/− mice were clearly smaller in size compared with the Opn+/− Rag2−/− mice at 13 wk old (Supplemental Fig. 1A) and started dying at ~10 wk of age (Fig. 1A). We performed pathology analyses on 14-wk-old mice. Although the lungs from Opn+/− Rag2−/− mice appeared normal (Fig. 1B, left panel), 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 panels). 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 the lungs of Opn−/− Rag2−/− mice (Fig. 1C).

Pneumocystis cannot be cultured; therefore, it is not possible to assess the fungal loads by CFU. We confirmed Pneumocystis loads 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-wk-old Opn−/− Rag2−/− mice showed much higher expression levels of both transcripts than the 4-wk-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 from the 14-wk-old Opn−/− Rag2−/− mice. In contrast, lungs from single gene-deficient mice (i.e., Opn−/− Rag2−/− and Opn−/− Rag2−/− mice) at the age of 14 wk 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 Opn−/− Rag2−/− mouse data 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 immunocompromised hosts, but OPN is dispensable in the presence of adaptive immunity.
Colocalization 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 colocalization 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 WT macrophages stimulated with *Pneumocystis*. Thirty minutes after *Pneumocystis* treatment, cells showed colocalization of iOPN, TLR2, dectin-1, and MR upon contact with *Pneumocystis* (Fig. 2A, 2B, Supplemental Fig. 2A). In contrast, *Opn−/−* macrophages failed to colocalize the PRR cluster (Fig. 2C, 2D, Supplemental Fig. 2B), suggesting that iOPN is essential for PRR clustering.

To elucidate a role of iOPN in downstream signaling pathway 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 Pam3CSK4, 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 (Fig. 2F). It should be noted that whole-cell lysates include both iOPN and sOPN (i.e., OPN that are to be secreted). 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 with WT macrophages (Fig. 3A, Supplemental Fig. 3A). We next evaluated which OPN, sOPN, or iOPN, is involved in phagocytosis of *Pneumocystis* by Ab-mediated sOPN depletion in WT macrophages and lentivirus-mediated iOPN expression in *Opn−/−* macrophages. sOPN depletion with OPN Ab (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). 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 Ab (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*. A previous study showed that deficiency of p91phox (Nox2), a component of the Nox2 NADPH oxidase that produces ROS, did not affect tissue damage in *Pneumocystis*-infected CD4+ T cell-depleted mice (26). Because Sasaki et al. (27)
WT cells and iOPN expression in B
ternal Fig. 3 of DHE staining in the initial 1 h after
rophages were cultured with
macrophages. *p
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zymosan stimulation.

**FIGURE 3.** Involvement of iOPN in phagocytosis, ROS generation, and fungal clearance by macrophages. 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 iOPN Ab (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 was calculated as mean fluorescence intensity value increase (percent) 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 iOPN expression in Opn−/− cells, as described in B. E. Pneumocystis clearance was evaluated with the levels of Pneumocystis rRNA in macrophages. *p < 0.05.

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. Deficiency in killing was demonstrated by the high levels of Pneumocystis rRNA and PCMe2 mRNA expression, which was detected in Opn−/− macrophages after phagocytosing Pneumocystis (Fig. 3E, Supplemental 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.

**FIGURE 4.** Involvement of iOPN in signal transduction triggered by zy- mosan. A. Expression of IL-1β and IL-10 by WT and Opn−/− macrophages stimulated with (Z) or without (N) 100 μg/ml zymosan for 24 h. Macrophage culture supernatants were analyzed by ELISA. B. Induction of IL-1β and IL-10 production by lentiviral iOPN expression in 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. *p < 0.05.

**iOPN enhances zymosan-induced cytokine production in macrophages**

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 (Supplemental 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 used zymosan, a glucan from fungal cell walls. Zymosan simultaneously ligates dectin-1 and TLR2, which are essential for the detection of Pneumocystis by host cells (5, 28–30). Although the expression levels of TNF-α and IFN-γ were not altered (Supplemental Fig. 3E), Opn−/− macrophages had significantly reduced production of both IL-1β and IL-10 (Fig. 4A) 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 induces 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 (29). Opn−/− macrophages had reduced ERK activation compared with WT macrophages (Fig. 4C), and iOPN expression in Opn−/− macrophages restored ERK activation (Fig. 4D). 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−/−Rag2−/− mice are extremely susceptible to spontaneous Pneumocystis infection. iOPN is essential for generating antifungal innate immune responses in pathogen 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, to our knowledge, by which a novel OPN isotype, iOPN, plays a critical role in anti-fungal responses. Lack of iOPN in the innate immune system may explain the extremely susceptible phenotype of Opn−/−Rag2−/− mice to opportunistic Pneumocystis infection.

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**Disclosures**

The authors have no financial conflicts of interest.
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