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

Makoto Inoue,* Yasuhiro Moriwaki,,*† Tomohiro Arikawa,*‡ Yu-Hsun Chen,* Young Joo Oh, * Timothy Oliver,§ and Mari L. Shinohara*‡

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: 19–23.

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

*Department of Immunology, †Department of Cell Biology, and ‡Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710; †Department of Pharmacology, Faculty of Pharmacy, Keio University, Minato-ku, Tokyo; and ‡Department of Immunology and Immunopathology, Faculty of Medicine, Kagawa University, Kitasato, Kagawa, Japan

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Address correspondence and reprint requests to Dr. Mari L. Shinohara, Department of Immunology, DUMC-3010, Duke University Medical Center, Durham, NC 27710. E-mail address: mari.shinohara@duke.edu

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Abbreviations used in this paper: DC, dendritic cell; DKO, double knockout; iOPN, intracellular osteopontin; IRAK1, 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 $-\Delta\Delta C_t$ 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 Ab 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 also detected by immunofluorescence. Levels of IL-1 were detected by immunoblotting.

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 Pamp-CSK, 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 (BD Pharmingen, San Diego, CA).

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Analysis of phagocytosis, reactive oxygen species production, and Pneumocystis clearance

*Pneumocystis* was labeled as previously reported (21), except for using *Pneumocystis* ($\times 10^6$ cysts) with 2 μl 10 mg/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 *Pneumocystis* was present, and showed 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.

**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-wk-old Opn+/− Rag2−/− mice and Opn−/− Rag2−/− mice. The image in a yellow square in the middle panel is enlarged in the right panel. Scale bars, 50 μm. Original magnification ×60. C, *Pneumocystis rRNA* was detected by quantitative PCR, normalized with β-actin mRNA. The first column (not seen) denotes *Pneumocystis rRNA* levels in lungs of 4-wk-old Opn+/− Rag2−/− (DKO) mice; otherwise, all the samples were from 14-wk-old mice of DKO and single KO of Rag2−/− or Opn−/−. The last column denotes values from lung draining lymph nodes from 14-wk-old DKO mice.
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<sup>-/-</sup> 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 IFN-β induction (14, 24). TLR2 stimulation with Pam<sub>3</sub>CSK<sub>4</sub>, 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 (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<sup>-/-</sup> macrophages, and phagocytosis of *Pneumocystis* by macrophages was detected by flow cytometry. Phagocytosis was significantly reduced in Opn<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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 p91<sub>phox</sub> (Nox2), a component of the Nox2 NADPH oxidase that produces ROS, did not affect tissue damage in *Pneumocystis*-infected CD4<sup>+</sup> T cell-depleted mice (26). Because Sasaki et al. (27)
We found that iOPN could compensate ROS generation in Nox1-deficient bone marrow macrophages/microbes, the Nox1 NADPH oxidase may compensate resistance against *Pneumocystis* in Nox2-deficient mice. We also found that *Pneumocystis* was not effectively killed in Opn<sup>−/−</sup> macrophages as in WT macrophages. Deficiency in killing was demonstrated by the high levels of *Pneumocystis* rRNA and PCMei2 mRNA expression, which was detected in Opn<sup>−/−</sup> 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.

### 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> macrophages had reduced ERK activation compared with WT macrophages (Fig. 4C), and iOPN expression in Opn<sup>−/−</sup> 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<sup>−/−</sup>–Rag<sup>−/−</sup> 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 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<sup>−/−</sup>–Rag<sup>−/−</sup> mice to opportunistic *Pneumocystis* infection.

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### Disclosures

The authors have no financial conflicts of interest.
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