Alveolar Macrophage–mediated Killing of *Pneumocystis carinii* f. sp. *muris* Involves Molecular Recognition by the Dectin-1 β-Glucan Receptor

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Abstract

Innate immune mechanisms against *Pneumocystis carinii*, a frequent cause of pneumonia in immunocompromised individuals, are not well understood. Using both real time polymerase chain reaction as a measure of organism viability and fluorescent deconvolution microscopy, we show that nonopsonic phagocytosis of *P. carinii* by alveolar macrophages is mediated by the Dectin-1 β-glucan receptor and that the subsequent generation of hydrogen peroxide is involved in alveolar macrophage–mediated killing of *P. carinii*. The macrophage Dectin-1 β-glucan receptor colocalized with the *P. carinii* cyst wall. However, blockage of Dectin-1 with high concentrations of anti–Dectin-1 antibody inhibited binding and concomitant killing of *P. carinii* by alveolar macrophages. Furthermore, RAW 264.7 macrophages overexpressing Dectin-1 bound *P. carinii* at a higher level than control RAW cells. In the presence of Dectin-1 blockage, killing of opsonized *P. carinii* could be restored through FcγRII/III receptors. Opsonized *P. carinii* could also be efficiently killed in the presence of FcγRII/III receptor blockage through Dectin-1–mediated phagocytosis. We further show that Dectin-1 is required for *P. carinii*–induced macrophage inflammatory protein 2 production by alveolar macrophages. Taken together, these results show that nonopsonic phagocytosis and subsequent killing of *P. carinii* by alveolar macrophages is dependent upon recognition by the Dectin-1 β-glucan receptor.

Key words: lung • leukocyte • innate • yeast • chemokine

Introduction

Pneumonia caused by *Pneumocystis carinii* (now *Pneumocystis jiroveci* in humans) continues to be a predominant cause of morbidity and mortality in immunocompromised individuals, especially those infected with HIV (1). Although the advent of highly active antiretroviral therapy has decreased the overall incidence of *P. carinii* pneumonia (2), the mortality rate of those requiring hospitalization remains high (2). Furthermore, previous *P. carinii* pneumonia significantly predisposes HIV^+ individuals to bacterial pneumonia (3), currently the most common cause of pneumonia in this population (4).

Innate host defenses, mediated primarily by alveolar macrophages, against *P. carinii* have been studied extensively. It is widely reported that phagocytosis by alveolar macrophages is the predominant mechanism of *P. carinii* clearance from the lungs (5–7). This clearance is thought to involve the recognition of mannose-containing residues on the major surface glycoprotein A, gpA, of *P. carinii* by the macrophage mannose receptor (MR; 8). β-glucans, a major component of the *P. carinii* cell wall, have also been shown to elicit critical proinflammatory cytokines such as TNF-α and chemokines such as macrophage inflammatory protein (MIP)-2 (9–11) from alveolar macrophages. However, the

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Abbreviations used in this paper: MIP, macrophage inflammatory protein; MnTMPyP, manganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; MR, mannose receptor.

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mechanisms by which macrophages phagocytose and kill *P. carinii* are not completely understood. It is hypothesized that upon phagocytosis, the oxidative burst by macrophages, primarily the generation of hydrogen peroxide and superoxide, has a direct cytotoxic effect on *P. carinii* (12–14). Indeed, studies have shown impaired hydrogen peroxide and superoxide production by alveolar macrophages from HIV^+ individuals with <200 $CD4^+$ T cells/mm^3 with and without *P. carinii* infection (15).

A major limitation in investigations of *P. carinii* is the lack of the ability of the organism to be cultured in vitro (16). Due to this limitation, traditional in vitro assays using radiometric labeling of *P. carinii* have not provided robust measurements of organism viability (6, 17). In this study, using quantitative real time PCR for *P. carinii* ribosomal RNA integrity, which correlates with organism viability (18, 19), we demonstrate that in vitro killing of nonopsonized *P. carinii* by alveolar macrophages involves recognition by the newly described Dectin-1 β-glucan receptor, a small, type II transmembrane protein that functions as the predominant β-glucan pattern recognition receptor on macrophages (20, 21). Moreover, recognition by Dectin-1 mediates release of the proinflammatory CXC chemokine MIP-2 by alveolar macrophages.

Materials and Methods

Mice. Male C57BL/6 mice, 6–8 wk of age, were purchased from the National Cancer Institute, National Institutes of Health. MR-deficient ($MR^{-/-}$) mice were generated on the 129SvJ × C57BL/6 background and were backcrossed to the C57BL/6 strain for seven generations (22). All animals were housed in a specific pathogen-free facility and handled according to institutionally recommended guidelines.

Peritoneal and Alveolar Macrophage Isolation. To isolate peritoneal macrophages, male C57BL/6 mice were administered 3% sterile thioglycollate intraperitoneally and allowed to rest for 5–7 d. Thereafter, animals were killed and a peritoneal lavage was performed using 10 ml RPMI 1640 (Invitrogen). The lavage fluid was centrifuged at 300 *g* for 10 min and the subsequent cell pellets were enumerated using a hemacytometer. For alveolar macrophage isolation, male C57BL/6 or $MR^{-/-}$ mice were anesthetized with intraperitoneal pentobarbital and killed by exsanguination. Thereafter, lungs were lavaged through an intratracheal catheter with prewarmed (37°C) calcium and magnesium-free PBS supplemented with 0.6 mM EDTA. A total of 10 ml was used in each mouse in 0.5-ml increments with a 30-s dwell time. The lavage fluids were pooled and centrifuged at 300 *g* for 10 min, and the cells were collected for the coculture assay. To ensure that each cell preparation was enriched for macrophages, 25,000 cells were cytospun onto slides and stained with hematoxylin and eosin. Cell preparations were generally >98% enriched for peritoneal or alveolar macrophages.

P. carinii f. sp. muris Isolate. A preparation of *P. carinii* was prepared as previously described (23, 24). In brief, CD40 ligand knockout mice previously inoculated with *P. carinii* were injected with a lethal dose of pentobarbital, and the lungs were aseptically removed and frozen for 30 min at −70°C in 1 ml PBS. Frozen lungs were thereafter homogenized in 10 ml PBS (Model 80 Stomacher; Tekmar Instruments), filtered through sterile gauze, and pelleted at 500 *g* for 10 min at 4°C. The pellet was resuspended in PBS and the number of *P. carinii* cysts were enumerated microscopically using a modified Giemsa stain (Diff-Quik; Baxter). The preparation was adjusted to 2×10^6 cysts/ml and 50 μl aliquots were placed into tubes containing 200 μl 90% fetal bovine serum supplemented with 10% dimethylsulfoxide and stored at −80°C. Using this storage method, stable *P. carinii* viability, as determined by quantitative real time PCR (18), can be maintained for >1 yr.

P. carinii Viability Assay. Macrophages (10^6/ml) in a volume of 100 μl were cocultured with *P. carinii* (10^4 cysts/ml, 100 μl) for 16 h at 37°C, 5% CO_2. Controls include *P. carinii* incubated with medium alone. The contents of each well were collected and pelleted at 800 *g* for 5 min. The supernatants were discarded and total RNA was isolated from the cell pellets using TRIZOL™ reagent (Invitrogen). Viability of *P. carinii* was analyzed through real time PCR measurement of rRNA copy number and quantified by using a standard curve of known copy number of *P. carinii* rRNA as previously described (18). This methodology detects viable *P. carinii* organisms as evidenced by the absence of detectable *P. carinii* rRNA in samples subjected to heat inactivation or exposure to trimethoprim/suflamethoxazole. Percent killing was defined as: 1 − rRNA copy number from wells containing macrophages + *P. carinii* rRNA copy number from wells containing *P. carinii* alone × 100.

Analysis of Phagocytosis and Oxidative Killing Mechanisms. In experiments to determine whether phagocytosis was critical for macrophage killing of *P. carinii*, macrophages were pretreated with 10 μM cytochalasin D for 30 min at 37°C (Sigma-Aldrich). Thereafter, the macrophages were washed twice and cocultured with *P. carinii*. To address oxidative killing mechanisms, macrophages and *P. carinii* were cocultured in the presence or absence of 5,000 U/ml catalase, 1 mM Nω-nitro-L-arginine methyl ester (both from Sigma-Aldrich), or 100 μM manganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP; Calbiochem). There was no effect of any reagent on macrophage viability. Controls included *P. carinii* cultured in the presence or absence of each reagent.

β-Glucan and MR Carbohydrate Inhibition Experiments. In experiments to determine the effects of β-glucan and MR blockage on the killing of *P. carinii*, macrophages were preincubated with medium alone or 100–600 μg/ml glucan or mannan for 30 min at 37°C (all from Sigma-Aldrich), and *P. carinii* was added thereafter. Controls included *P. carinii* cultured in the presence or absence of glucan or mannan.

FITC Labeling of P. carinii. *P. carinii* was labeled with FITC as previously described (25). In brief, 5-ml suspensions of *P. carinii* (4×10^5 cysts/ml) were incubated with 0.1 mg/ml FITC in PBS (Sigma-Aldrich) for 30 min at 37°C. Thereafter, the suspension was washed three times for 10 min at 1,300 *g*. The final pellet was resuspended in 1 ml medium and a small aliquot was viewed under fluorescent microscopy for confirmation of FITC labeling. Both cyst and trophozoite forms were labeled and viability was not affected.

Analysis of Dectin-1. Initial experiments determined that alveolar macrophages expressed high levels of Dectin-1. In experiments to determine the effects of blocking the Dectin-1 β-glucan receptor on macrophage-mediated killing, macrophages were preincubated with 2.0 μg/ml of the monoclonal antibody 2A11 for 30 min at 37°C, a rat anti–mouse Dectin-1 IgG2b antibody (20), and thereafter cocultured with *P. carinii*. Controls included macrophages cultured with an irrelevant IgG2b antibody and *P. carinii* cultured in the presence or absence of either antibody.

In Vitro Binding Assay. RAW 264.7 cells (American Type Culture Collection) overexpressing Dectin-1 (RAW-Dectin) or control cells (RAW-FB) were cultured as previously described (26). These cells or 1–2 × 10^5 C57BL/6 primary alveolar macrophages in a volume of 200 μl were adhered to sterile 13-mm round glass coverslips for 2–3 h at 37°C (24). The coverslips were washed to remove nonadherent cells and 1–2 × 10^5 FITC-conjugated *P. carinii* cysts in a volume of 200 μl was added to each coverslip for 1 h at 37°C. Thereafter, the coverslips were washed to remove unbound/unphagocytosed FITC-*P. carinii*. In specific experiments, adherent macrophages were preincubated with low (0.1 μg/ml, which does not inhibit binding and killing) or high (2.0 μg/ml, which does inhibit binding and killing) concentrations of the anti–Dectin-1 monoclonal antibody 2A11 before the addition of FITC-*P. carinii*.

Analysis of P. carinii Cyst Binding by Fluorescent Deconvolution Microscopy. Adherent alveolar macrophages were incubated with Alexa Fluor 568–conjugated goat anti–rat IgG2b (Molecular Probes) for detection of the anti–Dectin-1 monoclonal antibody 2A11 for 30 min at room temperature. Thereafter, adherent macrophages were fixed with 4% formaldehyde and counterstained with 0.4 μg/ml DAPI (Molecular Probes) nucleic acid stain for 2 min at room temperature followed by application of Prolong™ (Molecular Probes) mounting media. The coverslips were analyzed on a Leica DMRXA automated upright epifluorescent microscope (Leica) equipped with a Sensicam QE CCD digital camera (Cooke Corporation) and filter sets for FITC (exciter HQ480/20, dichroic Q495LP, and emitter HQ510/20m), Alexa 568 (exciter 560/55x, dichroic Q595LP, and emitter HQ645/75m), and DAPI (exciter 360/40x, dichroic 400DCLP, and emitter GG420LP). Slidebook™ deconvolution software (Intelligent Imaging Innovations) mathematical algorithms were applied to isolate and calculate macrophage surface area binding over 20 fields at ×400. Data was expressed as the amount of alveolar macrophage surface area interacting with *P. carinii* (surface area in μm^2).

In specific experiments using RAW 264.7 macrophages, fluorescence superimposition over Nomarski optics (differential interference contrast microscopy) was used to enumerate *P. carinii* binding to macrophages. Images were captured and deconvolved using Slidebook™ software and enumerated. Data was expressed as percent of cells with *P. carinii* bound.

In Vitro Phagocytosis Assay. For Alexa Fluor labeling of *P. carinii*, *P. carinii* was resuspended in PBS at 10^8 organisms/ml and 50 mM sodium carbonate was added to increase the pH to 8.5. The organisms were then fluorescently labeled by adding Alexa Fluor 488 succinimidyl ester (Molecular Probes) at a final concentration of 0.4 mg/ml. The *P. carinii* preparation was mixed and rocked at room temperature for 30 min, and then washed three times in cold PBS. The fluorescently labeled particles were recounted and resuspended at 10^8 organisms/ml and placed at 4°C until use in the phagocytosis assay.

Alveolar macrophages were isolated from C57BL/6 or $MR^{-/-}$ mice as described above. 3 × 10^5 macrophages in a volume of 100 μl were aliquoted into polypropylene tubes and incubated with isotype control antibody or 2.0 μg/ml of the anti–Dectin-1 monoclonal antibody 2A11 for 30 min at 37°C. Thereafter, 100 μl Alexa fluorescently labeled *P. carinii* was added at a ratio of five *P. carinii* organisms to one macrophage. This coculture was incubated at 37°C for 80–90 min with occasional mixing. The macrophages were washed once, resuspended in 200 μl PBS. The red fluorescent carbocyanine lipid probe DiI (for macrophage labeling; Molecular Probes) was then added at a final concentration of 50 μg/ml. After 10 min at room temperature, the macrophages were washed, resuspended in 200 μl cold PBS, and placed on ice until analysis with confocal laser scanning microscopy. For microscopic examination, aliquots of the cells were placed individually into a glass-bottom 96-well plate and analyzed with a Zeiss LSM 510 Scanning Microscope. 15 fields were analyzed in each well, with 20 optical sections (each 0.8–1.0-μm thick) taken through the depth of the cells. Alexa Fluor–labeled *P. carinii* were determined to be bound to the surface of the macrophages or internalized by examining their position in relation to the DiI-labeled macrophage membranes throughout the entire depth of individual cells. Data was expressed as the mean number of internalized *P. carinii* organisms per alveolar macrophage.

Analysis of MIP-2 Production. Alveolar macrophages or RAW-Dectin and RAW-FB cells (2 × 10^5) were cocultured with *P. carinii* at macrophage to total *P. carinii* organism ratios of 1:10 and 1:100 in the presence of rat IgG or 2A11 for 16 h in a 96-well plate at 37°C, 5% CO_2. Controls included alveolar macrophage cultured in medium alone. Thereafter, the contents of each well were collected and the supernatants were analyzed for MIP-2 concentrations using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical Analysis. Data were analyzed using StatView statistical software (Brainpower Inc.). Comparisons between groups were made with analyses of variance and appropriate ad hoc testing. The Mann-Whitney U test or the Wilcoxon paired sample test was used to make ordinal comparisons. Significance was accepted at $P < 0.05$.

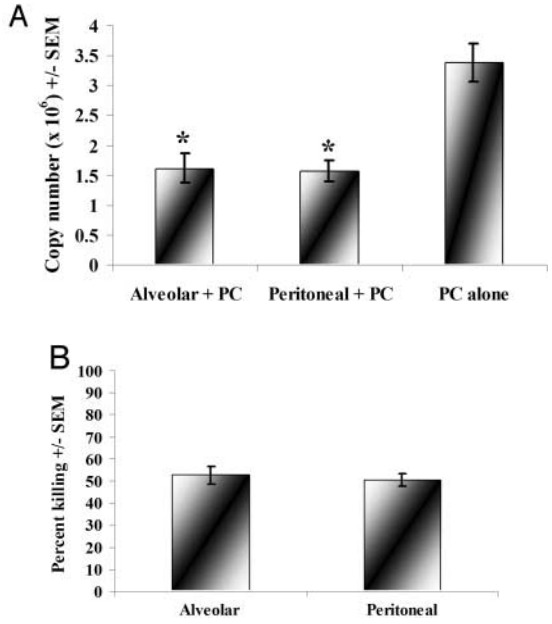

Figure 1. Reduction in *P. carinii* viability during coculture with alveolar and peritoneal macrophages. Alveolar or peritoneal macrophages were isolated from 6–8-wk-old, male C57BL/6 mice and cocultured overnight with a constant number of *P. carinii* organisms. Controls included *P. carinii* cultured in the absence of macrophages. Thereafter, RNA was isolated from the contents of each well and quantitative real time PCR for *P. carinii* rRNA copy number was performed. Cumulative results from six separate experiments for *P. carinii* rRNA copy number (A) and percent killing (B) are shown. *, significant differences between alveolar macrophages or peritoneal macrophages compared with *P. carinii* (PC) alone ($P = 0.0013$ and 0.0017 for alveolar and peritoneal macrophages, respectively; A). Data are expressed as mean copy number (A) or mean percent killing (B) ± SEM.

Results

Reduction in P. carinii Viability During Coculture with Alveolar or Peritoneal Macrophages. To better understand interactions and the recognition of *P. carinii* by tissue macrophages, we used both thioglycollate-elicited peritoneal macrophages as a control for an elicited macrophage population and alveolar macrophages as a source of resident tissue macrophages. Various ratios of alveolar and peritoneal macrophages were cocultured with a constant number of *P. carinii* organisms in a 96-well tissue culture plate. *P. carinii* was cultured alone as a control for 100% viability. Thereafter, the contents of each well were collected, RNA was isolated, and real time quantitative PCR for *P. carinii* rRNA was performed. The ED_{50} of both populations of macrophages to mediate a 50% reduction in *P. carinii* rRNA integrity, a measure of organism viability, was similar at a ratio of 100 macrophages to 1 *P. carinii* cyst (Fig. 1, A and B), which correlates with a 1:1 macrophage to total *P. carinii* (cysts plus trophozoites) in our *P. carinii* preparations (27).

Alveolar Macrophage–mediated Killing of P. carinii Requires Phagocytosis and Involves Generation of Reactive Oxygen Species. To determine putative mechanism(s) by which alveolar macrophages killed *P. carinii*, we investigated the role of phagocytosis and the subsequent release of reactive oxygen/nitrogen species. After pretreatment with cytochalasin D, alveolar macrophages lost their ability to phagocytose the organism, and thus their ability to reduce *P. carinii* rRNA copy number in vitro compared with those pretreated with medium alone (Fig. 2 A). To confirm that internalization of *P. carinii* was essential for the detected reduction in viability, we performed an in vitro binding/phagocytosis assay using FITC-conjugated *P. carinii* cysts and used fluorescent deconvolution microscopy to determine whether untreated or cytochalasin D–treated alveolar macrophages phagocytosed *P. carinii*. The representative micrograph in Fig. 2 B shows the engulfment of the cyst form of *P. carinii* by an untreated alveolar macrophage. *P. carinii* organisms were not observed to be associated or internalized in cytochalasin D–treated alveolar macrophages (unpublished data).

To address the role of superoxide in *P. carinii* killing by alveolar macrophages, we cocultured macrophages and *P. carinii* in the presence of MnTMPyP, a cell-permeable superoxide dismutase mimetic (28). Although superoxide has been implicated in alveolar macrophage function against *P. carinii* (13), alveolar macrophage–mediated killing of *P. carinii* remained intact in the presence of superoxide dismutation (Fig. 2 C). In contrast, decomposition of hydrogen peroxide by catalase significantly attenuated alveolar macrophage–mediated killing of *P. carinii* (Fig. 2 C). Additional studies showed that *P. carinii* incubated overnight in the presence of physiologic concentrations of hydrogen peroxide (29) had a reduction in viability (26.4 ± 1.1% and 42.8 ± 2.1% killing for 10 and 100 μM H_2O_2, respectively). Inhibition of nitric oxide synthesis had no effect on alveolar macrophage–mediated killing of *P. carinii* (Fig. 2 C).

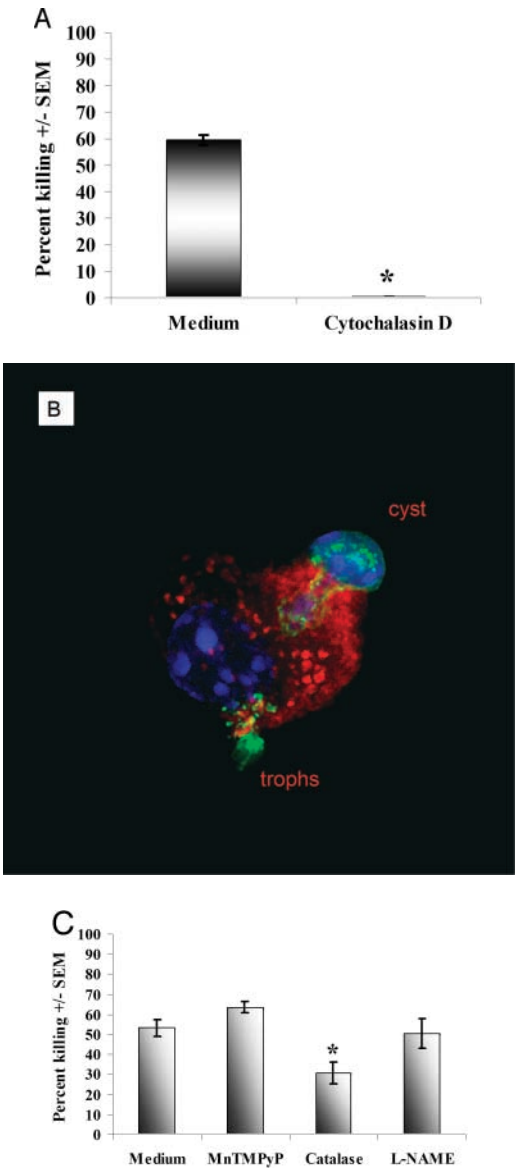

Figure 2. Alveolar macrophage–mediated killing of *P. carinii* requires phagocytosis and generation of reactive oxygen species. Alveolar macrophages were isolated from 6–8-wk-old, male C57BL/6 mice and were pretreated with 10 μM cytochalasin D for 30 min at 37°C, washed, and cocultured overnight with *P. carinii* at a macrophage to *P. carinii* cyst ratio of 100:1 (A). Controls included *P. carinii* cultured in the absence of macrophages. In B, an in vitro phagocytosis assay was performed with alveolar macrophages and FITC-labeled *P. carinii*. A representative micrograph (×630) shows phagocytosis of *P. carinii* cyst and trophozoite forms (red arrows). Blue indicates DAPI-stained nucleic acid, green indicates FITC on the *P. carinii* surface, and red indicates phalloidin staining for F-actin. In C, alveolar macrophages were cocultured overnight with *P. carinii* at a macrophage to PC cyst ratio of 100:1 in the presence of 100 μM MnTMPyP, 5,000 U/ml catalase, 1 mM Nω-nitro-L-arginine methyl ester (L-NAME), or 100 and 10 μM hydrogen peroxide. Controls include *P. carinii* cultured in the absence of macrophages but in the presence of each compound separately. Thereafter, RNA was isolated from the contents of each well and quantitative real time PCR for *P. carinii* rRNA copy number was performed. Cumulative results from four separate experiments (A and C) are shown. *, significant differences between untreated and cytochalasin D–treated alveolar macrophages ($P = 0.003$; A) and untreated and catalase-treated alveolar macrophages ($P = 0.005$; C). Data are expressed as mean percent killing ± SEM.

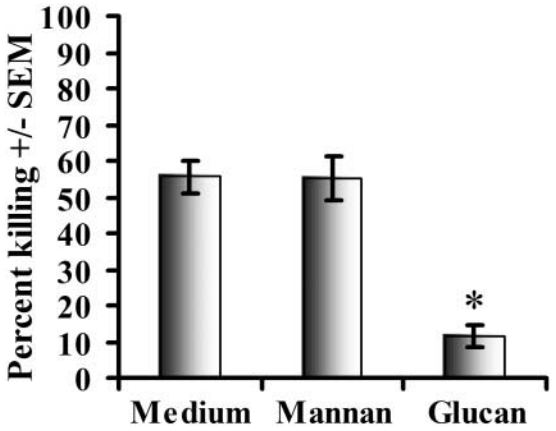

Figure 3. Effects of mannose and β-glucan receptor blockage on alveolar macrophage–mediated killing of *P. carinii*. Alveolar macrophages were isolated from 6–8-wk-old, male C57BL/6 mice and were pretreated with 100–600 μg/ml mannan or glucan for 30 min and thereafter added to *P. carinii* overnight at a final macrophage to *P. carinii* cyst ratio of 100:1. Controls include *P. carinii* cultured in the absence of macrophages but in the presence of mannan or glucan. Thereafter, RNA was isolated from the contents of each well and quantitative real time PCR for *P. carinii* rRNA copy number was performed. Cumulative results from four separate experiments are shown. *, significant differences between untreated and glucan-treated alveolar macrophages ($P = 0.008$). Data are expressed as mean percent killing ± SEM.

Effects of MR and β-Glucan Receptor Blockage on Alveolar Macrophage–mediated Killing of P. carinii. Macrophages express multiple molecular recognition receptors that function in the nonopsonic uptake of pathogenic organisms and in the activation of inflammatory responses (30). Previous studies using rat-derived *P. carinii* cocultured with human macrophages support a role for the macrophage MR as a critical molecular recognition receptor required for the uptake of *P. carinii* by alveolar macrophages (24). In addition, β-glucan receptors have been reported to be critical in the generation of macrophage proinflammatory responses in response to *P. carinii* cell wall β-glucans (11). However, the role of β-glucan and MRs in the specific killing of *P. carinii* has not been directly determined. The results illustrated in Fig. 3 show that competitive carbohydrate blockage of the macrophage MR with *Saccharomyces cerevisiae*–derived mannan did not reduce the ability of alveolar macrophages to kill *P. carinii* in vitro. In contrast, blockage of β-glucan receptors by preincubation of alveolar macrophages with glucan from *S. cerevisiae* results in a significant decrease in alveolar macrophage–mediated killing of *P. carinii*.

The Role of the Dectin-1 β-Glucan Receptor in Pattern Recognition and Alveolar Macrophage–mediated Killing of P. carinii. Due to the observation that carbohydrate blockage of β-glucan receptors, but not the macrophage MR, abrogated killing of *P. carinii* by alveolar macrophages, we sought to further characterize the role of β-glucan receptors in the killing of *P. carinii*. Recently, the macrophage β-glucan receptor that recognizes zymosan, *S. cerevisiae*, and *Candida albicans* was identified as Dectin-1 (20, 26). We questioned whether Dectin-1 was responsible for nonopsonic alveolar macrophage–mediated killing of *P. carinii*. Initial studies were performed to determine whether Dectin-1 was expressed on the surface of alveolar macrophages. Using fluorescent deconvolution microscopy and a novel anti–Dectin-1 monoclonal antibody (2A11; reference 20), we show that alveolar macrophages expressed significant levels of the Dectin-1 β-glucan receptor (Fig. 4 A). Previous studies have shown that high concentrations of 2A11 (100 μg/ml) can inhibit the binding of zymosan to macrophages (20). Using 50-fold less concentrations of 2A11 (2.0 μg/ml) resulted in a significant abrogation of alveolar macrophage–mediated killing of *P. carinii* (Fig. 4 B).

Next, we sought to confirm that the Dectin-1 β-glucan receptor was required for recognition and binding of *P. carinii* by using an in vitro binding assay containing FITC-labeled *P. carinii* cysts that used fluorescent deconvolution microscopy for visualization and quantification of *P. carinii* binding to alveolar macrophages. Using a low concentration of 2A11 (0.1 μg/ml), which did not block *P. carinii* killing by alveolar macrophages (unpublished data) but efficiently labeled Dectin-1 on alveolar macrophages, did not affect the ability of alveolar macrophages to bind *P. carinii* (Fig. 4 C, left). Similar data were observed with unstained cells (unpublished data). Using Slidebook™ analysis software, we calculated the amount of *P. carinii* binding to alveolar macrophages pretreated with low and high concentrations of 2A11. As shown in Fig. 4 D, alveolar macrophages pretreated with 0.1 μg/ml 2A11 had a higher surface area bound by *P. carinii* compared with those pretreated with 2.0 μg/ml. We also used Slidebook™ analysis software to perform pseudocolor rendering analysis to determine whether the Dectin-1 β-glucan receptor colocalized with the *P. carinii* cell wall. The high powered micrograph illustrated in Fig. 4 E shows significant colocalization of Dectin-1 with the cell wall *P. carinii* (green/yellow area between two gray nuclei; left, alveolar macrophage; right, *P. carinii* cyst). In contrast, using concentrations of 2A11, which blocked alveolar macrophage–mediated killing of *P. carinii* (2.0 μg/ml; Fig. 4 B), completely abrogated binding and internalization of *P. carinii* by alveolar macrophages (Fig. 4 C, right, and D).

Internalization of P. carinii by Alveolar Macrophages from Macrophage MR-deficient ($MR^{-/-}$) Mice. As previously stated, the macrophage MR is thought to mediate initial recognition and ultimately clearance of *P. carinii*. To determine the role of the MR in alveolar macrophage–mediated recognition of *P. carinii*, we performed an in vitro phagocytosis assay using Alexa Fluor–conjugated *P. carinii* cocultured with alveolar macrophages isolated from $MR^{-/-}$ or wild-type control mice. Results presented in Fig. 5 show that alveolar macrophages isolated from $MR^{-/-}$ mice can internalize *P. carinii* at levels equal to that of alveolar macrophages isolated from wild-type control mice (solid bars). More importantly, when alveolar macrophages from both $MR^{-/-}$ and wild-type strains were pretreated with 2.0 μg/ml 2A11, a significant reduction in *P. carinii* internalization was observed (gray bars). Analysis of alveolar macrophage Dectin-1 expression by flow cytometry showed no differences between $MR^{-/-}$ and wild-type mice (unpublished data).

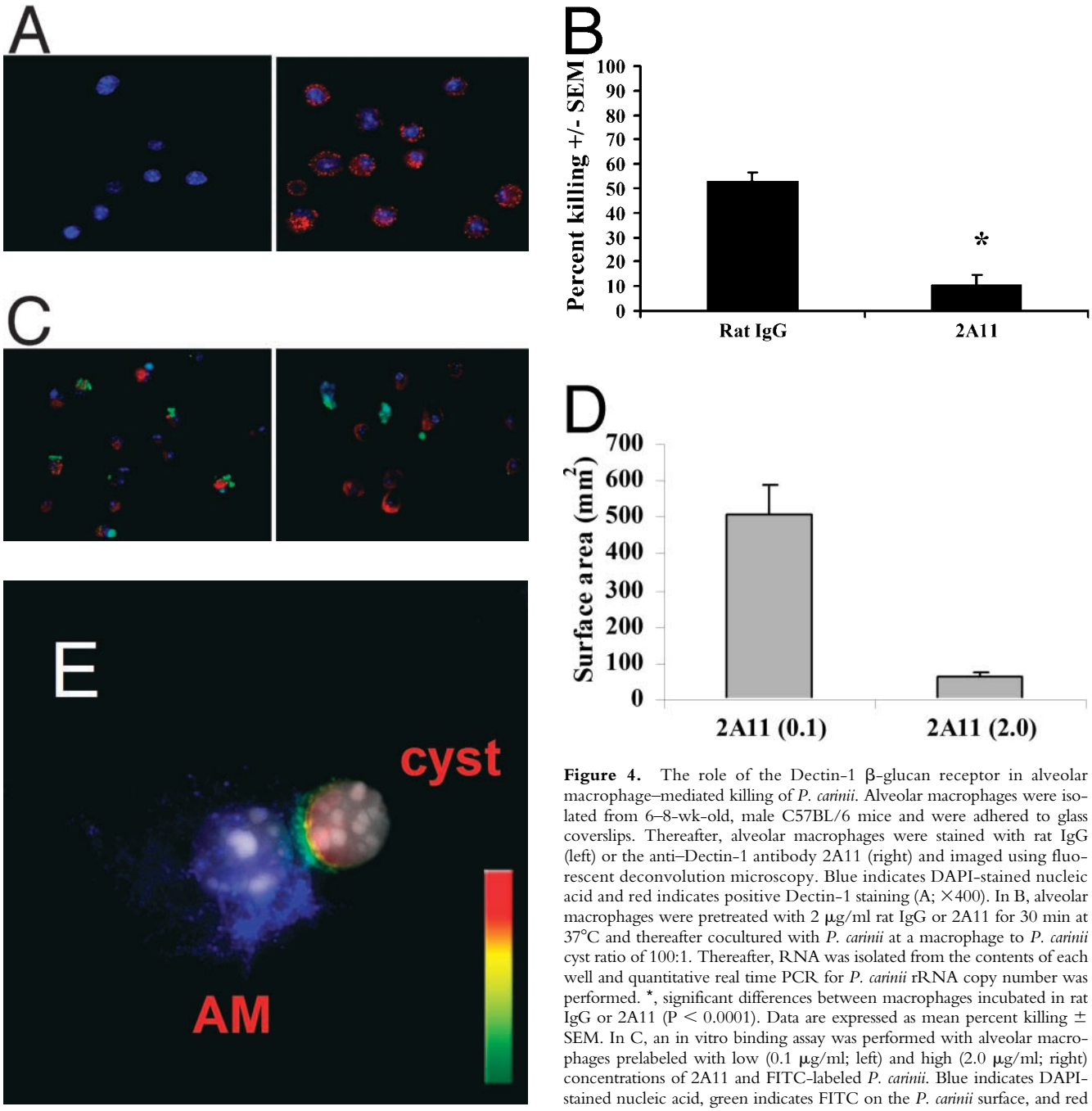

Figure 4. The role of the Dectin-1 β-glucan receptor in alveolar macrophage–mediated killing of *P. carinii*. Alveolar macrophages were isolated from 6–8-wk-old, male C57BL/6 mice and were adhered to glass coverslips. Thereafter, alveolar macrophages were stained with rat IgG (left) or the anti–Dectin-1 antibody 2A11 (right) and imaged using fluorescent deconvolution microscopy. Blue indicates DAPI-stained nucleic acid and red indicates positive Dectin-1 staining (A; ×400). In B, alveolar macrophages were pretreated with 2 μg/ml rat IgG or 2A11 for 30 min at 37°C and thereafter cocultured with *P. carinii* at a macrophage to *P. carinii* cyst ratio of 100:1. Thereafter, RNA was isolated from the contents of each well and quantitative real time PCR for *P. carinii* rRNA copy number was performed. *, significant differences between macrophages incubated in rat IgG or 2A11 ($P < 0.0001$). Data are expressed as mean percent killing ± SEM. In C, an in vitro binding assay was performed with alveolar macrophages prelabeled with low (0.1 μg/ml; left) and high (2.0 μg/ml; right) concentrations of 2A11 and FITC-labeled *P. carinii*. Blue indicates DAPI-stained nucleic acid, green indicates FITC on the *P. carinii* surface, and red indicates Dectin-1 (×200). In D, Slidebook™ analysis software algorithms were used to calculate macrophage surface area that was bound with *P. carinii* between alveolar macrophages pretreated with low (0.1 μg/ml; C, left) and high (2.0 μg/ml; C, right) concentrations of 2A11. In E, pseudocolor rendering analysis was performed by Slidebook™ analysis software to determine colocalization of Dectin-1 and the *P. carinii* cell wall (green/yellow area between two gray nuclei; left, alveolar macrophage; right, *P. carinii* cyst; ×630).

MIP-2 Production by Alveolar Macrophages in Response to P. carinii Is Mediated by Dectin-1. As mentioned previously, purified β-glucan from the cyst cell wall of *P. carinii* elicits MIP-2 production by alveolar macrophages (10). However, it remains unclear whether β-glucan recognition of whole organisms is required for MIP-2 production. To this end, we pretreated alveolar macrophages with 2A11 or an isotype control antibody and thereafter cocultured them with *P. carinii* at a macrophage to total *P. carinii* (cysts plus trophozoites) ratio of 1:10 and 1:100. After 16 h, MIP-2 levels in coculture supernatants were quantified. As shown in Fig. 6, Dectin-1 blockage significantly reduced MIP-2 production by alveolar macrophages in response to *P. carinii*.

Binding of P. carinii and MIP-2 Production by RAW 264.7 Macrophages Overexpressing Dectin-1. RAW-264.7 cells, a murine macrophage cell line, express low endogenous lev-

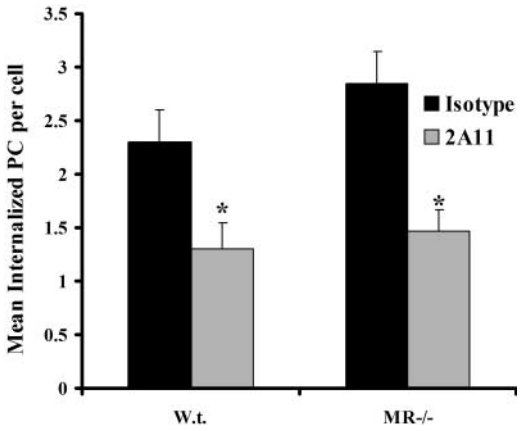

Figure 5. Internalization of *P. carinii* by alveolar macrophages collected from macrophage MR-deficient ($MR^{-/-}$) mice. Alveolar macrophages were isolated from 6–8-wk-old, male C57BL/6 or $MR^{-/-}$ mice and pretreated with 2.0 μg/ml 2A11 (gray bars) or isotype control antibody (solid bars). Alexa Fluor 488 succinimidyl ester–labeled *P. carinii* cysts and trophozoites were added at a macrophage to *P. carinii* ratio of 1:5 in an in vitro phagocytosis assay. Thereafter, aliquots of macrophage/*P. carinii* cocultures were subjected to confocal microscopy for determination of *P. carinii* internalization. *, significant differences between cocultures of isotype and 2A11 ($P < 0.05$). Data are expressed as the mean number of internalized *P. carinii* organisms per alveolar macrophage.

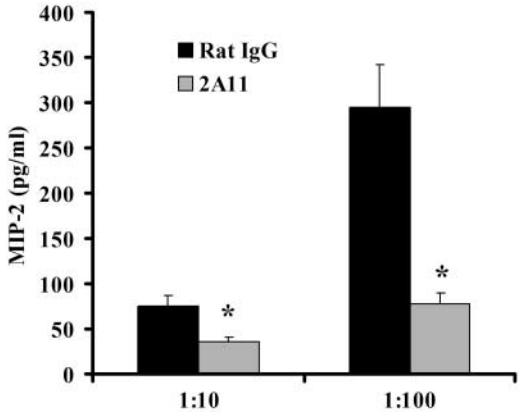

Figure 6. MIP-2 production by alveolar macrophages in response to *P. carinii* is mediated by Dectin-1. Alveolar macrophages were isolated from 6–8-wk-old, male C57BL/6 mice and cocultured for 16 h in the presence of rat IgG or 2A11 with *P. carinii* at macrophage to total *P. carinii* organism ratios of 1:10 and 1:100. Controls included alveolar macrophages cultured in medium alone. Thereafter, MIP-2 concentrations in coculture supernatants were determined by ELISA. Cumulative results from four separate experiments are shown. *, significant differences between cocultures of rat IgG and 2A11 ($P < 0.05$ and $P = 0.011$ for 1:10 and 1:100, respectively). Data are expressed as mean MIP-2 pg/ml ± SEM.

els of Dectin-1 (26). To confirm by additional means that Dectin-1 was indeed the receptor required for recognition and binding of *P. carinii*, we used RAW 264.7 macrophages transduced with a retrovirus encoding the murine Dectin-1 cDNA (RAW-Dectin cells; reference 26). Thereafter, we performed an in vitro binding assay using FITC-conjugated *P. carinii* and quantified the level of *P. carinii* binding. As shown in the micrographs in Fig. 7 A,

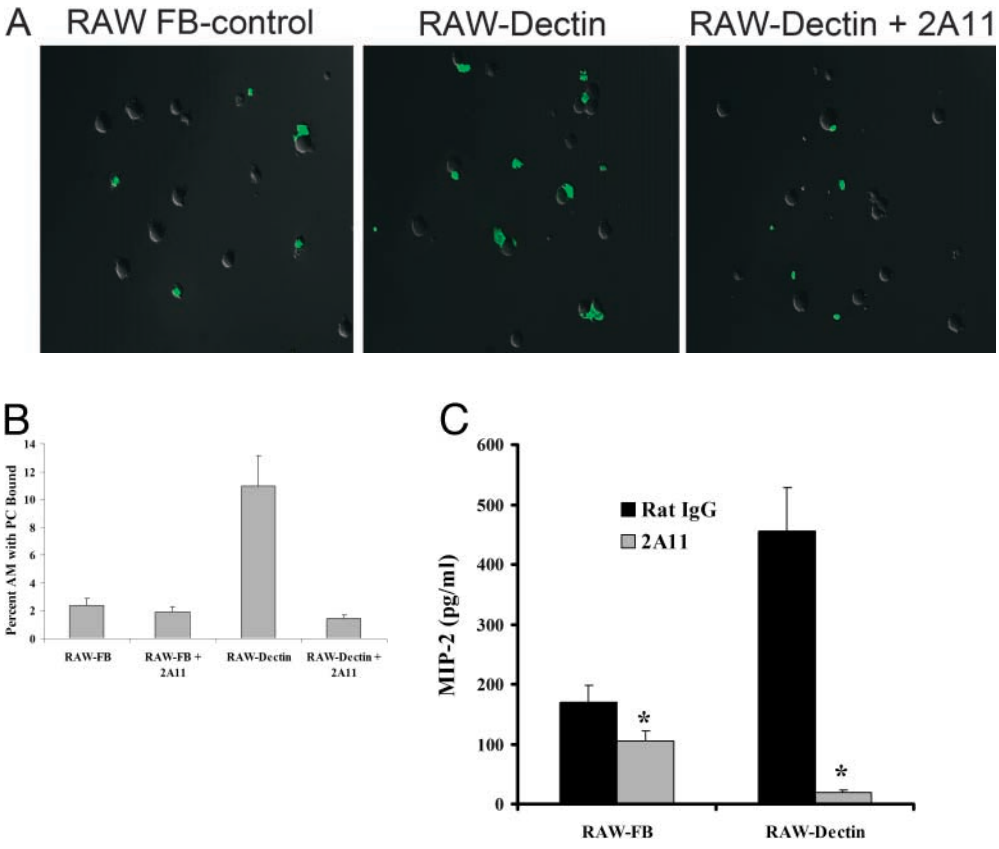

Figure 7. *P. carinii* binding and MIP-2 production by RAW 264.7 macrophages overexpressing Dectin-1. In A, an in vitro binding assay was performed with RAW-FB or RAW-Dectin macrophages prelabeled with an isotype control antibody or 2.0 μg/ml 2A11 and FITC-labeled *P. carinii*. Slidebook™ analysis software was used to capture, deconvolve, and superimpose FITC-*P. carinii* images with Nomarski macrophage images. In B, numbers of macrophages bound with *P. carinii* were enumerated using Slidebook™ software. In C, RAW-FB or RAW-Dectin macrophages were cocultured for 16 h in the presence of rat IgG or 2A11 with *P. carinii* at a macrophage to total *P. carinii* organism ratio of 1:100. Controls included macrophages cultured in medium alone. Thereafter, MIP-2 concentrations in coculture supernatants were determined by ELISA. Cumulative results from three separate experiments are shown. *, significant differences between cocultures of rat IgG and 2A11 ($P < 0.05$). Data are expressed as mean MIP-2 pg/ml ± SEM.

there was little to no binding or association of *P. carinii* cysts with RAW-FB macrophages transduced with a control plasmid (Fig. 7 A, left), whereas RAW-Dectin macrophages overexpressing Dectin-1 were capable of binding *P. carinii* (Fig. 7 A, middle). Furthermore, when macrophages overexpressing Dectin-1 were pretreated with 2.0 μg/ml 2A11, a significant reduction in binding of *P. carinii* was observed (Fig. 7 A, right). Quantitative data of the microscopic analysis is presented in Fig. 7 B.

We also further confirmed that MIP-2 production in response to *P. carinii* was associated with Dectin-1 recognition. As illustrated in Fig. 7 C, RAW-Dectin macrophages overexpressing Dectin-1 produced significantly higher levels of MIP-2 than RAW-FB control macrophages. Furthermore, pretreatment with 2.0 μg/ml 2A11 abrogated MIP-2 production by Dectin-1 overexpressing RAW macrophages.

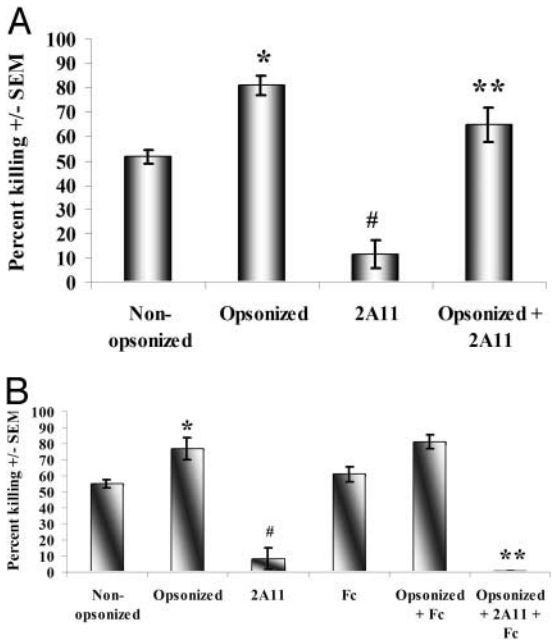

Figure 8. The effects of Dectin-1 blockage in the presence of FcγRII/III-mediated phagocytosis. Alveolar macrophages were isolated from 6–8-wk-old, male C57BL/6 mice and added to *P. carinii* in the presence of nonopsonizing or opsonizing sera (diluted 1:100; A), pretreated with 2 μg/ml 2A11 for 30 min at 37°C with or without opsonizing sera (A), or pretreated with 2 μg/ml 2A11 and anti-CD16/CD32 (Fc) for 30 min at 37°C with and without opsonizing sera (B), and added to *P. carinii* overnight for a final macrophage to *P. carinii* cyst ratio of 100:1 (A). Controls included *P. carinii* cultured without macrophages in the presence of nonopsonizing or opsonizing sera alone or containing the specific antibody. Thereafter, RNA was isolated from the contents of each well and quantitative real time PCR for *P. carinii* rRNA copy number was performed. Cumulative results from four separate experiments are shown. In A: *, significant differences between nonopsonizing versus opsonizing sera ($P = 0.0002$); #, significant differences between nonopsonizing sera and 2A11 ($P = 0.0001$); **, significant differences between 2A11 and opsonizing sera plus 2A11 ($P = 0.0005$). In B: *, significant differences between nonopsonizing versus opsonizing sera ($P = 0.007$); #, significant differences between nonopsonizing sera and 2A11 ($P = 0.0001$); **, significant differences between 2A11 and opsonizing sera plus 2A11 plus Fc Block (Fc; $P < 0.0001$). Data are expressed as mean percent killing ± SEM.

Nonopsonic Dectin-1–mediated Phagocytosis Can be Circumvented by FcγRII/III-mediated Phagocytosis. Antibody-mediated immunity plays a significant role in protection against *P. carinii* (31–33). Because Dectin-1 is required for nonopsonic phagocytosis of *P.* carinii, we questioned whether Dectin-1 was required for opsonic phagocytosis. The addition of anti–*P. carinii* IgG-containing sera, which we have previously shown to be protective in adoptive transfer experiments (18), to the macrophage/*P. carinii* coculture resulted in a significant enhancement in alveolar macrophage–mediated *P. carinii* killing (Fig. 8 A). Complement was not a factor contributing to the enhanced killing of *P. carinii* as heat inactivation of immune sera at 56°C for 30 min had no effect on the enhanced killing (74 and 84% for immune and immune heat inactivated, respectively). Next, we questioned whether blockage of nonopsonic Dectin-1–mediated phagocytosis had an effect on opsonic phagocytosis. To this end, we preincubated alveolar macrophages with 2A11 and thereafter added anti–*P. carinii* IgG-containing sera in conjunction with *P. carinii*. Opsonized *P. carinii* were efficiently killed by alveolar macrophages, despite the macrophage's inability to recognize *P. carinii* through the Dectin-1 β-glucan receptor (Fig. 8 A). To investigate whether this killing was mediated through FcγRII/III receptors, alveolar macrophages were pretreated with both 2A11 and anti-CD16/CD32 antibodies followed by the addition of anti–*P. carinii* IgG and *P. carinii*. Blockage of both FcγRII/III receptors and Dectin-1 completely abrogated the killing of opsonized *P. carinii* (Fig. 8 B). Interestingly, blockage of CD16/CD32 had no effect on alveolar macrophage–mediated killing of opsonized *P. carinii* (Fig. 8 B), suggesting intact Dectin-1–mediated recognition of opsonized *P. carinii*.

Discussion

Although cellular interactions with *P. carinii* are well reported in the literature, significant limitations in the analysis of *P. carinii* in vitro exist and therefore need to be taken into account when evaluating results. First and foremost, *P. carinii* cannot be cultured in vitro (16). To date, many experimental designs have used methods to radiolabel *P. carinii*, which can take up to 24 h before the organism is sufficiently labeled (6). Furthermore, additional protocols that use the incorporation of a radioactive compound (17) are based on the ability of an organism to proliferate in vitro, which is clearly not the case when dealing with *P. carinii*. Another peculiar characteristic of *P. carinii* is its species specificity. It has been reported that isolates of *P. carinii* originally obtained from a human specimen will in general not be pathogenic to rodents and vice versa (16, 34). However, studies to date that have often characterized innate recognition of *P. carinii* have largely relied on these nonsyngeneic systems whereby *P. carinii* from one species is cocultured with cells from another species (7, 15, 35, 36), which may lead to aberrant results. Taken together, there exists a precedent whereby a better coculture system can be used.

Here we report the use of a syngeneic in vitro coculture system that uses murine alveolar macrophages cocultured with murine *P. carinii* that quantifies specific changes in *P. carinii* viability using real time PCR for *P. carinii* rRNA copy number. Using this system, we were able to evaluate multiple experimental manipulations to macrophage functions and determine their effects on killing of *P. carinii*. Using an optimal macrophage to *P. carinii* cyst ratio of 100:1, we consistently observed a 50% reduction in *P. carinii* viability using both peritoneal and alveolar macrophages. A limitation to the real time PCR method of detection we used in this study is that it does not distinguish between the cyst and trophozoite forms of *P. carinii*. Although the macrophage to *P. carinii* cyst ratios in the viability assay were based on a known amount of *P. carinii* cysts, we estimate that our *P. carinii* preparations contain 75–100-fold higher numbers of trophozoites, therefore the ratio of macrophage to total *P. carinii* organisms is likely to be significantly lower (i.e., 1:1) than that represented in the figures.

We attributed the killing of *P. carinii* by alveolar macrophages to be a product of phagocytosis because inhibition of actin filament polymerization by cytochalasin D treatment rendered the macrophage completely unable to kill *P. carinii*. Furthermore, cocultures performed in the presence of catalase, which converts hydrogen peroxide to water and molecular oxygen, had significantly more detectable *P. carinii* rRNA, and thus more viable organisms, compared with those performed in the absence of catalase. These results implicate hydrogen peroxide in the direct killing of *P. carinii* by alveolar macrophages. In support of this, we detected less *P. carinii* rRNA from organisms that were cultured in the presence of physiologic concentrations of hydrogen peroxide. Interestingly, we were unable to detect a role for superoxide as dismutation with a cell-permeable superoxide dismutase mimetic did not induce a concomitant reduction in alveolar macrophage–mediated killing of *P. carinii*. However, it has been hypothesized that *P. carinii* may synthesize its own superoxide dismutase (14), thereby potentially rendering it somewhat resistant to superoxide-mediated damage. This may suggest that superoxide-mediated killing plays a minor role in alveolar macrophage defense against *P. carinii*. Nevertheless, previous studies have reported a role for both reactive oxygen species in the killing of *P. carinii*. Some reports have shown that both hydrogen peroxide and superoxide are directly toxic to *P. carinii* (12–14). Furthermore, studies have shown that *P. carinii* can elicit an oxidative burst of both hydrogen peroxide and superoxide from macrophages (12, 13). However, our study is the first to specifically inhibit the induction or activity of hydrogen peroxide, generated by the interaction between alveolar macrophages and *P. carinii*, and demonstrate a specific effect on killing of the organism. Although the reduction in killing in the presence of catalase was statistically significant, the observed decrease was modest. Therefore, we cannot exclude the possibility that additional intracellular molecules, such as proteases, are also playing a role in the killing of *P. carinii* (37).

The primary focus of this study was to evaluate the role of putative molecular recognition receptors in alveolar macrophage–mediated killing of *P. carinii*. For this, we performed competitive carbohydrate inhibition studies whereby alveolar macrophages were pretreated with yeast-derived mannan to block the macrophage MR and thereafter cocultured with *P. carinii*. In contrast to previous publications with nonautologous *P. carinii*/macrophage systems that investigated the binding and phagocytosis of *P. carinii* but not the killing, we observed no reduction in alveolar macrophage–mediated killing of *P. carinii* in the presence of MR blockage. The MR was previously identified as the first molecular recognition receptor mediating the binding and uptake of *P. carinii* (24). Additional studies have shown that the glycoprotein A of *P. carinii* specifically binds to the MR (8). Finally, defects in MR expression and function have been reported in HIV^+ individuals with and without *P. carinii* infection (7, 15). Although these studies report the importance of the MR in the recognition of *P. carinii*, our results indicate that the killing of *P. carinii* by alveolar macrophages may occur independently of MR recognition. Because we used a syngeneic coculture of murine *P. carinii* and murine alveolar macrophages in our experimental design, differences in our results with others may potentially be explained through the use of nonsyngeneic cocultures (i.e., rat-derived *P. carinii* cocultured with human macrophages) in the previous analyses (7, 15). Although it has been reported that all *P. carinii*, regardless of their host of origin, express mannose-containing surface glycoproteins (38), taking into account the species specificity of *P. carinii* isolates (16, 34), it is conceivable that the carbohydrate composition of the *P. carinii* cell wall might be different between isolates from different species. Finally, MR-deficient mice were recently shown to have no defects in phagocytosis and no overt susceptibility to *C. albicans* (39), a fungal organism containing a mannose-enriched cell wall that is recognized by the MR (40). However, phagocytosis of *C. albicans* was inhibited by β-glucan, suggesting alternative receptors responsible for phagocytosis of this organism. To specifically address the role of the macrophage MR, we isolated alveolar macrophages from MR-deficient ($MR^{-/-}$) mice (22) and cocultured them with fluorescently labeled *P. carinii* in an in vitro phagocytosis assay. We observed no defects in the ability of alveolar macrophages from $MR^{-/-}$ mice to internalize both *P. carinii* cysts and trophozoites compared with alveolar macrophages from wild-type control mice. Furthermore, Swain et al. (41) have recently reported that $MR^{-/-}$ mice clear *P. carinii* from the lungs similar to wild-type control mice. Taken together, these data suggest that the macrophage MR is not required for the binding and killing of murine *P. carinii*.

Our studies support a role for other receptors mediating the killing of *P. carinii* by alveolar macrophages, namely β-glucan receptors. Analysis of the *P. carinii* cell wall has shown that β-glucans constitute the majority of carbohydrate moieties (42, 43). As well, studies have shown a dis-

tinct role for β-glucan receptors in the alveolar macrophage inflammatory response (10, 11). Recently, the major β-glucan receptor on macrophages was identified as Dectin-1 (20, 21). Dectin-1 is a 28-kD, type II transmembrane receptor containing one lectin-like carbohydrate recognition domain, which recognizes β1,3-linked and β1,6-linked glucans and intact fungal organisms. Therefore, we performed studies to address the role of β-glucan receptors in alveolar macrophage–mediated killing of *P. carinii*. Initial competitive carbohydrate inhibition studies showed poor killing of *P. carinii* by alveolar macrophages in the presence of *S. cerevisiae*–derived glucan, suggesting that a β-glucan receptor was critical in nonopsonic killing of *P. carinii*. Subsequent studies using immunofluorescent microscopy showed high levels of Dectin-1 β-glucan receptor expression on alveolar macrophages. Preincubation of alveolar macrophages with an anti–Dectin-1 monoclonal antibody significantly abrogated the killing of *P. carinii*. We confirmed the interaction of Dectin-1 on alveolar macrophages and *P. carinii* by using fluorescent deconvolution microscopy that showed alveolar macrophages binding *P. carinii* in the presence of low concentrations of anti–Dectin-1 antibody but not in the presence of 20-fold higher concentrations. We were also able to demonstrate colocalization of Dectin-1 interacting with the *P. carinii* cell wall. Additional studies showed that RAW 264.7 macrophages overexpressing Dectin-1 bound *P. carinii* at higher levels than control macrophages and the binding was significantly inhibited when Dectin-1 was blocked. Finally, confirming the importance of Dectin-1 in recognition and internalization of *P. carinii*, alveolar macrophages from $MR^{-/-}$ mice pretreated with anti–Dectin-1 antibody have significant reductions in *P. carinii* internalization. Taken together, our results support the discovery of the Dectin-1 β-glucan receptor as a critical receptor responsible for the nonopsonic recognition and subsequent inhibitory function by alveolar macrophages against *P. carinii*.

In addition, we report here that elicitation of the proinflammatory chemokine MIP-2 from alveolar macrophages by whole *P. carinii* organisms is also dependent on Dectin-1 as a >75% reduction in MIP-2 production was observed from alveolar macrophages pretreated with anti–Dectin-1 monoclonal antibody and exposed to *P. carinii*. Moreover, RAW 264.7 macrophages overexpressing Dectin-1 produced more MIP-2 than control macrophages when cocultured with *P. carinii* and the elicitation of MIP-2 was completely abrogated when these cells were pretreated with anti–Dectin-1 antibody. Dectin-1 was initially reported to contain an immunoreceptor tyrosine-based activation motif in the cytoplasmic tail (44). A recent study reported production of TNF-α in response to zymosan, *C. albicans*, and *S. cerevisiae* by the RAW macrophage cell line that overexpressed Dectin-1 (26). Furthermore, zymosan-induced TNF-α production was significantly blunted in RAW cells transfected with truncated Dectin-1 or with Dectin-1 containing a mutated immunoreceptor tyrosine-based activation motif (26). Although alveolar macrophages have been shown to produce both TNF-α and MIP-2 in response to purified *P. carinii* cell wall β-glucans, we were unable to elicit significant levels of TNF-α using whole *P. carinii* organisms (unpublished data). Nevertheless, we show that alveolar macrophage–mediated proinflammatory chemokine responses to *P. carinii* requires Dectin-1.

We also examined alveolar macrophage–mediated killing of *P. carinii* through opsonic pathways and determined whether Dectin-1 was required. Opsonized *P. carinii* could be killed by alveolar macrophages either through Dectin-1–mediated phagocytosis or FcγRII/III-mediated phagocytosis. However, upon blockage of both Dectin-1 and FcγRII/III, alveolar macrophages were completely unable to kill opsonized *P. carinii*.

In conclusion, our data indicates that nonopsonic macrophage-mediated killing of *P. carinii* is specifically dependent on recognition of *P. carinii* by the Dectin-1 β-glucan receptor leading to phagocytosis and subsequent killing of the organism by hydrogen peroxide. Furthermore, we report that the Dectin-1 β-glucan receptor is required for efficient elicitation of proinflammatory chemokines, such as MIP-2, by alveolar macrophages in response to *P. carinii*. β1,3-linked and β1,6-linked glucan moieties are prevalent in the cell walls of such medically important fungi as *Aspergillus fumigatus* and *C. albicans* (45). Further investigations into the role of Dectin-1 in these fungal diseases will provide invaluable insight as to how these organisms are recognized by the innate immune system. Finally, although it is not currently known whether HIV infection affects Dectin-1 expression, our results support the importance of developing antibody-based vaccination strategies (18) for *P. carinii* as anti–*P. carinii* IgG not only significantly enhanced alveolar macrophage host defense against *P. carinii*, but also restored macrophage phagocytosis in the presence of Dectin-1 blockage.

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