Dectin immunoadhesins and Pneumocystis pneumonia

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The opportunistic pathogen *Pneumocystis jirovecii* is a significant cause of disease in HIV-infected patients and others with immunosuppressive conditions. *Pneumocystis* can also cause complications in treatment following antiretroviral therapy or reversal of immunosuppressive therapy, as the newly reconstituted immune system can develop a pathologic inflammatory response to remaining antigens or a previously undetected infection. To target β-(1,3)-glucan, a structural component of the *Pneumocystis* cell wall with immune-stimulating properties, we have developed immuno-adhesins consisting of the carbohydrate-binding domain of Dectin-1 fused to the Fc regions of the 4 subtypes of murine IgG. These immuno-adhesins bind β-glucan with high affinity, and precoating the surface of zymosan with Dectin-1:Fc can reduce cytokine production by macrophages in an *in vitro* stimulation assay. All Dectin-1:Fc variants showed specificity of binding to the asci of *P. murina*, but effector activity of the fusion molecules varied depending on Fc subtype. Dectin-1:mIgG2a Fc was able to reduce the viability of *P. murina* in culture through a complement-dependent mechanism, whereas previous studies have shown the mIgG1 Fc fusion to increase macrophage-dependent killing. In an *in vivo* challenge model, systemic expression of Dectin-1:mIgG1 Fc significantly reduced ascus burden in the lung. When administered post-infection in a model of immune reconstitution inflammatory syndrome (IRIS), both Dectin-1:mIgG1 and mIgG2a Fc reduced hypoxemia despite minimal effects on fungal burden in the lung. Taken together, these data indicate that molecules targeting β-glucan may provide a mechanism
for treatment of fungal infection and for modulation of the inflammatory response to *Pneumocystis* and other pathogens.
Pneumocystis is an opportunistic fungal pathogen that causes pneumonia in immunocompromised hosts, including those with human immunodeficiency virus (HIV) and immunosuppression secondary to chemotherapy or organ transplantation. Despite a significant decline in the incidence of Pneumocystis pneumonia (PCP) following the introduction of PCP chemoprophylaxis and potent combination antiretroviral therapy (ART), PCP remains a leading opportunistic infection in HIV+ adults and children worldwide (1–3). In addition, as more potent immunotherapies are developed and the number of patients receiving immunosuppressive therapy and antitumor chemotherapeutic agents increases, the prevalence of PCP in non-HIV infected patients continues to grow (4, 5).

The depletion or dysfunction of CD4+ T cells is the primary risk factor for host susceptibility to Pneumocystis infection, and HIV+ patients with CD4+ T cell counts below 200 cells/µl are highly susceptible to PCP if not receiving preventive therapy (6, 7). Animal models have also demonstrated the fundamental role of CD4+ T cells in protective immunity against Pneumocystis. Selective depletion of CD4+ T cells can render mice susceptible to persistent pulmonary infection (8, 9), and SCID, Rag1−/−, and Rag2−/− mice which lack functional B and T cells are also highly susceptible to PCP (10, 11). In HIV-infected patients, the goal of ART treatment is to increase the population of CD4+ T cells and restore protective immunity against pathogens such as
Pneumocystis, but this restoration may be associated with a paradoxical clinical deterioration and the development of a severe inflammatory response against specific foreign or self-antigens (12–14). This immune reconstitution inflammatory syndrome (IRIS), also termed immune reconstitution disease (IRD) or immune reconstitution syndrome (IRS), has become a major challenge in the clinical treatment of HIV, affecting from 8-33% of ART responders (15–18). Development of IRIS has also been reported in non-HIV patient populations following withdrawal of immunosuppressive therapy, such as transplant recipients (19) and patients receiving tumor necrosis factor antagonists (20).

IRIS in the context of Pneumocystis infection has been described in both HIV-infected patients (21–24) and for those with non-HIV immunosuppressive conditions (25, 26). PCP-associated IRIS has been modeled in murine hosts using selective transfer of lymphocyte subsets (27) and depletion of regulatory T-cells from these cell populations can exacerbate lung injury (28). In further support of effector T-cells mediating lung injury in Pneumocystis IRIS, it has also been shown that anti-CD3 antibodies can ameliorate lung injury in the adoptive transfer model (29).

Carbohydrate antigens such as β-glucans in the Pneumocystis cell wall are believed to play a significant role in both protective immunity and the pathologic inflammatory response of IRIS (30, 31). Signaling of the pattern recognition receptor Dectin-1 in response to β-glucan can regulate expression of innate response genes, linking innate to adaptive immunity (32). Dectin-1 signaling can
promote activation of dendritic cells, rendering them competent to prime Th1 and Th17 responses as well as cytotoxic T lymphocyte responses (33, 34). Prior studies in the lab demonstrated that administration of a fusion protein that encodes the extracellular domain of the β-glucan receptor Dectin-1 fused to a murine IgG Fc can opsonize Pneumocystis organisms and can reduce organism burden in the lungs of P. murina infected SCID mice (35). In this study we investigated the role of the IgG Fc fusion isotype in killing of Pneumocystis in vitro as well as reduction of organism burden in vivo. Moreover, we examined the effect of these molecules on Dectin-1 signaling and the ability of Dectin-1:Fc to ameliorate lung injury in the IRIS adoptive transfer model.

**MATERIALS AND METHODS**

Mice. Six- to 8-wk-old, wild type C57BL/6J mice and immunodeficient B6.129S7-Rag1tm1Mom/J (Rag1-/-) mice were obtained from The Jackson Laboratory (Bar Harbor, MN). Immunodeficient B10;B6-Rag2tm1Fwa Ii2rgtm1Wjl (Rag2-/-Il2rg-/-) mice were obtained from Taconic (Hudson, NY). Animals were housed in a pathogen-free environment and given food and water ad libitum. All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Monoclonal abs and Fc-fusion proteins. Anti-Dectin-1 mAb, clone 2A11, rat IgG2b (AbD Serotec, Raleigh, NC) was used as previously described (36). Construction of recombinant Dectin-1:mlG1 Fc was previously described (35).
Additional Dectin-1 fusion vectors containing an IgK leader sequence to facilitate secretion, the extracellular domain of the Dectin-1 receptor (amino acids 69–244), and hexapeptide linker with thrombin cleavage site cloned in frame with the Fc regions of murine IgG2a, IgG2b, and IgG3 were constructed using the parent Dectin-1:mlG1 Fc vector and the vectors pFUSE-mlG2a-Fc, pFUSE-mlG2b-Fc, and pFUSE-mlG3-Fc, respectively (Invivogen, San Diego, CA).

**Molecular interaction analysis.** Real-time surface plasmon resonance experiments were performed on a BIAcore 3000 Instrument with CM5 sensor chips (GE Healthcare Life Sciences, Piscataway, NJ) at 25°C. Amine coupling with N-hydroxysuccinimide (NHS)/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was used to covalently immobilize rabbit anti-mouse capture antibodies onto the chip surface (Mouse Antibody Capture Kit, GE Healthcare Life Sciences). Affinity purified Dectin-1:Fc was captured by injection at a flow rate of 5 μl/min (total 35 μl). Laminarin (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS and diluted in running buffer (10 mM HEPES (pH 8.0), 150 mM NaCl, 0.002% Tween 20), injected by KINJECT and allowed to reach equilibrium, after which only running buffer was applied. Immobilized rabbit anti-mouse Abs or an irrelevant IgG2a mAb served as a control surface and nonspecific binding of laminarin was subtracted from the signal in the active flow cell. The association rate constant ($k_a$) and dissociation rate constant ($k_d$) were calculated and the equilibrium association ($K_A$) and dissociation ($K_D$) constants were determined using BIAevaluation 4.1 software.
**In vitro macrophage stimulation.** Zymosan (Invivogen) was depleted of TLR-activating ligands by boiling in hot alkali as previously described (37) and optionally pretreated with 100 µg/ml Dectin-1:Fc for 30 minutes at 37°C. RAW264.7 cells cultured in DMEM + 10% FBS were plated into 96-well flat-bottom plates at a density of 1×10^5 cells per well and stimulated with 50 µg/ml zymosan. A control group of RAW cells were also pretreated for 30 minutes with 5 µg/ml of mAb 2A11 to block Dectin-1 binding to zymosan. Cell culture supernatants were harvested 16 hours after stimulation and assayed for G-CSF and TNFα production using a custom multiplex bead assay (EMD Millipore, Billerica, MA).

**Pneumocystis isolation and inoculum.** *Pneumocystis murina* (PC) organisms were isolated from the lung tissue of *Rag2⁻/⁻Il2rg⁻/⁻* mice previously inoculated with PC. The inoculum for infectious challenge was prepared by differential centrifugation as previously described (38). Briefly, *Rag2⁻/⁻Il2rg⁻/⁻* mice with *Pneumocystis pneumonia* were sacrificed, and the lungs were aseptically removed and frozen in 1 ml of sterile phosphate-buffered saline (PBS) at -80°C. Frozen lungs were thawed, homogenized through a 70 µM filter, and pelleted by centrifugation at 800×g for 10 minutes at 4°C. The pellet was resuspended in 1 ml PBS, and a 1:10 dilution was stained with Hema-3 modified Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA). The number of PC asci (cyst forms) was quantified microscopically (8), and the inoculum concentration was adjusted to 2×10^6 asci per ml. 100 µl of this inoculum, corresponding to 2×10^5 asci per mouse, was given by oropharyngeal aspiration using the tongue-pull technique.
Briefly, mice were lightly anesthetized using 2-3% isoflurane, suspended by their front incisors, and the tongue was gently extended using forceps. The inoculum was pipetted into the trachea, and the tongue was held until two breaths were completed. For ascus-enriched preparations of PC, asci were isolated from trophic forms using sucrose gradient density centrifugation, according to a method previously described (39).

**Pneumocystis viability assay.** *P. murina* (1×10⁴ asci per well, estimated 1:10 ascus to trophic form ratio) was cultured in 96 well round bottom plates in DMEM plus 10% fetal bovine serum (FBS). Serum was treated by heat inactivation for 30 minutes at 56°C to deplete complement activity (HI-FBS) or left untreated (Non-HI FBS). *P. murina* was treated with affinity purified Dectin-1:Fc at various concentrations and cultured for 24 hours. A viability control of PC incubated with control medium was included. Following incubation, the contents of the wells were collected and total RNA was isolated using TRIzol-LS reagent (Life Technologies, Carlsbad, CA). Viability of PC was analyzed with real-time PCR measurement of rRNA copy number as described below.

**Flow cytometry.** For comparison of different Fc isotypes, *P. murina* organisms were stained with affinity purified Dectin-1:Fc and FITC-conjugated Fcγ fragment specific (subclass 1+2a+2b+3) secondary antibody (Jackson Immunoresearch, West Grove, PA) and analyzed using an LSRll flow cytometer (BD Biosciences, San Jose, CA). To assess the specificity of Dectin-1:Fc to specific forms of *Pneumocystis*, ascus-enriched or total preparations of PC were stained with
Dectin-1:Fc directly conjugated to PerCP-Cy5.5 (Abcam, Cambridge, MA) and analyzed with an LSRII flow cytometer. Secondary analysis was performed using FlowJo software (Tree Star, Ashland OR).

**Immunofluorescence.** Pneumocystis samples were heat-fixed onto slides and further fixed and permeabilized by immersion in ice cold methanol. Slides were washed with PBS and blocked using 5% mouse serum and 1% bovine serum albumin in PBS. Samples were stained with AlexaFluor555-conjugated Dectin-1:mlgG1 and Dectin-1:mlgG2a Fc (APEX antibody labeling kit, Invitrogen). DAPI-containing mounting medium (Vector Labs, Burlingame, CA) was used to coverslip samples, which were visualized at 60X magnification.

**Hydrodynamic injection of plasmid DNA.** Expression of Dectin-1 Fc fusion proteins was achieved *in vivo* by systemic administration of plasmid DNA according to the method of Liu et al. (40). Mice were injected with 10 µg endotoxin-free plasmid DNA in isotonic saline (0.9% w/v NaCl) or Ringer’s solution (0.9% NaCl, 0.03% KCl and 0.016% CaCl₂). A total injection volume of 1 ml per 10 g mouse bodyweight was injected via tail vein within 5 to 10s.

**Purification of T cell subsets and adoptive transfer.** Spleens from naïve C57BL/6J mice were collected, teased apart, and filtered through a 70 µM cell strainer under sterile conditions. CD4⁺CD25⁻ cells for adoptive transfer were purified by magnetic bead separation using only the negative selection step of a CD4⁺CD62L⁺ T cell isolation kit to deplete non-CD4 cells (Miltenyi Biotec, Auburn, CA). To induce immune reconstitution syndrome, $3 \times 10^5$ purified
CD4⁺CD25⁻ cells were injected intravenously via tail vein to WT or Rag1⁻/⁻ mice, 25 days post infection with 2×10⁵ *P. murina* asci and 3 days post hydrodynamic injection. Reconstituted mice were sacrificed 11 days post adoptive transfer.

**BAL fluid, serum, and lung tissue collection.** At time of sacrifice, mice were anesthetized with ketamine/xylazine and serum was collected from the posterior vena cava. Tracheas were cannulated and 1 ml of PBS without calcium and magnesium (PBS-free) was instilled through the cannula and aspirated. Bronchoalveolar lavage (BAL) fluid cells were pelleted by centrifugation at 500×g and the supernatant was stored at -80°C for further analysis. Following the collection of lavage fluid, lungs were harvested for RNA isolation, cytokine analysis, and histology. After tying off the right bronchus, one lobe of the right lung was excised and placed into 1 ml TRizol (Life Technologies), homogenized, and stored at -80°C until RNA isolation. The remaining lobes of the right lung were placed into 1 ml PBS-free containing complete protease inhibitor (Roche, Indianapolis, IN), then homogenized and centrifuged at 12,000×g for 15 minutes at 4°C. The supernatant was stored at -80°C for later cytokine analysis. For histological study, the left lung was inflated with buffered zinc formalin (Z-fix, Anatech, Battle Creek, MI) and placed into fixative. Paraffin-embedded sections were stained with Gomori-methenamine silver and scored blindly for intensity of infection as previously described using a semi-quantitative scale, ranging from 1 (rare asci per high-power field) to 4 (asci throughout most alveoli with foamy exudate) (38).
RNA isolation and TaqMan probes and primers for Pneumocystis rRNA.

The assay for determination of *P. murina* copy number per whole lung has been previously described (38). Briefly, cDNA was synthesized with iScript reverse transcription reagents (Bio-Rad, Hercules, CA), and real-time PCR was performed using primers for PC large subunit ribosomal RNA gene with SsoFast Probes Supermix (Bio-Rad). The threshold cycle values were converted to rRNA copy number by using a standard curve of known copy number of *Pneumocystis* rRNA as previously described (41).

**Pulse oximetry and parameters of lung injury.** Blood oxygen saturation was measured using a MouseOx pulse oximeter with a tail sensor (Starr Life Sciences, Oakmont, PA). Following anesthesia with 100 mg/kg intraperitoneal ketamine, the tail sensor was placed at the base of the tail and measurements were recorded using MouseOx software. One minute readings were taken from each mouse and an average value of 10-20 stable, error-free measurements over this interval are reported. Total protein in the BAL fluid was assayed with a BCA protein assay kit (Pierce, Rockford, IL) as per manufacturer’s instructions. Lactate dehydrogenase (LDH) levels in the BAL fluid were analyzed by an LDH activity assay kit (BioVision, Milpitas, CA) as per manufacturer’s instructions. Expression levels of Dectin-1:Fc proteins in serum and BALF of *Rag1*+/− mice were measured using isotype-specific IgG ELISA quantitation sets (Bethyl Laboratories, Montgomery, TX).

**Cytokine analysis.** BAL fluid and lung homogenate samples were analyzed for
protein levels of G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα using a MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) on a Bio-Plex 200 instrument (Bio-Rad). The data were analyzed using Bio-Plex Manager software (Bio-Rad). Data are reported as mean ± SEM.

**Statistical analysis.** GraphPad Prism (GraphPad Software, La Jolla, CA) was used to calculate $p$ values using one-way ANOVA with a Holm-Sidak multiple comparisons posttest. For testing of non-parametric data with three or more groups, such as PC ascus burden, Kruskal-Wallis test was performed with a Dunn multiple comparisons posttest. For comparison of HI and non-HI FBS in Dectin-1:Fc treatment on PC viability, multiple T test comparisons were performed with a Holm-Sidak correction. A value of $p \leq 0.05$ was considered statistically significant.

**RESULTS**

**Characterization of Dectin-1:Fc protein reactivity and structure.** A recombinant Dectin-1 receptor Fc fusion protein containing the extracellular domain of the murine β-glucan receptor Dectin-1 (amino acids 69-244), a thrombin-sensitive hexapeptide linker, and the murine IgG1 hinge and CH₂ through CH₃ domains was previously shown to increase macrophage-
dependent killing of PC in vitro and enhance host recognition and clearance of PC in immunodeficient SCID mice (35). As these findings suggested that FcγR-based targeting of PC via cell wall carbohydrate recognition can promote resistance against PC pneumonia in the immunodeficient host, we sought to understand the relative contribution of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) by creating Dectin-1 fusions containing the Fc regions of murine IgG2a, IgG2b, and IgG3. The resulting vectors Dectin-1:mIgG2a, Dectin-1:mIgG2b, and Dectin-1:mIgG3 were used to produce recombinant proteins in transfected HEK293-T cells, and we assessed the binding of these Fc fusion proteins to β-(1,3)D-glucan using surface plasmon resonance measurements (Fig 1).

Initially, varying amounts of each affinity-purified Dectin-1 Fc protein were captured with immobilized anti-mouse antibodies on a CM5 sensor chip (GE Healthcare Life Sciences, Piscataway, NJ) and measured for response to laminarin, a glucan molecule consisting of primarily β-(1,3) linked glucan. The ratio of laminarin-binding response units (RU) per Dectin-1:Fc protein RU loaded was similar for all Fc subtypes, indicating that none of the Fc subtypes grossly interfered with the carbohydrate recognition domain of Dectin-1. (Fig 1A, Table 1). We further characterized the binding of Dectin-1:mIgG2a and Dectin-1:mIgG2b by performing kinetic and affinity analyses with laminarin in 2-fold dilution series from 20 mM to 0.156 mM (Fig 1B,C). The rate of association (kₐ), the rate of dissociation (k₅), and the equilibrium constants of association (Kₐ) and dissociation (K₅) were calculated (Table 2), demonstrating high-affinity binding of
Dectin-Fc proteins to laminarin, similar in quality to our previous results with Dectin-1:mIgG1Fc (35) and to that of various carbohydrate-directed Abs or lectin receptors for their ligands. In addition, these affinity analyses demonstrate the generation of a stable interaction between laminarin and immobilized Dectin-1:Fc over the course of the kinetic analysis. This binding phenomenon may affect the calculation of the equilibrium constants, as the multiple glucan subunit interactions on each laminarin chain stabilize each other and prevent dissociation of laminarin from the Dectin-1 complex on the surface of the chip.

**Dectin-1:Fc masking of β-(1,3)-glucan residues reduces zymosan-induced stimulation of macrophages.** To address the ability Dectin-1:Fc to modify recognition of particles containing β-(1,3)-D-glucan, such as the cell wall surface of *Pneumocystis* and other fungi, we evaluated whether Dectin-1:mIgG1 and Dectin-1:mIgG2a could affect RAW264.7 macrophage stimulation by particulate zymosan. Zymosan is an insoluble cellular wall polysaccharide derived from *S. cerevisiae*, composed mostly of β-glucan and mannan (42). To ensure the specificity of stimulation to Dectin-1 in the assay, we removed all TLR2-activating components of zymosan by treating zymosan with hot alkali to make depleted zymosan (37). RAW cells were stimulated with depleted zymosan (Zym) with or without precoating with a molar excess of Dectin-1:Fc and compared to cells pretreated with the Dectin-1 receptor blocking monoclonal antibody 2A11 (43). After 18h of stimulation, production of granulocyte colony-stimulating factor (G-CSF) and tumor necrosis factor (TNFα) in the supernatant was measured (Fig. 3).
The results indicate decreased stimulation by zymosan in the Dectin-1:Fc treated groups, with significantly lower production of G-CSF than both zymosan alone and zymosan plus 2A11 mAb. Dectin-1:Fc treatment of zymosan also significantly reduced macrophage production of TNFα.

Dectin-1:mlgG2a Fc binds *Pneumocystis* organisms and enhances macrophage-independent killing. Our previous studies have demonstrated that Dectin-1:mlgG1 Fc binds specifically to the surface of *Pneumocystis* asci (cyst forms) using fluorescent deconvolution microscopy and flow cytometry (35). In this study, we tested and confirmed the binding of the novel Dectin-1:IgG2a, :IgG2b, and :IgG3 Fc fusion proteins to *P. murina* organisms by flow cytometry (Fig. 3A). When subpopulations of asci and trophic forms were analyzed using flow cytometry, Dectin-1 Fc proteins showed an increased binding to *P. murina* asci, indicating a greater availability of β-(1,3)-D-glucan in the cell wall of the ascus (Fig 3B,C). Immunofluorescence staining of whole *P. murina* with fluorochrome-labeled Dectin-1 Fc also showed specific binding to the ascus (Fig 3D,E).

The Dectin-1:mlgG1 Fc protein was previously shown to enhance the macrophage-dependent killing of PC *in vitro* (35). Preopsonization of PC organisms with Dectin-1:mlgG1 Fc diminished overall copy numbers by 3-fold in studies with thioglycollate-elicited peritoneal macrophages and by up to 10-fold with alveolar macrophages (35). We sought to determine if Dectin-1 Fc proteins could enhance PC killing *in vitro* without macrophages through a complement-
dependent cytotoxic mechanism. We studied decreases in absolute quantities of PC mitochondrial large-subunit rRNA (PC mtLSU rRNA) copy numbers as a correlate of in vitro PC killing, a methodology validated by previous work (41, 44). PC organisms were isolated from infected murine lung homogenates and were treated with Dectin-1:Fc in media supplemented with fetal bovine serum that was untreated or heat-inactivated for 30 minutes at 56°C to remove complement activity (HI). Dectin-1:mlgG1 Fc did not reduce PC rRNA copies in vitro in the absence of macrophages (Fig. 4A). In contrast, Dectin-1:IgG2a exhibited potent PC killing activity at 35 and 14 ug/ml, but only in media supplemented with non-heat-inactivated serum, indicating a role for complement fixation in the killing process (Fig 4B). These results are consistent with previous observations that mouse IgG2a is the most efficient IgG at fixing complement and exhibits the most CDC (45).

Sustained expression of Dectin-1:Fc via hydrodynamic injection can reduce P. murina asci in B- and T cell-deficient Rag1−/− mice. Following our previous reports of increased macrophage-dependent killing of PC in vitro and protection in vivo in a SCID model with Dectin-1:mlgG1 Fc (35) and our current observations with macrophage-independent in vitro killing of PC by Dectin-1:mlgG2a Fc, we sought to compare the effects of these molecules in an immunodeficient mouse model of PC infection. For this model, we used Rag1−/− mice, which lack B and T cells, and chose to express the Dectin-1:Fc fusion proteins through hydrodynamic injection of plasmid DNA via tail vein. Groups of
Rag1−/− mice were injected with Dectin-1:mlgG1 Fc, Dectin-1:mlgG2a Fc, or buffer control on day 0 and inoculated with PC on day 1. Blood samples were collected at baseline, 4, 7, and 10 days to assess protein production, and all mice were sacrificed on day 15 to assess PC lung and ascus burden (Fig. 5A). Hydrodynamic injection of 10 µg plasmid DNA resulted in sustained high level expression of Dectin-1:Fc proteins during the course of infection (Fig. 5B). At 14 days post infection, total PC lung burden assessed by realtime RT-PCR for PC mtLSU rRNA was not significantly different between any groups (Fig. 5C). However, this assay does not discriminate between asci or trophic forms of the organism. In contrast, quantification of asci by histological scoring of Gomori methenamine silver (GMS) stained lung sections revealed a significant decrease in the P. murina ascus burden in Dectin-1:mlgG1 Fc-injected mice (Fig. 5D).

Dectin-1:Fc decreases markers of inflammation in an immune reconstitution inflammatory syndrome model of PC infection. As fungal β-(1,3)-glucan is known to trigger potent inflammatory responses, altering glucan surface content of an infectious organism may have strong potential to modulate the host immune response. In the case of Pneumocystis, we have demonstrated that Dectin-1:Fc has an increased binding affinity of for the P. murina ascus, which contains a greater proportion of β-(1,3)-glucan in the cell wall (46). We have also shown that macrophages exhibit decreased production of inflammatory cytokines when these glucan residues are masked by soluble Dectin-1:Fc in an in vitro stimulation assay. We hypothesized that these properties along with the
increased PC killing activity shown by Dectin-1:mIgG1 and Dectin-1:mIgG2a Fc via macrophage- and complement-dependent mechanisms may provide a therapeutic benefit in a mouse model of immune reconstitution inflammatory syndrome by reducing the generation of damaging inflammatory responses in the lung.

To test this hypothesis, WT or Rag1\(^{-/-}\) mice were inoculated with PC at day 0 and allowed to progress for 3 weeks of infection, followed by hydrodynamic injection treatment at day 22. WT mice received buffer control only, and Rag1\(^{-/-}\) mice were treated with either buffer control, IgG1 Fc control, IgG2a Fc control, Dectin-1:mIgG1 Fc, or Dectin-1:mIgG2a Fc (Fig. 6A). Dectin-1:Fc protein expression was assayed 3 days later, with expression levels in serum averaging 22 and 14 µg/ml for Dectin-1:mIgG1 and Dectin-1:mIgG2a, respectively (Fig. 6B). At this time point, 3×10\(^5\) purified CD4\(^+\)CD25\(^-\) T cells were adoptively transferred to induce immune reconstitution syndrome as previously described (28). At 11 days post transfer, mice were sacrificed and assessed for markers of inflammation and PC burden. Oxygen saturation was measured under anesthesia at the time of sacrifice using a rodent pulse oximeter (Starr Life Sciences, Holliston, MA). Data are reported as percent oxygen saturation relative to uninfected WT controls (Fig. 6C). Reconstituted Rag1\(^{-/-}\) mice in the control injection group showed significantly lower oxygen saturation levels at the time of sacrifice compared to reconstituted Dectin-1-treated Rag1\(^{-/-}\) mice and WT mice that underwent the same infection and adoptive transfer procedure, indicating more severe inflammation and lung pathology (Fig. 6C). To further assess lung injury, total protein content and
lactate dehydrogenase (LDH) activity were measured in the bronchoalveolar lavage (BAL) fluid. Levels of total protein and LDH were significantly higher in all *Rag1*−/− groups regardless of treatment with Dectin-1:Fc (Fig. 6D,E). BAL fluid levels of Dectin-1:Fc proteins were also measured at 7 and 14 days post hydrodynamic injection, and ranged from 10-20 ng/ml. We further assessed lung inflammation by measuring inflammatory cytokine and chemokine levels in the BAL fluid using a bead-based multiplex assay. Following adoptive transfer, immune competent WT mice expressed the lowest levels of total protein, LDH, and inflammatory cytokine levels in the BAL fluid. Total lung PC burden and histological PC ascus burden were not significantly different among *Rag1*−/− groups (Fig. 6F,G) regardless of Dectin-1 Fc treatment. However, several inflammatory cytokines and chemokines were significantly reduced by administration of Dectin-1 Fc in immune reconstituted *Rag1*−/− mice, including IL12p40 in Dectin-1:mIgG1 treatment, and reduction of KC and MIP2 to for both Dectin-1:Fc subtypes (Fig. 6H). MCP-1 also showed a trend towards reduction with Dectin-1:mIgG1 treatment, but levels of TNFα were not reduced for either construct (Fig. 6H). We also assessed immune cell numbers and phenotype in the lung during IRIS using intracellular staining of PMA-ionomycin-stimulated lung cells collected at day 11 post adoptive transfer. The predominant T cell response after stimulation appeared to be Th1, with stimulated T cells showing an increase in the percent and total number of IFN-g positive cells but not IL-17 or IL-13 (Supplemental Fig. S1 A). Dectin-1:Fc treatment did not result in a significant decrease of this IFN-g+ population.
The β-1,3-glucan receptor Dectin-1 plays a pivotal role in recognition and clearance of fungal pathogens such as *Pneumocystis* (41, 47), *Candida albicans* (48, 49), *Aspergillus fumigatus* (36, 50), and *Coccidioides* species (51, 52). β-glucans are major structural components of the fungal cell wall, but exposure of β-glucan to the immune system can be masked by mannoproteins and cell wall composition can differ depending on the life cycle stage of the organism (53). Dectin-1 has been shown to specifically bind to swollen conidia and early germlings of *Aspergillus* and to the yeast form of *Candida albicans* (36, 48). In the case of *Pneumocystis*, the ascus or cyst form has been shown to exhibit a thicker cell wall with higher β-glucan content and greater availability of β-glucan to host binding by Dectin-1 (35, 46). Previous rodent studies have indicated that a purified *Pneumocystis* asci preparation induces inflammation and a fulminant PCP infection (54), whereas echinocandin treatment can selectively ablate the cyst population and prevent transmission of infection (55). Another recent publication by Linke et al. has shown that murine hosts mount unique immune responses to the different life forms of *P. murina*, and that the ascus can contribute to the detrimental inflammatory response associated with infection and immune reconstitution syndrome (56). As β-glucan recognition is a critical component of fungal immunity, we have sought to test the effect of systemic expression of recombinant Dectin-1:Fc fusion proteins on host defense and inflammatory response in a murine model of *P. murina* infection.
To investigate the contributions of different IgG Fc subtypes on efficacy of protection in our model, we constructed fusion proteins of the Dectin-1 carbohydrate recognition domain with the Fc regions of murine IgG1, IgG2a, IgG2b, and IgG3 to form antibody-like molecules that specifically bind β-glucan. We then compared the ability of the different Fc fusions in their ability to bind laminarin by surface plasmon resonance and found that Fc subtype did not affect carbohydrate binding for any of the constructs (Fig. 1). Next, we tested the ability of Dectin-1:Fc to modify the stimulation of macrophages by zymosan, a β-glucan-rich, particulate cell wall preparation. Precoating TLR-agonist-depleted zymosan with a molar excess of Dectin-1:Fc decreased production of G-CSF and TNF-α upon exposure to RAW cells (Fig. 2). This reduction may result from competition of Dectin-1:Fc with macrophage Dectin-1 for binding of available β-glucan on the surface of zymosan. This competition could not completely abrogate cytokine production, however, as Fcγ receptor-mediated recognition of Dectin-1:Fc-opsonized zymosan can activate macrophages. Previous studies in our lab indicate that blockade of macrophage FcγRI and FcγRIII results in diminished binding and recognition of Dectin-1:Fc coated particulates (35). Other Dectin-1-independent mechanisms may also contribute to stimulation, as treatment with Dectin-1 blocking mAb 2A11 did not completely reduce cytokine production by unopsonized zymosan. These results are consistent with observations of normal cytokine production in Dectin-1 deficient mice infected with *Pneumocystis* (47).

Our previous studies in have shown that Dectin-1:mlG1 Fc can specifically bind to the cell wall of *Pneumocystis* and increase killing of *P. murina* by...
macrophages in vitro (35). In our current study, we found that all Dectin-1:Fc fusion isotypes bound *Pneumocystis* equally, and increased binding of Dectin-1:Fc to the asci-enriched population indicated specific recognition of the ascus, which contains a significant portion of β-(1,3)-D-glucan in its cell wall (Fig. 3). Other studies have shown that Dectin-1 deficient mice infected with *P. murina* harbor a greater ascus burden in the lung early in infection, particularly in immune-compromised conditions, indicating the importance of carbohydrate recognition to killing of the ascus (47).

In a direct killing assay, a Dectin-1:mlG1 Fc fusion did not mediate killing in the absence of macrophages, whereas Dectin-1:IgG2a Fc could reduce the viability of *P. murina* in culture through a complement-dependent process (Fig. 4). Our *P. murina* preparation assumes a 1:10 ascus to troph ratio, but there may be some variation in the total number of trophic forms (57). In addition, asci contain 8 ascospores with separate nuclei and mitochondria, and potentially reduction of 1 ascus can reduce mitochondrial RNA subunit transcript levels as much as the reduction of 8 trophic forms. We have tested RNA from ascus-enriched and trophic form-enriched populations and found their mitochondrial RNA transcript levels to be roughly equivalent for the same amount of total input RNA. This indicates that the assay has the potential to report killing of both life forms, but that it may be weighted more towards a reduction of asci. In addition, trophic forms may contain low levels of β-glucan in their cell walls, although at a level that is much reduced from asci and less accessible to Dectin-1 binding. The process of culturing *P. murina* during the killing assay may result in more
exposure of β-glucan by trophic forms, rendering them more accessible to killing by Dectin-1 immunoadhesins. The macrophage-independent process of PC killing by Dectin-1:IgG2a is dependent on an intact complement system, as heat inactivation of complement proteins in the fetal bovine serum used for the killing assay abrogates killing. However, the membrane attack complex formed by the complement proteins C6 through C9 has not been shown to directly cause lysis of Pneumocystis and other fungi with large structural cell walls, such as Cryptococcus and Candida spp. It may be possible that the membrane attack complex or another component of the complement system, such as C3a, are able to sufficiently perforate or perturb the membrane integrity of Pneumocystis and induce killing, but it is unlikely that the ascus will be completely lysed. Trophic forms may be more susceptible to complement-mediated lysis, which we are continuing to investigate.

In contrast to the in vitro studies, the IgG1 isotype fusion of Dectin-1 was more effective in reducing ascus burden in vivo, with the IgG2a isotype showing a trend toward a greater reduction in total lung burden as measured by P. murina rRNA copy number (Fig. 5). The Dectin-1:IgG2a Fc construct showed a greater in vitro killing efficacy, perhaps in part due to differences in concentration of the target molecule. Previous studies have shown that systemic administration of a recombinant Fc conjugated TNF inhibitor can result in functional levels of protein in the lung (58). We have tested blood and BAL samples at the time of sacrifice in the IRIS experiment (14 days post hydrodynamic injection), and levels of both Dectin-1:IgG1 and IgG2a Fc are waning in the blood and BALF. At an earlier time
point of 7 days post hydrodynamic injection, there are higher levels of Dectin-1:Fc in the alveolar lumen (10-20 ng/ml), but protein availability through the course of a severe infection may contribute to lack of efficacy in vivo. Complement-dependent killing induced by Dectin-1:IgG2a Fc may require a higher threshold of expression in vivo, which may be overcome through an alternate method of therapeutic delivery.

We also observed a greater reduction in ascus burden by the IgG1 isotype when it was administered pre-challenge as opposed to administration after established infection. This may be due to better efficacy of the molecule when ascus burden is low in the lung. Both constructs were effective in reducing hypoxemia in the IRIS model despite minimal effects on fungal burden in the lung (Fig. 6). These data indicate that β-glucan- and Dectin-1-mediated signals contribute to hypoxemia during Pneumocystis IRIS, consistent with recent observations implicating the β-glucan-rich ascus in producing a proinflammatory response (56). Possible mechanisms of inflammatory exacerbation include induction of IL-8 secretion and activation of the IL-23/IL-17 axis by Pneumocystis β-glucan (59, 60), as well as the effects of Dectin-1 signaling on Th1 and Th17 differentiation of CD4+ T cells (33, 56, 61, 62), which have been shown to contribute to risk for IRIS (63, 64). Although intracellular staining of PMA and ionomycin-stimulated lung cells did not reveal a strong Th17 signature (Fig. S1A), antigen-specific stimulation of lung or draining lymph node cells may exhibit greater induction of IL-17. Another potential method for Dectin-1:Fc to exert anti-inflammatory activity
is through a recently discovered interaction between FcγRIIB and Dectin-1, in which galactosylated IgG1 immune complexes suppress complement receptor-mediated inflammation (65). However, this mechanism has not been described in antibodies of the IgG2a isotype. Administration of control Fc only vectors did not improve hypoxemia in our IRIS model, indicating that observed differences with the administration of Dectin-1:Fc were specific to the Dectin-1 moiety (Fig 6C).

Despite improved hypoxemia, the Dectin immunoadhesins did not affect markers of cell death (LDH) or vascular leak (total protein). There are several possible explanations for these data. One possibility is that these markers of lung injury are Dectin-1 and β-glucan independent. Secondly, since we did not achieve a significant reduction of *P. murina* asci prior to reconstitution in this model, the retained ascus burden may be sufficient to mediate lung injury. Lastly, as these molecules do not affect burden of the trophic form in the lung, this life form may also contribute to lung injury in this model. In summary, these data provide evidence that immunoadhesins targeting β-glucan may provide a mechanism for treatment of fungal infection and for modulation of the inflammatory response to *Pneumocystis* and other pathogens.
REFERENCES


FIGURE 1.

Surface plasmon resonance analysis of the interaction between Dectin-1:Fc and β-glucan. Binding of laminarin to immobilized Dectin-1:Fc was studied in real-time using a Biacore 3000 instrument. Rabbit anti-mouse antibodies were chemically immobilized on CM5 chips and used as a capture surface for Dectin-1:Fc. Nonspecific binding was measured using a control flow cell consisting of immobilized capture Ab alone or with an irrelevant IgG2a mAb and was subtracted from each laminarin binding curve. A, Dectin-1:mIgG1, Dectin-1:mIgG2a, Dectin-1:mIgG2b, and Dectin-1:mIgG3 were captured at various concentrations and measured for response to 10 mM laminarin. Response units (RU) to laminarin were proportional to RU Dectin-1:Fc loaded for each Ig subtype, indicating similar β-glucan binding capacity (Table 1). Detailed kinetic and affinity analysis was performed for Dectin-1:mIgG2a (B) and Dectin-1:mIgG2b (C) using a 2-fold dilution series of laminarin ranging from 20 mM to 0.156 mM. The rate of association (k_a), the rate of dissociation (k_d), and the equilibrium constants of association (K_A) and dissociation (K_D) were calculated (Table 2).

FIGURE 2.

Effect of Dectin-1:Fc pretreatment of zymosan on stimulation of macrophages. RAW264.7 cells were stimulated with 50 µg/ml untreated TLR agonist-depleted
zymosan (Zym) or with depleted zymosan pretreated with 10 µg/ml Dectin-1:Fc. RAW cells were also pretreated with 5 µg/ml of the Dectin-1 blocking mAb 2A11 as an inhibition control. Cell culture supernatant was assayed at 24 hours for the production of granulocyte colony-stimulating factor (A) and tumor necrosis factor (B). Data are expressed as mean pg/ml ± SEM for n=3 per group. *, p < 0.05, **, p < 0.01, ***, p < 0.001, using one-way ANOVA with a Holm-Sidak multiple comparisons test.

FIGURE 3.
Dectin-1:Fc binds the cell wall of Pneumocystis asci. A, PC organisms were stained with Dectin-1:Fc fusion proteins and a FITC-conjugated anti-mouse IgG secondary and measured using flow cytometry. Staining of each Fc subtype fusion protein is shown as labeled compared to unstained PC (underlying histogram). Non-specific staining of isotype controls was minimal for each subtype (data not shown). Binding affinity of Dectin-1:mIgG1 (B) and Dectin-1:mIgG2a (C) to P. murina asci was also determined by flow cytometry. Populations of ascus-enriched PC (black line) and whole PC (gray dashed line) were prepared by rate-zonal centrifugation over a sucrose gradient and stained with Dectin-1:Fc directly conjugated to PerCP-Cy5.5. Unstained whole PC (gray shaded histogram) was indistinguishable from unstained ascus-enriched PC (not shown). Immunofluorescence staining was performed on whole PC using Alexa Fluor 555-conjugated Dectin-1:mIgG1 (D) and Dectin-1:mIgG2a (E) and DAPI. Dectin-1:Fc (red) stains the P.murina asci (white arrows), which contain 8
individual DAPI-positive ascospore nuclei (blue). Trophic forms (white arrowheads) are represented by lone nuclei absent of Dectin-1:Fc staining.

**FIGURE 4.**

*Pneumocystis* killing activity of Dectin-1:Fc fusion proteins *in vitro*. Dectin-1:mlgG1 (A) and Dectin-1:mlgG2a (B) Fc fusion proteins were tested for PC killing in media containing 10% heat-inactivated (HI) or untreated (Non-HI) fetal bovine serum (FBS). 2×10^5 PC asci were cultured per well in media alone (PC only) or with varying amounts of Dectin-1:Fc. After 24 hours, total RNA was harvested and viability was assessed through real-time RT-PCR measurement of PC mitochondrial large subunit (mtLSU) rRNA copy number. All data are reported as mean ± SEM for n = 3 per group. *, p < 0.05, **, p < 0.01 using multiple t tests with a Holm-Sidak correction.

**FIGURE 5.**

*Pneumocystis* infection in *Rag1*^-/-^ mice expressing Dectin-1:mlgG1 and Dectin-1:mlgG2a Fc fusion proteins. A, *Rag1*^-/-^ mice were treated with hydrodynamic injection of 10 µg Dectin-1:Fc plasmid DNA (Dectin-1:mlgG1,2a) or buffer control (*Rag1*^-/-^) on day 0, infected with PC on day 1, and sacrificed at day 15 to assess PC burden. B, Dectin-1:Fc protein levels in serum of *Rag1*^-/-^ mice at 4, 7, and 10 days post hydrodynamic injection of 10 µg plasmid DNA. Data are reported as median values plus error. C, PC organism burden at two weeks post-infection determined by real-time RT-PCR reported as mtLSU copy number/µg lung RNA. Data are reported as mean ± SEM. D, PC ascus burden determined by
qualitative scoring of GMS-stained histological sections of lung tissue. Horizontal
lines denote median values. E, Representative micrographs of lung sections
stained with GMS. n = 5 per group. *, p < 0.05, **, p < 0.01, ***, p < 0.001, using
non-parametric Kruskal-Wallis test with Dunn multiple comparison posttest (D).

FIGURE 6.
Effect of systemic Dectin-1:mlgG1 and Dectin-1:IgG2a Fc expression in a model
of Pneumocystis-associated immune reconstitution inflammatory syndrome. A,
WT C57BL6 and Rag1⁻/⁻ mice were infected with PC on day 0 and treated on day
22 with hydrodynamic injection. WT mice received buffer control, and Rag1⁻/⁻
mice received buffer control or 10 µg plasmid injection. Plasmid groups included
IgG1 Fc control, IgG2a Fc control, Dectin-1:IgG1 Fc, and Dectin-1:IgG2a Fc.
Immune reconstitution syndrome was induced by adoptive transfer of 3×10⁵
purified CD4⁺CD25⁻ naïve T cells on day 25, and mice were sacrificed at day 36
to assess PC burden and markers of inflammation and lung damage. B, Levels of
Dectin-1:Fc protein expression in serum at day 25 (3 days post hydrodynamic
injection). C, Oxygen saturation levels in anesthetized mice measured by pulse
oximeter at time of sacrifice on day 36, reported as percentage of uninfected
control. D, Total protein concentration in bronchoalveolar lavage (BAL) fluid
measured by BCA assay. E, Lactate dehydrogenase (LDH) activity levels in BAL
fluid. F, PC organism burden at 36 days post-infection determined by real-time
RT-PCR reported as mtLSU copy number/µg lung RNA. G, PC ascus burden
determined by qualitative scoring of GMS-stained histological sections of lung
tissue. H, protein levels of KC, MCP-1, IL-12 p40, MIP-2, and TNFα in BAL fluid
measured by multiplex bead array. Data are reported as mean ± SEM (B-F, H) or median values (G) for $n = 4-5$ per group. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, using non-parametric Kruskal-Wallis test with Dunn multiple comparisons posttest (G) or one-way ANOVA with Holm-Sidak multiple comparisons test (C-E, H).
Table 1. Dectin-1:Fc Binding of Different Isotypes

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<th>Protein</th>
<th>RU loaded</th>
<th>RU Laminarin</th>
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