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 constituted immune system can develop a pathological 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 immunoadhesins consisting of the carbohydrate binding domain of Dectin-1 fused to the Fc regions of the 4 subtypes of murine IgG (mIgG). These immunoadhesins bind β-glucan with high affinity, and pre-coating 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 ascii of *Pneumocystis 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 postinfection in a model of immune reconstitution inflammatory syndrome (IRIS), both Dectin-1:mIgG1 and Dectin-1: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.
ing 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 carries the extracellular domain of the β-glucan receptor Dectin-1 fused to a murine IgG (mlgG) Fc can opsonize Pneumocystis organisms and can reduce organism burden in the lungs of Pneumocystis 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-week-old, wild-type (WT) C57BL/6J mice and immunodeficient B6.129S7-Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> (Rag<sup>−/−</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Immunodeficient B10.B6:Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup> Fc (Rag<sup>−/−</sup>) 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.

MAbs and Fc fusion proteins. Anti-Dectin-1 mononclonal antibody (MAb), clone 2A11, rat IgG2b (AbD Serotec, Raleigh, NC), was used as previously described (36). Construction of recombinant Dectin-1:mlgG1 Fc was previously described (35). Additional Dectin-1 fusion vectors containing an IgG1 leader sequence to facilitate secretion, the extracellular domain of the Dectin-1 receptor (amino acids 69 to 244), and a hexapeptide linker with a thrombin cleavage site cloned in frame with the Fc domain of the Dectin-1 receptor (amino acids 69 to 244), and a hexapeptide linker with a thrombin cleavage site cloned in frame with the Fc region of murine IgG2a, IgG2b, and IgG3 were constructed using the parent Dectin-1:mlgG1 Fc vector and the vectors pFUSE-mlgG2a-Fc, pFUSE-mlgG2b-Fc, and pFUSE-mlgG3-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 phosphate-buffered saline (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.

In vitro macrophage stimulation. Zymosan (InvivoGen) was depleted of TLR-activating ligands by being boiled in hot alkali as previously described (37) and optionally pretreated with 100 μg of Dectin-1:Fc for 30 min at 37°C. RAW 264.7 cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum were plated onto 96-well flat-bottom plates at a density of 1 × 10<sup>5</sup> cells per well and stimulated with 50 μg/ml zymosan. A control group of RAW cells were also pretreated for 30 min with 5 μg/ml of MAb 2A11 to block Dectin-1 binding to zymosan. Cell culture supernatants were harvested 16 h after stimulation and assayed for granulocyte colony-stimulating factor (G-CSF) and tumor necrosis factor alpha (TNF-α) production using a custom multiplex bead assay (EMD Millipore, Billerica, MA).

Pneumocystis isolation and inoculum. Pneumocystis murina organisms were isolated from the lung tissue of Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice previously inoculated with P. murina. The inoculum for infectious challenge was prepared by differential centrifugation as previously described (38). Briefly, Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> 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 min 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 P. murina asci (cyst forms) was quantified microscopically (8), and the inoculum concentration was adjusted to 2 × 10<sup>5</sup> ascii per ml. One hundred microliters of this inoculum, corresponding to 2 × 10<sup>5</sup> ascii per mouse, was given by oropharyngeal aspiration using the tongue-pull technique. Briefly, mice were lightly anesthetized using 2 to 3% isoflurane and 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 P. murina, asci were isolated from trophic forms using sucrose gradient density centrifugation, according to a method previously described (39).

Pneumocystis viability assay. P. murina (1 × 10<sup>5</sup> 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 min 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 h. A viability control of P. murina 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). The viability of P. murina was analyzed with real-time PCR measurement of RNA copy number as described below.

Flow cytometry. For comparison of different Fc isoforms, P. murina organisms were stained with affinity-purified Dectin-1:Fc and fluorescent isothiocyanate (FITC)-conjugated Fcγ fragment-specific (subclass 1 + 2a + 2b + 3) secondary antibody (Jackson ImmunoResearch, West Grove, PA) and analyzed using an LSRII 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 P. murina were stained with Dectin-1:Fc directly conjugated to peridinin chlorophyll protein (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 Alexa Fluor 555-conjugated Dectin-1:mlgG1 and Dectin-1:mlgG2a Fc (ApeX antibody labeling kit; Invitrogen). DAPI (4′,6-diamidino-2-phenylindole)-containing mounting medium (Vector Laboratories, Burlingame, CA) was used to coverslip samples, which were visualized at an ×60 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% [wt/vol] 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 body weight was injected via tail vein within 5 to 10 s.

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<sup>+</sup> CD25<sup>−</sup> cells for adoptive transfer were purified by magnetic bead separation using only the negative selection step of a CD4<sup>+</sup> CD26<sup>−</sup> T cell isolation kit to deplete non-CD4 cells (Miltenyi Biotec, Auburn, CA). To induce immune recon-
The rate of dissociation (K_D) of laminarin ranging from 20 mM to 0.156 mM. The rate of association (K_A) was calculated (Table 2).

**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 intraperitoneal ketamine/kg of body weight, 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 average values of 10 to 20 stable, error-free measurements over this interval are reported. Total protein in the BALF was assayed with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) per the manufacturer’s instructions. Lactate dehydrogenase (LDH) levels in the BALF were analyzed by an LDH activity assay kit (BioVision, Milpitas, CA) per the manufacturer’s instructions. Details of the statistical analysis, experiments, and data processing have been described (41).

**Cytokine analysis.** BALF and lung homogenate samples were analyzed for protein levels of G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), interleukin-10 (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, monocyte chemoattractant protein 1 (MCP-1), MIP-1α, MIP-1β, MIP-2, RANTES, and TNF-α using a MillexMap mouse cytokine/chemokine magnetic bead panel (EMD Milipore) on a Bio-Plex 200 instrument (Bio-Rad). The data were analyzed using Bio-Plex Manager software (Bio-Rad). Data are reported as means ± standard errors of the means (SEM).

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

**TABLE 1** Dectin-1:Fc binding of different isotypes

<table>
<thead>
<tr>
<th>Protein</th>
<th>RU loaded</th>
<th>RU for laminarin</th>
<th>Response ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dectin-1:mlgG1</td>
<td>549.9</td>
<td>34.3</td>
<td>16.03</td>
</tr>
<tr>
<td>Dectin-1:mlgG2a</td>
<td>1,011.8</td>
<td>59.1</td>
<td>17.12</td>
</tr>
<tr>
<td>Dectin-1:mlgG2b</td>
<td>1,460.4</td>
<td>85.4</td>
<td>17.1</td>
</tr>
<tr>
<td>Dectin-1:mlgG3</td>
<td>485.2</td>
<td>24.1</td>
<td>20.1</td>
</tr>
</tbody>
</table>
TABLE 2 Rate and affinity constants of Dectin-1:Fc

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_d (M^{-1} s^{-1})$</th>
<th>$k_s (s^{-1})$</th>
<th>$K_A (M^{-1})$</th>
<th>$K_D (M)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dectin-1:mIgG2a</td>
<td>2.10E+03</td>
<td>4.40E-06</td>
<td>4.80E+08</td>
<td>2.10E-09</td>
</tr>
<tr>
<td>Dectin-1:mIgG2b</td>
<td>2.10E+03</td>
<td>1.10E-06</td>
<td>1.90E+09</td>
<td>5.40E-10</td>
</tr>
</tbody>
</table>

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 to 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 *P. murina* in vitro and enhance host recognition and clearance of *P. murina* in immunodeficient SCID mice (35). As these findings suggested that FcyR-based targeting of *P. murina* via cell wall carbohydrate recognition can promote resistance against *P. murina* 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 HEK293T cells, and we assessed the binding of these Fc fusion proteins to β-(1,3)-β-glucan using surface plasmon resonance measurements (Fig. 1).

Initially, various 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 ratios of laminarin binding response units (RU) per Dectin-1:Fc protein RU loaded were similar for all Fc subtypes, indicating that none of the Fc subtypes grossly interfered with the carbohydrate recognition domain of Dectin-1 (Fig. 1A and 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 and C). 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), demonstrating high-affinity binding of Dectin-Fc proteins to laminarin, similar in quality to our previous results with Dectin-1:mIgG1 Fc (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)-β-glucan residues reduces zymosan-induced stimulation of macrophages. To address the ability of Dectin-1:Fc to modify recognition of particles containing β-(1,3)-β-glucan, such as the cell wall surface of *Pneumocystis* and other fungi, we evaluated whether Dectin-1:mIgG1 and Dectin-1:mIgG2a could affect RAW 264.7 macrophage stimulation by particulate zymosan. Zymosan is an insoluble cellular wall polysaccharide derived from *Saccharomyces 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 18 h of stimulation, production of granulocyte colony-stimulating factor (G-CSF) and tumor necrosis factor alpha (TNF-α) in the supernatant was measured (Fig. 2A and B). The results indicate decreased stimulation by zymosan in the Dectin-1:Fc-treated groups, with significantly lower production of G-CSF than in those treated with both zymosan alone and zymosan plus 2A11 MAb. Dectin-1:Fc treatment of zymosan also significantly reduced macrophage production of TNF-α.
Dectin-1:mIgG2a Fc binds Pneumocystis organisms and enhances macrophage-independent killing. Our previous studies using fluorescent deconvolution microscopy and flow cytometry have demonstrated that Dectin-1:mIgG1 Fc binds specifically to the surface of Pneumocystis asci (cyst forms) (35). In this study, we tested and confirmed the binding of the novel Dectin-1:mIgG2a, Dectin-1:mIgG2b, and Dectin-1:mIgG3 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 \( \beta-1,3 \)-D-glucan in the cell wall of the ascus (Fig. 3B and C). Immunofluorescence staining of whole P. murina with fluorochrome-labeled Dectin-1 Fc also showed specific binding to the ascus (Fig. 3D and E).

The Dectin-1:mIgG1 Fc protein was previously shown to enhance the macrophage-dependent killing of P. murina in vitro (35). Preopsonization of P. murina organisms with Dectin-1:mIgG1 Fc diminished overall copy numbers by 3-fold in studies with thioglycolate-elicited peritoneal macrophages and up to 10-fold with alveolar macrophages (35). We sought to determine if Dectin-1 Fc proteins could enhance P. murina killing in vitro without macrophages through a complement-dependent cytotoxic mechanism. We studied decreases in absolute quantities of P. murina mitochondrial large-subunit rRNA (PC mtLSU rRNA) copy numbers as a correlate of in vitro P. murina killing, a methodology validated by previous work (41,44). P. murina organisms were isolated from infected murine lung homogenates and were treated with Dectin-1:Fc in medium supplemented with fetal bovine serum that was untreated or heat inactivated for 30 min at 56°C to remove complement activity (HI). Dectin-1:mIgG1 Fc did not reduce P. murina rRNA copies in vitro in the absence of macrophages (Fig. 4A). In contrast, Dectin-1:mIgG2a exhibited potent P. murina killing activity at 35 and 14 \( \mu \)g/ml, but only in medium 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 P. murina \(\textit{in vitro}\) and protection \(\textit{in vivo}\) in a SCID model with Dectin-1:mlG1Fc (35) and our current observations with macrophage-independent \(\textit{in vitro}\) killing of P. murina by Dectin-1:mlG2aFc, we sought to compare the effects of these molecules in an immunodeficient mouse model of P. murina infection. As fungal \(\beta\)-(1,3)-glucan is known to trigger potent inflammatory responses, altering the glucan surface content of an infectious organism may have strong potential to modulate the host immune response. In the case of \textit{Pneumocystis}, we have demonstrated that Dectin-1:Fc has an increased binding affinity for the P. murina asci, which contains a greater proportion of \(\beta\)-(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 \(\textit{in vitro}\) stimulation assay. We hypothesized that these properties along with the increased P. murina-killing activity shown by Dectin-1:mlG1 and Dectin-1:mlG2a 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 P. murina 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:mlG1Fc, or Dectin-1:mlG2aFc (Fig. 6A). Dectin-1:Fc protein expression was assayed 3 days later, with expression levels in serum averaging 22 and 14 \(\mu\)g/ml for Dectin-1:mlG1 and Dectin-1:mlG2a, respectively (Fig. 6B). At this time point, 3 \(\times\) \(10^5\) purified CD4\(^+\)CD25\(^-\) T cells were adaptively transferred to induce immune reconstitution syndrome as previously described (28). At 11 days posttransfer, mice were sacrificed and assessed for markers of inflammation and P. murina 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 than did 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 fluid (BALF). Levels of total protein and LDH were significantly higher in all Rag1\(^{-/-}\) groups regardless of treatment with Dectin-1:Fc (Fig. 6D and E). BALF levels of Dectin-1:Fc proteins were also measured at 7 and 14 days after hydrodynamic injection and ranged from 10 to 20 ng/ml. We further assessed lung inflammation by measuring inflammatory cytokine and chemokine levels in the BALF using a bead-based multiplex assay. Following adoptive transfer, immunocompetent WT mice expressed the lowest levels of total protein, LDH, and inflammatory cytokine levels in the BALF. Total lung P. murina burdens and histological P. murina...
ascus burdens were not significantly different among Rag1−/− groups (Fig. 6F and 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 IL-12(p40) in Dectin-1:mIgG1 treatment, and reduction of KC and MIP-2 for both Dectin-1:Fc subtypes (Fig. 6H). MCP-1 also showed a trend toward reduction with Dectin-1:mIgG1 treatment, but levels of TNF-α 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 phorbol myristate acetate (PMA)-ionomycin-stimulated lung cells collected at day 11 after adoptive transfer. The predominant T cell response after stimulation appeared to be Th1, with stimulated T cells showing an increase in the percentage and total number of IFN-γ-positive cells but not IL-17 or IL-13 (see Fig. S1A in the supplemental material).

Dectin-1:Fc treatment did not result in a significant decrease of this IFN-γ+ population.

**DISCUSSION**

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 compositions 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.
FIG 6 Effect of systemic Dectin-1:mIgG1 and Dectin-1:mIgG2a Fc expression in a model of Pneumocystis (PC)-associated immune reconstitution inflammatory syndrome. (A) WT C57BL/6 and Rag1<sup>−/−</sup> mice were infected with P. murina on day 0 and treated on day 22 with hydrodynamic injection. WT mice received buffer control, and Rag1<sup>−/−</sup> mice received buffer control or a 10-μg plasmid injection. Plasmid groups included mIgG1 Fc control, mIgG2a Fc control, Dectin-1:mIgG1 Fc, and Dectin-1:mIgG2a Fc. Immune reconstitution syndrome was induced by adoptive transfer of 3 × 10<sup>5</sup> purified CD4<sup>+</sup> CD25<sup>−</sup> naive T cells.
to host binding by Dectin-1 (35, 46). Previous rodent studies have indicated that a purified Pneumocystis ascus 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 abilities 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 Fcy receptor-mediated recognition of Dectin-1:Fc-opsonized zymosan can activate macrophages. Previous studies in our lab indicate that blockade of macrophage FcγRII and FcγRII in vivo 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 mice have demonstrated that Dectin-1:mIgG1 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 ascus-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 under immunocompromised conditions, indicating the importance of carbohydrate recognition to killing of the ascus (47).

In a direct killing assay, a Dectin-1:mIgG1 Fc fusion did not mediate killing in the absence of macrophages, whereas Dectin-1:mlG2a 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-trophic-form 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 can 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 toward 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 that in 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 upregulation of the complement-dependent process of P. murina killing by Dectin-1:mlG2a 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 species. It may be possible that the membrane attack complex or another component of the complement system, such as C3a, is able to sufficiently perforate or perturb the membrane integrity of Pneumocystis and induce killing, but it is likely 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:mlG2a 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 BALF samples at the time of sacrifice in the IRIS experiment (14 days after hydrodynamic injection), and levels of both Dectin-1:mIgG1 and Dectin-1:mlG2a Fc were waning in the blood and BALF. At an earlier time point of 7 days after hydrodynamic injection, there are higher levels of Dectin-1:Fc in the alveolar lumen (10 to 20 ng/ml), but protein availability on day 25, and mice were sacrificed at day 36 to assess P. murina burden and markers of inflammation and lung damage. (B) Levels of Dectin-1:Fc protein expression in serum at day 25 (3 days after hydrodynamic injection). (C) Oxygen saturation levels in anesthetized mice, measured by pulse oximeter at time of sacrifice on day 36 and reported as a percentage of the uninfected control. (D) Total protein concentration in bronchoalveolar lavage fluid (BALF) measured by BCA assay. (E) Lactate dehydrogenase (LDH) activity levels in BALF. (F) P. murina organism burden at 36 days postinfection, determined by real-time RT-PCR and reported as mLSU copy number/μg lung RNA. (G) P. murina 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 TNF-α in BALF measured by multiplex bead array. Data are reported as means ± SEM (B to H) or median values (G) for n = 4 to 5 per group. *, P < 0.05; **, P < 0.01; ****, P < 0.001. Using a nonparametric Kruskal-Wallis test with a Dunn multiple-comparison posttest (G) or one-way ANOVA with a Holm-Sidak multiple-comparison test (C to E and H).
through the course of a severe infection may contribute to lack of efficacy in vivo. Complement-dependent killing induced by Dectin-1:Fc 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 prechallenge than when it was administered 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 P. murina.

mechanism for treatment of fungal infection and for modulation of the trophic form in the lung, this life form may also contribute to this model, the retained ascus burden may be sufficient to mediate pathways of IL-23 and 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 leakage (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. Second, since we did not achieve a significant reduction of P. murina asci before reconstitution in this model, the retained ascus burden may be sufficient to mediate lung injury. Lastly, as these molecules do not affect the 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


