Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus *Pneumocystis murina*

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Host defense against opportunistic fungi requires coordination between innate and adaptive immunity for resolution of infection. Antibodies generated in mice vaccinated with the fungus *Pneumocystis* prevent growth of *Pneumocystis* organisms within the lungs, but the mechanisms whereby antibodies enhance antifungal host defense are poorly defined. Nearly all species of fungi contain the conserved carbohydrates β-glucan and chitin within their cell walls, which may be targets of innate and adaptive immunity. In this study, we show that natural IgM antibodies targeting these fungal cell wall carbohydrates are conserved across many species, including fish and mammals. Natural antibodies bind fungal organisms and enhance host defense against *Pneumocystis* in early stages of infection. IgM antibodies influence recognition of fungal antigen by dendritic cells, increasing their migration to draining pulmonary lymph nodes. IgM antibodies are required for adaptive T helper type 2 (Th2) and Th17 cell differentiation and guide B cell isotype class-switch recombination during host defense against *Pneumocystis*. These experiments suggest a novel role for the IgM isotype in shaping the earliest steps in recognition and clearance of this fungus. We outline a mechanism whereby serum IgM, containing ancient specificities against conserved fungal antigens, bridges innate and adaptive immunity against fungal organisms.

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Abbreviations used: BALF, bronchoalveolar lavage fluid; DLN, draining LN; nAb, natural antibody; PRR, pattern recognition receptor; sIgM, secreted IgM; SPF, specific pathogen free.

Opportunistic fungal pathogens cause severe, often fatal, disease in immunosuppressed individuals, who have impaired adaptive immune responses to eradicate these infections (Corti et al., 2009). The number of individuals susceptible to opportunistic fungal infections is increasing globally, although currently there are no vaccines against any fungal pathogen in clinical use. *Pneumocystis jirovecii*, the fungal organism that causes Pneumocystis pneumonia in humans, is a common cause of morbidity and mortality in individuals with HIV-AIDS or other immunosuppressive states such as chronic treatment with bone marrow–suppressive chemotherapeutics or high-dose corticosteroids (Morris et al., 2004; Thomas and Limper, 2004). The mainstay of prevention and treatment of Pneumocystis pneumonia involves the use of antimicrobials such as trimethoprim-sulfamethoxazole, of which there is concern for evolving microbial resistance (Nahimana et al., 2003; Huang et al., 2004a; Crothers et al., 2005).

Although $CD4^+$ T cells and adaptive immune responses are required for host defense against *Pneumocystis*, little is understood regarding the evolution of protective cellular and humoral responses after exposure to fungi. Antibodies are not an absolute requirement for host resistance against *Pneumocystis*, although antibodies produced in mouse hosts vaccinated with *Pneumocystis* can prevent and clear existing infections

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(Roths and Sidman, 1993; Zheng et al., 2001). The specificities of protective antibodies, the relevance of particular isotypes, and the contributions of antibodies to an evolving host immune response against *Pneumocystis* are poorly characterized. Contributions of antibody isotype as a correlate of effector function have been explored in greater detail against other fungal pathogens. In *Cryptococcus neoformans* infection, for example, mAb IgG subtype plays a critical role in determining whether antibodies targeting the cell capsular antigen glucuronoxylomannan are protective or detrimental to host defense (Nussbaum et al., 1996). IgM antibodies against fungi can increase complement deposition and complement-dependent and -independent phagocytosis by APCs (Zhang et al., 1998; Han et al., 2001; Taborda and Casadevall, 2002), and some IgM specificities against fungi demonstrate direct antimicrobial effects (Xander et al., 2007). However, there has been little evaluation of the role of native IgM antibodies in the evolution of host responses to fungal infection. Humans with mutations in CD40L resulting in the X-linked hyper-IgM syndrome are susceptible to Pneumocystis pneumonia, suggesting that IgM in and of itself is not sufficient to provide host resistance against this infection.

Fungal organisms, of which there are an estimated 1.5 million different species, contain cell walls that are remarkably similar. It is estimated that ∼90% of the fungal cell wall consists of polysaccharides, and at the core of this cell wall exist two carbohydrates, β-glucan and chitin. Nearly all species of pathogenic fungi, including *Pneumocystis* sp., contain the conserved cell wall carbohydrates β-glucan, chitin, and mannan, which are emerging as targets of multiple host defense pathways (Ezekowitz et al., 1991; Roth et al., 1997; Steele et al., 2003; Latgé, 2007). The abundance of these antigens in fungi is underscored by evidence demonstrating that quantities of β-glucan in the serum are significantly elevated in patients infected with *P. jirovecii* and other opportunistic fungal pathogens (Marty et al., 2007; Persat et al., 2008).

Natural antibodies (nAbs) are predominantly of the IgM isotype, generated without the requirement for exogenous antigenic stimulation, and are primarily produced by the B-1 subset of B cells. In this study, we present evidence for the existence of natural IgM antibodies targeting the fungal cell wall carbohydrates β-glucan and chitin, which are specificities conserved over evolution. We show that nAbs are protective in the early stages of host defense against pulmonary mucosal infection with *Pneumocystis* and further demonstrate that IgM antibodies mediate processes of pulmonary DC migration and shape the generation of adaptive Th and induced antibody responses in response to pulmonary fungal infection. Collectively, these data indicate a novel role for IgM in host defense against fungi and suggest that selective pressure may exist on the host to encode native IgMs with specificity for fungal antigens.

RESULTS

Anticarbohydrate antibody responses during *Pneumocystis* infection

We first assessed the hypothesis that exposure to the opportunistic fungal pathogen *Pneumocystis* might lead to the generation of antibodies targeting conserved fungal cell wall carbohydrates β-glucan, chitin, and mannan. BALB/c mice were challenged with *Pneumocystis murina* intratracheally, and Ig responses were monitored. Unexpectedly, we observed significant quantities of IgM reactive with both laminarin, a primarily β-1,3 linked glucan (the predominant β-glucan linkage found in fungal cell walls), and chitosan/chitin, a polymer of 75–85% deacetylated chitin, before *Pneumocystis* challenge. These IgM levels rapidly increased in serum through 2 d after challenge and subsided to approach baseline levels by 7 d (Fig. 1 A). Of note, IgM in the serum was not reactive with α-1,6 linked mannan, underscoring the intrinsic specificity of the nAb repertoire (Mouthon et al., 1995; Yang et al., 1998). Quantities of IgG targeting β-glucan and chitin at baseline were at the lower limit of detection compared with quantities of IgM against these antigens. Using mouse reference serum, we found that the mean concentration of antichitin IgM in BALB/c mice was 451 ± 36 ng/ml, and anti–β-1,3 glucan IgM was 386 ± 18 ng/ml. Levels in C57BL/6 mice were similar with a mean antichitin IgM level of 228 ng/ml and anti–β-1,3 glucan IgM of 640 ng/ml. This ELISA reactivity is caused by the variable region, as irrelevant mouse IgMs such as DX-5 (which recognizes CD49b) or 11E10 (which recognizes lipopolysaccharide) had no reactivity in these assays (unpublished data). Additionally, serum from secreted IgM ($sIgM^{-/-}$) mice also lacked reactivity in these assays.

Anti–β-glucan and antichitin antibodies are present in germ-free mice and are evolutionarily conserved

As IgM specificities targeting β-glucan and chitosan/chitin are present in serum of mice reared in specific pathogen-free (SPF) conditions, we questioned whether adventitious exposure to environmental pathogens or colonization of the gut by bacterial or fungal organisms might induce differentiation of B cells producing these antibody specificities. Analysis of serum from C57BL/6 mice reared in germ-free conditions demonstrated similar quantities of IgM targeting β-glucan and chitosan/chitin compared with mice reared in SPF conditions (Fig. 1 B), suggesting that these IgMs are nAbs, produced in the absence of exogenous microbial stimulation. Because high quantities of IgMs targeting β-glucan and chitin were present in SPF mice, we assessed the localization of B cells spontaneously producing β-glucan–specific IgM. B-1 B cells are considered the predominant B cell subset involved in the production of natural IgM antibodies and are present in pleural and peritoneal spaces in mice (Boes, 2000). Comparing cells derived from peritoneal lavage, spleen, bone marrow, and lung for the ability to produce IgM reactive with β-glucan by ELISPOT, we found that the primary site of cells producing IgM reactive with β-glucan was the spleen (Fig. 1 C). It was previously identified that peritoneal B-1 cells migrate into the spleen and other secondary lymphoid organs upon stimulation by toll-like receptor ligands (Kawahara et al., 2003; Ha et al., 2006; Yang et al., 2007), where they differentiate into antibody-secreting cells. Upon purification of $CD5^{+}IgM^{+}$ B cells and $CD5^{-}$ cells from the spleen, the majority of the

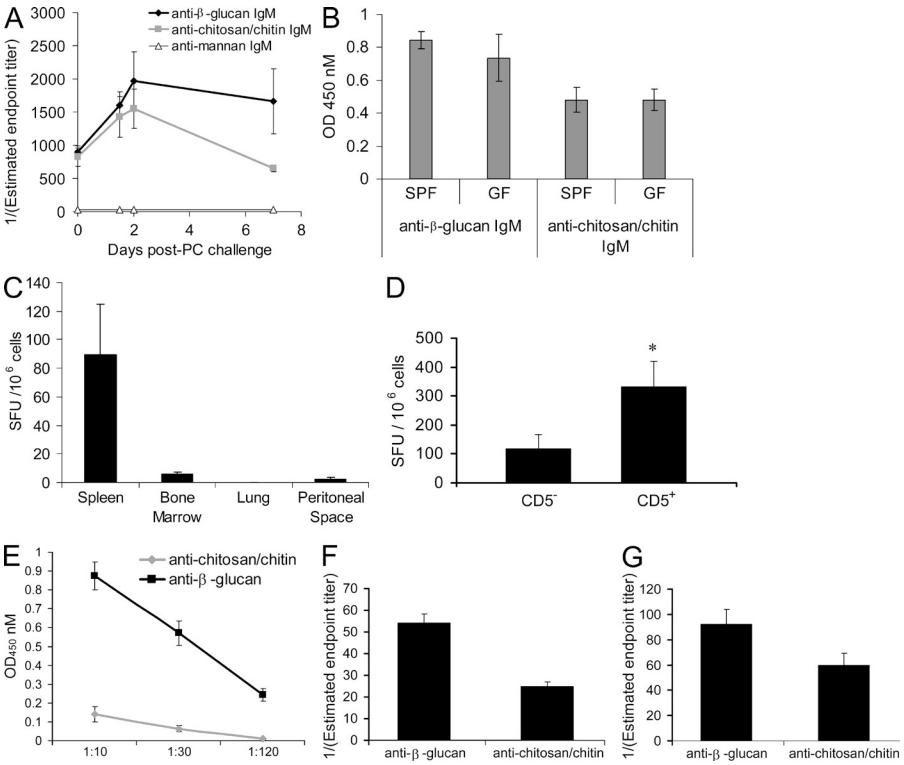

Figure 1. nAbs against conserved fungal cell wall carbohydrates are produced in diverse species, without requirement for exogenous antigenic stimulation. (A) Characterization of serum IgM antibodies in BALB/c mice before and after intratracheal challenge with *Pneumocystis* (PC) against fungal cell wall carbohydrates laminarin (primarily β-1,3 linked glucan), chitosan/chitin, and mannan by ELISA (n = 5–6 mice per time point from two independent experiments). (B) Relative quantity of IgM specific for β-glucan and chitosan/chitin in C57BL/6 mice reared under conventional SPF or germ-free (GF) conditions was measured by ELISA (n = 6 pooled from two independent experiments). Data are shown as OD_{450} with the background of secondary antibody alone subtracted. In all cases, the secondary antibody alone yielded an OD_{450} value of <0.050. (C) ELISPOT detection of individual anti–β-glucan IgM-producing B cells at various tissue sites in BALB/c mice (n = 6 per condition pooled from two independent experiments). (D) Anti–β-glucan IgM-producing $CD5^+$ and $CD5^-$ B cells sorted from the spleen of BALB/c mice and detected by ELISPOT (n = 6 per condition pooled from two independent experiments; *, $P < 0.05$ by Mann-Whitney test). SFU, spot-forming unit. (E–G) Reactivity of tetrameric IgM homologue in catfish sera (E), human cord blood IgM (F), or rhesus macaque cord blood IgM (G) with β-glucan and chitosan/chitin was measured by ELISA (n = 6–8 per group pooled from two independent experiments). All error bars represent SEM.

spontaneous anti–β-glucan IgM production was confined to the $CD5^+$ population (Fig. 1 D), suggesting that the cells generating anti–β-glucan IgM are within the B-1a subset, which are known for their spontaneous production of nAbs reactive with self and microbial antigens. Our data support experiments suggesting that the spleen is a common site for nAb-secreting cells such as those secreting IgM reactive with fungal cell wall carbohydrates (Yang et al., 2007).

As nAbs have been demonstrated to play a critical role in host defense against bacterial and viral infections (Boes et al., 1998b; Ochsenbein et al., 1999), likely acting at the earliest moments upon pathogen exposure much like membrane-bound pattern recognition receptors (PRRs), it was hypothesized that nAb specificities have been selected over evolution partly as a function of their ability to defend against infection (Ochsenbein and Zinkernagel, 2000). We questioned whether antibodies targeting fungal cell wall carbohydrates are present among the earliest evolved animals with adaptive, combinatorial immune systems. The serum of the channel catfish, *Ictalurus punctatus*, which contains a tetrameric homologue of IgM, was assessed for IgM reactivity against β-glucan and chitosan/chitin. Antibodies with these reactivities were present in catfish serum, with quantities of β-glucan–specific IgM higher than chitosan/chitin-specific IgM (Fig. 1 E). As mice raised in germ-free conditions may still be exposed to fungal carbohydrates, we determined whether these IgM specificities are present in the preimmune repertoire by assessing human and nonhuman primate umbilical cord blood for IgM reactivity against β-glucan and chitosan/chitin. As IgM does not cross the placenta (Mostov, 1994; Ben-Hur et al., 2005), IgM present in umbilical cord blood represents specificities generated in the preimmune repertoire by the fetus. We observed the presence of anti–β-glucan and antichitosan/chitin IgM antibodies in both human cord blood (Fig. 1 F) as well as cord blood from rhesus macaque (Fig. 1 G). Similar to mouse serum, the reactivity of human serum depends on the variable region, as purified human IgM Fc fragments or myeloma IgM lacking carbohydrate specificity had no reactivity in these assays (Fig. S1). As further evidence that these IgMs exist within the nAb repertoire, chitin-binding IgM^+ B cells were sorted by FACS, and single-cell PCR was performed to determine the sequences across the V_H chain. Genetic analysis revealed that several of the high chitin-binding clones cluster with E06, a canonical nAb recognizing oxidized antiphosphatidylcholines and capsular polysaccharide (Fig. S2; Chou et al., 2009). Thus, IgM specificities exist that target important structural carbohydrates present in most fungal cell walls. These IgM specificities are conserved across multiple species and do not require exogenous microbial stimulation for their production. Additionally, the genetic rearrangements encoding these IgMs in mice appear similar to those seen for other nAbs.

Natural IgM binds to fungal cell walls, and binding capacity is influenced by growth stage

We next assessed whether natural IgM antibodies are reactive with the cell walls of diverse fungal species. Zymosan, a particulate derived from the cell wall of *Saccharomyces cerevisiae*, consisting primarily of β-1,3 glucan, was incubated with diluted serum from SPF mice and then assessed for binding of IgM by flow cytometry. We observed marked binding of mouse IgM from SPF serum to zymosan (Fig. 2 A, I), and this phenotype was also present, although somewhat diminished, when assessing human cord blood IgM reactivity (Fig. 2 A, II). Preincubation of serum with laminarin partly diminished the ability of mouse SPF serum IgM to bind zymosan (Fig. 2 B), demonstrating specific binding of natural IgM to fungal cell wall β-1,3 glucan. Additionally, a cyst-enriched preparation of *P. murina* demonstrated high quantities of natural IgM binding when incubated with mouse SPF serum (Fig. 2 A, III). To extend our observation that diverse fungal cell walls are recognized by natural IgM, we compared the ability of natural IgM to bind another opportunistic pulmonary fungal pathogen, *Aspergillus fumigatus*. Resting conidia, which have decreased quantities of surface β-1,3 glucan, were poorly reactive with natural IgM, whereas conidia matured to a swollen, prehyphal state, leading to cell wall remodeling, and the display of high quantities of surface β-1,3 glucan (Hohl et al., 2005; Steele et al., 2005) bound significant quantities of IgM (Fig. 2 A, IV, top). IgG present in mouse SPF serum was significantly less reactive with swollen *A. fumigatus* conidia than IgM was (Fig. 2 A, IV, bottom) and approached that seen with secondary control antibody staining alone. These data suggest that natural IgM antibodies found in serum and mucosal secretions are a common opsonin for diverse species of fungi. Additionally, specific antigens targeted by nAbs may be more abundant or accessible during different stages of the fungal life cycle and may be mutually recognized by both membrane-bound PRR and natural IgM.

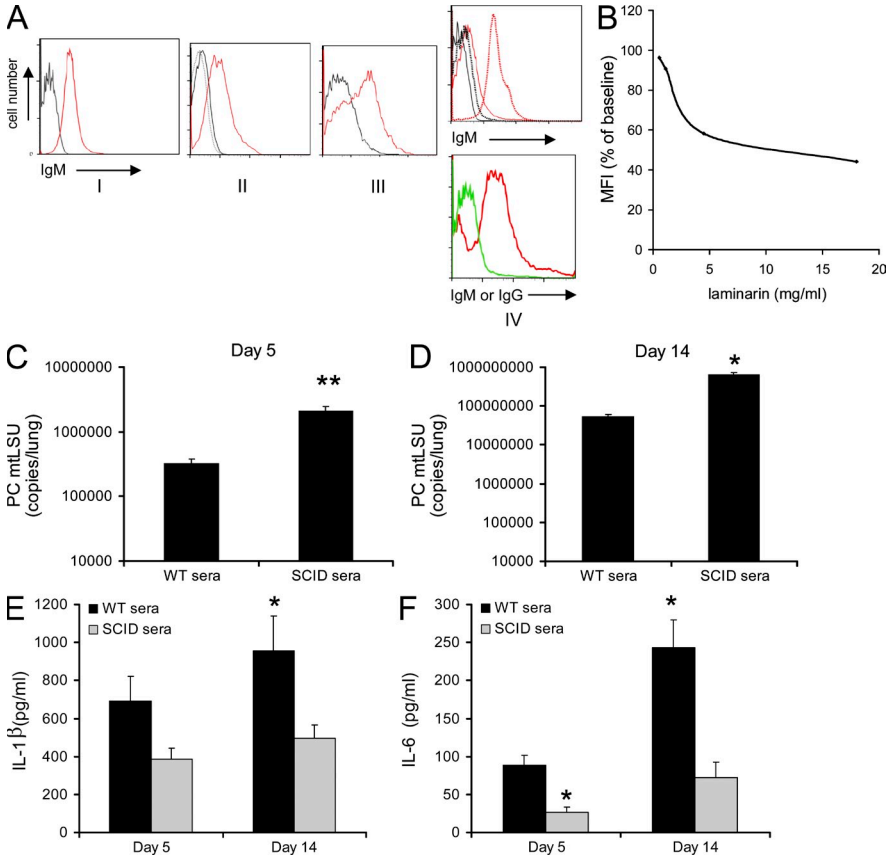

Figure 2. Natural IgM antibodies bind fungal cell walls, and transfer of nAb-containing serum from WT SPF mice enhances immune responses and impairs growth of *Pneumocystis* in the lungs of susceptible SCID mice. (A) Representative histograms of IgM binding. Fungal cells or fungal cell walls were incubated with serum diluted 1:4 in 2% BSA/PBS, washed, and then probed with anti-Ig Cy3 conjugates and studied by flow cytometry. Serum from BALB/c SPF mice (I) or human cord blood (II) was incubated with zymosan particles and then probed with anti–mouse IgM or anti–human IgM conjugate antibodies, respectively (red line). Black line indicates primary incubation with SCID sera (I) or anti–human IgM conjugate antibody alone (II; the dotted line in II indicates unstained control). (III) Cyst-enriched *Pneumocystis* preparation incubated with BALB/c SPF sera followed by anti–mouse IgM conjugate antibody. (IV, top) *A. fumigatus* resting (solid lines) and swollen conidia (dotted lines) incubated with serum from SCID (black lines) or BALB/c SPF mice (red lines), followed by anti–mouse IgM conjugate antibody. (bottom) Relative binding of mouse sera natural IgG (green line) versus natural IgM (red line) from WT SPF mice to *A. fumigatus* swollen conidia. Secondary antibody staining with anti–mouse IgG conjugate antibody was not different than when SCID sera control was used as primary (not depicted). FACS plots are representative of three independent experiments. (B) BALB/c SPF serum diluted 1:4 in 2% BSA/PBS was preincubated with laminarin before exposure to zymosan (laminarin final concentration is expressed). Particles were washed and then probed with anti–mouse IgM conjugate antibody. (C and D) Cohorts of SCID mice received a total of 400 µl of serum (100 µl of serum i.v. and 300 µl of serum i.p.), from either SPF WT mice or SCID mice, and 1 h thereafter were challenged with *Pneumocystis* intratracheally. Pathogen burden was determined at 5 (C) and 14 d (D) by assessing lungs for total *Pneumocystis* (PC) mitochondrial large subunit ribosomal RNA (mtLSU) subunit copy numbers by real-time PCR. Data are plotted as mean ± SEM (n = 6–8 pooled from three independent experiments; *, $P < 0.05$; **, $P < 0.01$). (E and F) Kinetics of IL-1β (E) and IL-6 (F) production in the lung homogenate of infected mice were determined by Luminex assay (n = 6 pooled from two independent experiments; *, $P < 0.05$). Error bars represent SEM.

Natural IgM modulates innate and adaptive immune responses to *Pneumocystis*

We assessed the relevance of the IgM isotype, the predominant isotype of nAb, in host defense against *Pneumocystis* infection. SCID mice, which permit the growth of *P. murina* in the lungs, were transferred with complement-inactivated SPF serum from either WT or SCID mice. Serum was administered in a combination of i.v. and i.p. routes, and 1 h later, mice were challenged intratracheally with *Pneumocystis*. Serum containing nAbs significantly suppressed the growth of *Pneumocystis* infection in SCID mice at early and intermediate time points after infection (5 d and 14 d, respectively; Fig. 2, C and D). The transfer of serum also enhanced production of cytokines IL-1β and IL-6 in the lungs (Fig. 2, E and F). It has previously been demonstrated that signaling by the IL-1 receptor is required for host defense against Pneumocystis pneumonia (Chen et al., 1992), and IL-6 is implicated in regulating recruitment of inflammatory cells to the lungs, diminishing numbers of neutrophils recruited into the lungs after *Pneumocystis* challenge (Chen et al., 1993). Thus, serum containing nAb enhanced the production of cytokines important in the evolving host response to this infection. As host defense was enhanced in SCID hosts without T and B cells, nAb-containing serum has protective functions in the absence of adaptive immune mechanisms. However, despite the transfer of IgM-containing sera, SCID mice do not ultimately clear the infection (unpublished data), underscoring the requirement for adaptive immune responses in the clearance of infection.

To further confirm the unique functions of sIgM in host defense against *Pneumocystis*, we assessed infection in mutant mice unable to secrete IgM, sIgM$^{-/-}$. C57BL/6J sIgM$^{-/-}$ mice, previously reported and characterized, express B cell surface IgM and IgD and isotype class-switch normally to secrete other Ig isotypes (Boes et al., 1998a; Diamond et al., 2003). We infected WT and sIgM$^{-/-}$ mice and compared their relative ability to clear *Pneumocystis* infection at an intermediate time point in infection, 14 d after intratracheal challenge. sIgM$^{-/-}$ mice had substantially impaired clearance of *Pneumocystis* relative to WT mice (Fig. 3 A), underscoring the unique contribution of IgM in host defense against this infection. Additionally, sIgM$^{-/-}$ mice had diminished production of IL-1β and IL-6 (Fig. 3 B) in the lungs, cytokines which were similarly diminished in SCID mice transferred with SCID serum lacking nAbs (Fig. 2). IL-1β and IL-6 are cytokines known to be critical for the differentiation of Th17 cells, a subset of T cells important in antifungal host resistance (Bettelli et al., 2006; Sutton et al., 2006; Veldhoen et al., 2006). sIgM$^{-/-}$ mice also had enhanced production of IL-10 in the lungs (Fig. 3 B), a cytokine which inhibits the kinetics of *Pneumocystis*

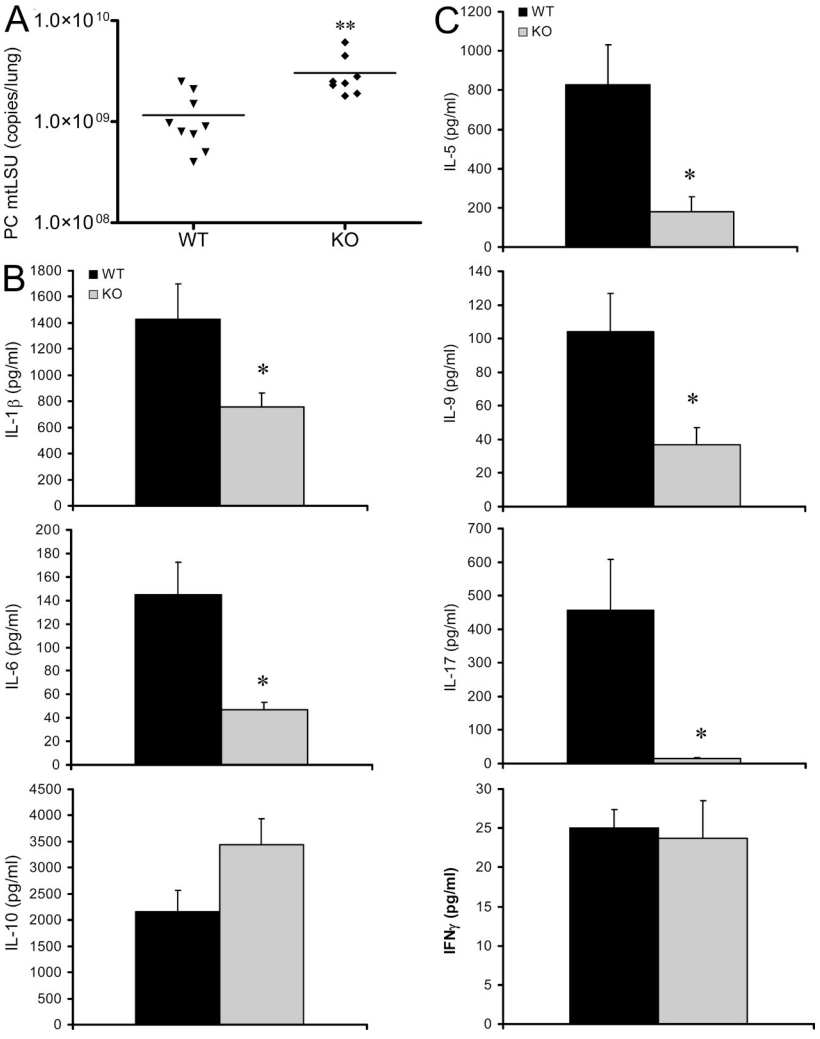

Figure 3. Mice unable to produce sIgM have impaired pulmonary clearance of *Pneumocystis* and altered inflammatory responses in local DLNs and in lung tissue. (A and B) C57BL/6J WT and sIgM-deficient (KO) mice were challenged with *Pneumocystis* intratracheally, and 14 d thereafter, pulmonary *Pneumocystis* (PC) burden (A) was assessed by real-time PCR. Symbols represent individual mice; horizontal lines represent the mean (n = 8–9 pooled from three independent experiments; **, $P < 0.01$ by Mann-Whitney test). mtLSU, mitochondrial large subunit ribosomal RNA. (B) IL-1β, IL-6, and IL-10 in lung homogenate were measured at 14 d (n = 6–8 pooled from two independent experiments; *, $P < 0.05$ by Mann-Whitney test). Cytokine concentrations in uninfected lungs were all <10 pg/ml. Error bars represent SEM. (C) Mediastinal DLNs isolated from WT or sIgM KO mice 14 d after intratracheal *Pneumocystis* challenge were gently teased apart, and cells from each LN were individually restimulated with or without *Pneumocystis* antigen and cultured for 3 d. Supernatants were assessed for production of IL-5, IL-9, IL-17, and IFN-γ by Luminex assay (n = 6 mice per group pooled from two independent experiments). Data are mean ± SEM (*, $P < 0.05$ by Mann-Whitney test). Cytokine responses without antigen stimulation were <25 pg/ml in these experiments.

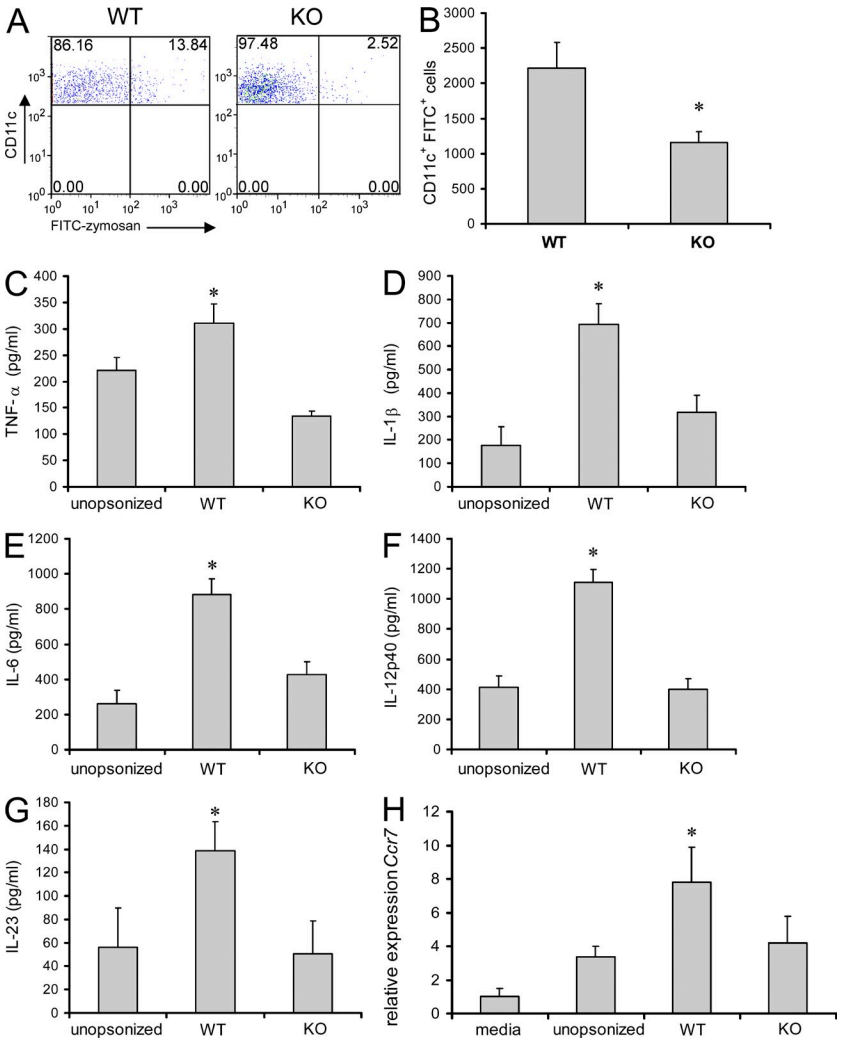

Figure 4. nAbs influence the earliest aspects of fungal antigen presentation and infiltration of inflammatory cells into the lungs. (A) C57BL/6J WT or sIgM$^{-/-}$ (KO) mice were intratracheally challenged with 500 µg FITC-zymosan delivered in 50 µl PBS. 18 h later, mediastinal DLNs were harvested and dissociated into single-cell suspensions. Cells were stained with CD11c-APC, and FITC association was assessed by flow cytometry. Representative WT or sIgM KO individual LNs are presented. (B) Recovery of CD11c$^+$ FITC^{hi} cells in LNs per cohort (n = 5–9 mice per group pooled from two independent experiments; *, $P < 0.05$ by Mann-Whitney test). (C–H) Bone marrow–derived DCs were grown in RPMI 1640 +10% FCS + GM-CSF + IL-4. After 6 d, DCs were collected, washed, and cultured in serum-free RPMI 1640. *Pneumocystis* cysts obtained from chronically infected SCID mice were unopsonized or opsonized with WT serum or serum from sIgM KO mice and added to DCs at a dose of 10 cysts per cell. Cell supernatants were assayed for TNF, IL-1β, IL-6, or IL-12p40 by Luminex or IL-23 by ELISA (n = 6 mice per group pooled from two independent experiments; *, $P < 0.05$). In H, total RNA was extracted, and relative expression of *Ccr7* was assayed by quantitative real-time PCR normalized to GAPDH (n = 6 mice per group pooled from two independent experiments; *, $P < 0.05$). Error bars represent SEM.

clearance (Qureshi et al., 2003). Thus, IgM antibodies have an important role in host defense at early stages of *Pneumocystis* infection, influencing inflammation and fungal burden in the lungs.

Given these alterations in inflammatory responses in the lung tissue, we next sought to determine whether immune responses in regional LNs, sites of antigen presentation and priming of adaptive immune responses, are affected by an environment deficient in sIgM. Mediastinal draining LNs (DLNs) were collected 14 d after *Pneumocystis* challenge of WT or sIgM$^{-/-}$ mice, and individual DLNs were restimulated with *Pneumocystis* antigen. We observed that sIgM$^{-/-}$ mice produced diminished quantities of the Th2 cytokines IL-5 and IL-9 and diminished quantities of IL-17 (Fig. 3 C). In contrast, we observed minimal differences in the induction of the Th1 cytokine IFN-γ in sIgM$^{-/-}$ mice. These data indicate that fungal antigens presented in a sIgM-sufficient environment produce increased Th2 and Th17 cytokines within the lungs and demonstrate that sIgM is critical for in vivo Th2 and Th17 priming against *Pneumocystis* within pulmonary DLNs.

To address how antigen presentation at the very earliest aspects of fungal exposure in lung tissue is affected by nAbs, we assessed whether mice deficient in sIgM have impaired uptake or presentation of fungal cell wall antigen to CD11c$^+$ APCs of the lung (Vermaelen et al., 2001) in vivo. WT or sIgM$^{-/-}$ mice were intratracheally challenged with FITC-zymosan and, 16 h later, were assessed for the frequency of CD11c$^+$ cells that were FITC positive secondary to zymosan endocytosis in the lung parenchyma and in DLNs. In the lung tissue, we observed that the vast majority of CD11c$^+$ cells were FITC positive after FITC-zymosan challenge, and equivalent frequencies of FITC$^+$ CD11c$^+$ cells were observed in WT and sIgM$^{-/-}$ mice (Fig. S3), suggesting that sIgM is not required for CD11c$^+$ cell recognition of zymosan in lung tissue. However, when we compared quantities of CD11c$^+$ cells in the mediastinal DLNs 16 h after challenge, the number of recovered FITC$^+$ CD11c$^+$ cells was diminished in sIgM$^{-/-}$ mice, suggesting that migration of CD11c$^+$ APCs to the LNs is impaired in the absence of sIgM (Fig. 4, A and B). Thus, natural IgMs are involved in the very earliest aspects of fungal carbohydrate antigen recognition in the host, influencing trafficking of antigen-loaded CD11c$^+$ cells to regional LNs.

Purified β-1,3 glucan from *Pneumocystis carinii* is highly inflammatory and results in the elaboration of TNF, IL-1β, and MIP-2 from APCs (Hoffman et al., 1993; Vassallo et al., 2000; Carmona et al., 2006). However, purified whole intact

organism preparations of *Pneumocystis* are less inflammatory, suggesting that glucans may not be easily accessible in intact organisms by macrophage PRRs such as dectin-1 (Steele et al., 2003). To assess whether IgM opsonization affects the DC response to this fungal pathogen, we incubated *Pneumocystis* with WT serum or sIgM$^{-/-}$ serum before incubation with bone marrow–derived DCs. Incubation of DCs with unopsonized *Pneumocystis* organisms results in small increases in TNF, IL-1β, IL-6, IL-12p40, and IL-23 (Fig. 4, C–G). However, opsonization with WT mouse serum resulted in significant increases in these cytokines. Opsonization with sIgM$^{-/-}$ serum resulted in a significant attenuation of TNF, IL-1β, IL-6, IL-12p40, and IL-23 (Fig. 4, C–G). Thus, IgM antibodies reactive with *Pneumocystis* organisms enhance inflammatory responses by DCs, which may influence DC activation and Th priming pathways. As we had also observed a defect in DC homing in sIgM$^{-/-}$ mice in vivo, we sought to determine whether opsonization of *Pneumocystis* affected expression of messenger RNA for the lymphoid homing receptor CCR7. Of note, unopsonized *Pneumocystis* induced *Ccr7* expression; however, opsonization with WT serum, in contrast to sIgM$^{-/-}$ serum, induced the highest amount of *Ccr7* expression (Fig. 4 H).

As exposure to *Pneumocystis* in mice unable to produce sIgM altered production of Th2- and Th17-associated cytokines in mediastinal DLNs and sIgM influences fungal antigen presentation in DLNs, we further assessed the hypothesis that sIgM might influence pathways of Th differentiation within draining lung LNs at the single-cell level. We infected sIgM$^{-/-}$ and WT mice with *Pneumocystis* and 14 d thereafter assessed frequencies of LN cells producing cytokines associated with Th1 (IFN-γ), Th2 (IL-5), or Th17 (IL-17) responses from mice by ELISPOT. To determine frequencies without expanding the population ex vivo, LN cells were not restimulated with *Pneumocystis* antigen. We observed that LN cells from sIgM$^{-/-}$ mice challenged with *Pneumocystis* had significantly lower frequencies of IL-5– and IL-17–producing cells relative to WT mice, whereas frequencies of IFN-γ–producing LN cells, while trending lower in sIgM$^{-/-}$ mice, were not substantially different from those found in WT mice (Fig. 5 A). We also observed similar decreases in Th2 and Th17 responses but preserved Th1 responses in CD4$^+$ T cells from DLNs in sIgM$^{-/-}$ mice by intracellular staining for IL-4, IL-17, and IFN-γ, followed by analysis by flow cytometry (Fig. S4). These data show that sIgM is involved in the optimal production of IL-5 and IL-17, cytokines which are associated with Th2 and Th17 lineages, respectively, in response to fungal infection. Our data demonstrate

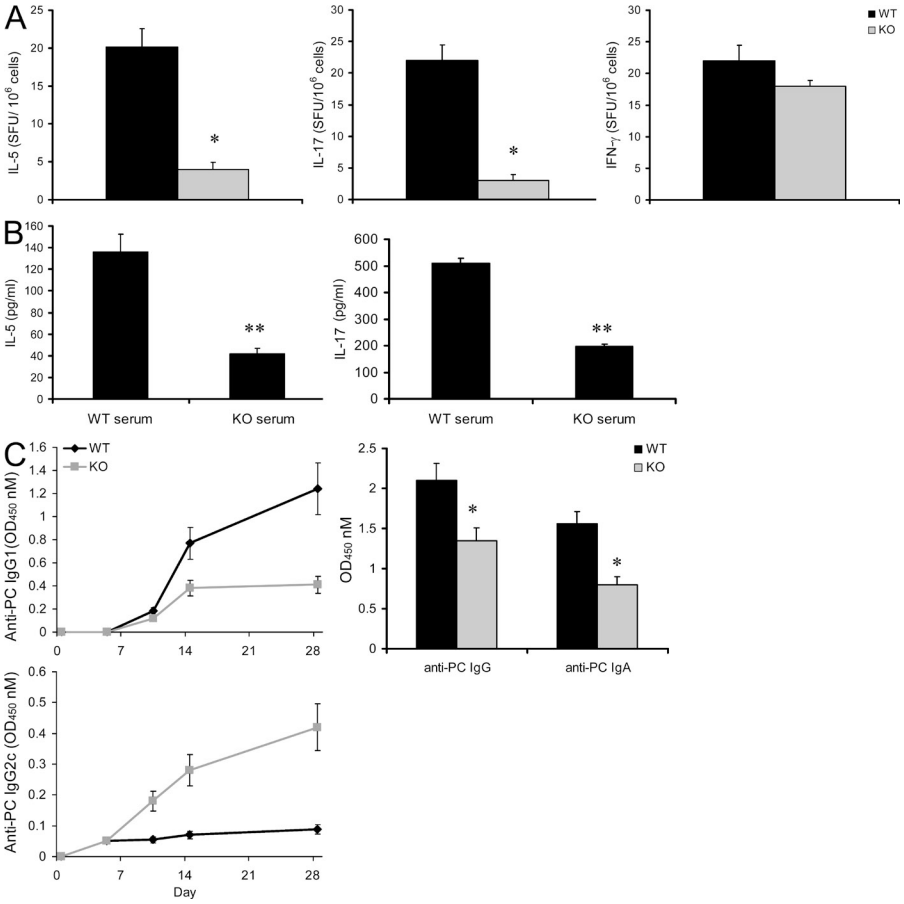

Figure 5. Mice unable to secrete IgM have diminished Th2 and Th17 responses in mediastinal LNs after *Pneumocystis* challenge and impaired Th2-type adaptive B cell responses. C57BL/6J WT and sIgM KO mice were challenged with *Pneumocystis* intratracheally. (A) 14 d after challenge, mediastinal LNs were harvested, and cells were dissociated into single-cell suspensions, enumerated, and assessed for production of IL-5, IL-17, and IFN-γ after 2-d culture by ELISPOT. In the absence of infection, spot frequencies are <5 spot-forming units (SFUs) per million cells (not depicted; $n = 6$ mice per group pooled from two independent experiments). (B) Before challenge, *Pneumocystis* inoculum was preopsonized with serum from either C57BL/6J WT or sIgM KO mice and thereafter administered to sIgM KO mice intratracheally. 14 d after infection, individual LNs were collected, and cells were dissociated into single-cell suspensions, enumerated, and stimulated with *Pneumocystis* antigen in culture for 2 d. IL-5 and IL-17 production was measured in tissue culture supernatants by Luminex assay ($n = 5$–6 pooled from two independent experiments). Data are mean ± SEM. (C) Serum was collected serially from WT and sIgM KO mice challenged intratracheally with *Pneumocystis* and probed for adaptive IgG1 and IgG2c responses against *Pneumocystis* antigen. BALF was collected at 28 d after infection and probed for total IgG and IgA responses against *Pneumocystis* antigen ($n = 5$–6 mice/condition/time point pooled from two independent experiments). Error bars represent SEM (*, $P < 0.05$; **, $P < 0.01$ by Mann-Whitney test).

that sIgM, which includes nAbs targeting fungal cell wall carbohydrate antigens, is capable of shaping Th-associated LN responses against fungal pathogens.

To address whether reconstitution of sIgM influences Th priming in LNs at the earliest time points after *Pneumocystis* exposure, *Pneumocystis* inoculum was preopsonized with either sIgM$^{-/-}$ or WT serum and was used to intratracheally infect sIgM$^{-/-}$ mice. 14 d thereafter, mediastinal LNs were collected and individually restimulated with *Pneumocystis* antigen. LNs from sIgM$^{-/-}$ mice challenged with *Pneumocystis* preopsonized with WT serum produced significantly higher quantities of total IL-5 and IL-17 in culture (Fig. 5 B). There was no difference in IFN-γ production with *Pneumocystis* restimulation with a level of 126 ± 31.6 pg/ml in WT mice and 107 ± 25.7 pg/ml in sIgM$^{-/-}$ mice (n = 6 each group; $P > 0.05$). This observation supports the hypothesis that opsonization of *Pneumocystis* with IgM directly promotes adaptive Th2 and Th17 responses in DLN inflammatory responses, at least in part by increasing fungal antigen trafficking to DLNs and altering pathways of APC activation.

As the role of sIgM has not been assessed in the evolving antibody response against any fungal organism, we evaluated the kinetics and isotype production of antibodies formed in sIgM$^{-/-}$ mice after exposure to *Pneumocystis*. Unexpectedly, we observed that the production of anti-*Pneumocystis* IgG1 was significantly impaired in sIgM$^{-/-}$ mice (Fig. 5 C). Of note, IgG1 is the predominant anti-*Pneumocystis* isotype produced after infection in mice (Garvy et al., 1997) and is an antibody isotype associated with Th2 immunity. In comparison, serum anti-*Pneumocystis* IgG2c, which is an isotype associated with Th1 immunity, was significantly increased in sIgM$^{-/-}$ mice (Fig. 5 C). Both trends emerged as early as 10 d after challenge and notably continued through 28 d after infection, with total quantities of IgG1 significantly lower and IgG2c higher in sIgM$^{-/-}$ mice. Additionally, production of anti-*Pneumocystis* IgG and IgA at mucosal surfaces was inhibited in the absence of sIgM at 28 d after infection (Fig. 5 C). Normal programming of B cell adaptive antibody responses against *Pneumocystis*, both systemically and at mucosal surfaces, requires sIgM.

DISCUSSION

We report on the significant functions of nAbs and the IgM isotype in host defense against the opportunistic fungal pathogen *P. carinii*. Although adaptive immune defense mechanisms involving CD4$^+$ T cells are central to host defense against Pneumocystis pneumonia, we have identified a significant function for sIgM in host defense against fungi and demonstrate that natural, innate antibodies and sIgM are involved in immunological responses at the earliest stages of fungal infection, enhance clearance of infection, and shape the evolving adaptive immune responses mounted by the host. Conserved natural IgMs target the abundant and conserved carbohydrate antigens present in fungal cell walls, are involved in the earliest stages of fungal recognition and host defense, and are of critical importance in promoting adaptive Th2 and Th17 type immune responses locally and systemically in response to *Pneumocystis* pulmonary infection. Routing of fungal antigen through sIgM-dependent pathways increased migration of pulmonary APCs to DLNs and enhanced inflammatory responses in the lungs and LNs, leading to more efficient clearance of pathogen in early stages of mucosal infection. Such alteration of antigen presentation and differential activation of APCs affects T cell priming and germinal center reactions, leading to changes in programming of B and CD4$^+$ T cell type adaptive immune responses.

Almost all fungi possess a cell wall with conserved composition, consisting of a core of β-1,3 linked glucan interlinked with chitin, which provides an important structural function for the fungal cell wall (Latgé, 2007). Although the manner in which these carbohydrates drive immune responses likely involves recognition by membrane-associated PRRs such as CD11b (Yan et al., 1999), dectin-1 (Brown and Gordon, 2001), and toll-like receptors and cellular enzymes such as chitinases (Elias et al., 2005; Reese et al., 2007; Da Silva et al., 2008), the role of innate antibodies in the identification of these structures has thus far not been evaluated. nAbs are molecules primarily of the IgM isotype, often with the capacity to bind more than one antigen, and are usually encoded by a limited set of germline genes without N-region additions (Baumgarth et al., 2005). Given their presence in the steady-state, their importance in immediate immunological responses such as delayed-type hypersensitivity (Szczepanik et al., 2003) and in defense against bacterial and viral infections has been studied (Boes et al., 1998b; Ochsenbein et al., 1999). The repertoire, although broad, is highly restricted (Mouthon et al., 1995), as for example, mice produce nAbs targeting the carbohydrate antigen Galα1-3Gal only after genetic deletion of an enzyme required for the production of this carbohydrate, and in our experiments, we note that mouse natural IgM is not reactive with the fungal cell wall carbohydrate mannan. We show that high quantities of IgM targeting β-glucan and chitin are present in the serum of naive mice and that mice reared in germ-free conditions contain these specificities. Binding of serum IgM to zymosan was inhibited by laminarin, although only partially, demonstrating a lack of 100% competition by laminarin, which may be partially explained by residual chitin in the zymosan preparation. As the nAb repertoire is likely to be the first representation of B cell immunity in evolution, we questioned whether more primitive organisms produce these specificities. Catfish, among the earliest species containing Igs derived from genetic recombination and containing a tetrameric homologue of IgM, possessed serum IgM reactivity against β-glucan and chitin, suggesting that the capacity to make these specificities is preserved between species. Natural IgM antibodies targeting β-glucan and chitin were also found in primate umbilical cord blood, demonstrating that these specificities are conserved and develop in the absence of exogenous antigenic stimulation. In the mouse, we observed that production of these antibodies predominates in the spleen, and that CD5$^+$ B cells purified from the

spleen were the predominant subset producing these antibodies. These data are consistent with findings made by other laboratories demonstrating that the spleen is a common site for nAb-secreting cells (Ochsenbein and Zinkernagel, 2000; Ha et al., 2006; Yang et al., 2007). Additionally, we found that chitin-specific IgM appears to be generated from genetic recombination events similar to those seen for the prototypic nAb for phosphatidylcholine, underscoring the limited genetic repertoire seen among nAbs. We observed that serum IgM binds cell wall preparations such as zymosan and that binding is partially inhibited by laminarin, suggesting that the carbohydrate components of fungal cell walls are specific targets of natural IgM. Additionally, natural IgM binds *Pneumocystis* organisms and growing *A. fumigatus* conidia, suggesting that certain antigens targeted by nAbs may be more accessible at certain stages of fungal cell growth. These data demonstrate that natural IgMs represent soluble PRRs for conserved fungal cell wall carbohydrates.

Transfer of SPF WT serum into SCID mice significantly limited the growth of *Pneumocystis* in the lungs at intermediate time points after infection and enhanced production of IL-1β and IL-6 in the lungs, cytokines which are important in host defense mechanisms against this infection. The existence of protective responses mediated by nAb-containing serum in the absence of B and T cells underscores an intrinsic innate effect of nAb on host inflammatory responses and their ability to recognize and to enhance clearance of fungal organisms. The hypothesized contribution of the IgM isotype in these responses was confirmed our experiments in the $sIgM^{-/-}$ mouse, which demonstrated impaired clearance of *Pneumocystis* at intermediate stages of infection and impaired production of IL-1β and IL-6 and enhanced production of IL-10 in the lungs. These data suggest that nAbs alter the routes of antigen presentation, perhaps partially inhibiting recognition of *Pneumocystis* directly through membrane-bound PRRs or through neutralizing inflammatory components such as shed β-glucan resulting from *Pneumocystis* degradation. Of note, recent work has demonstrated that *C. neoformans* infection in $sIgM^{-/-}$ mice leads to increased mortality and dissemination of infection to the brain, underscoring the capacity of the IgM isotype to limit fungal infection (Subramaniam et al., 2010).

As the earliest aspects of *Pneumocystis* recognition were altered in the absence of nAbs, we hypothesized that the absence of sIgM may potentially have consequences in the development of adaptive immune responses. We studied the interaction of zymosan with pulmonary $CD11c^+$ cells, as it is these cells which are hypothesized to rapidly migrate into the T cell zones of DLNs after instillation of antigen into the lungs (Vermaelen et al., 2001). By assessing how trafficking of the fungal cell wall carbohydrate particle zymosan occurs in the lungs in the absence of sIgM, we observed that pulmonary $CD11c^+$ cells were equivalently capable of internalizing zymosan. However, analysis of $CD11c^+$ cells in the mediastinal DLNs, DCs which have migrated secondary to activation and maturation, showed that the presence of DCs containing zymosan in inductive sites was impaired in the absence of sIgM. As DC migration is dependent on the process of cellular maturation and the expression of specific chemokine receptors mediating LN homing, which in turn is thought to be partly guided by the signaling pathways initiated by pathogen-associated molecular patterns, we believe that fungal cell wall carbohydrate antigens coated in IgM are processed through PRRs, which enhance the process of DC maturation. Not understood, however, is how sIgM-coated zymosan specifically mediates interactions with $CD11c^+$ cells leading to the promotion of migration. We demonstrate that DCs exposed to *Pneumocystis* opsonized with serum containing IgM produce higher quantities of TNF, IL-1β, IL-6, and IL-12p40 than DCs exposed to *Pneumocystis* opsonized with serum lacking IgM. DC chemokine responsiveness and migration to regional LNs have been demonstrated in models of *Mycobacterium tuberculosis* and *Yersinia pestis* to be dependent on IL-12p40 induction (Khader et al., 2006; Robinson et al., 2008). We postulate that defective IL-12p40 induction in the absence of sIgM may be one mechanism by which DC migration is adversely affected in $sIgM^{-/-}$ mice. Another area of future investigation is the role of complement and complement receptors as sIgM is a strong promoter of the classical complement pathway. sIgM may enhance complement deposition on fungi, leading to recognition and DC activation through signaling at complement receptors. Additionally, receptors that specifically identify IgM–antigen complexes such as the Fcα/μ receptor identified in mice and humans on hematopoietic and other cells (Shibuya et al., 2000; Sakamoto et al., 2001) could be preferentially involved in the recognition of IgM-opsonized fungi by DCs, shaping the cellular maturation program.

Although there is evidence that a Th2-type immune response is detrimental in host defense against various fungi, this has not been demonstrated to be the case in host defense against *Pneumocystis*, and notably, the Th2 response appears dominant in response to this infection (Garvy et al., 1997; Shellito et al., 2000). Additionally, the relevance of Th17 cells in fungal host defense is emerging. Mice treated with a neutralizing antibody against IL-17 have impaired clearance of *Pneumocystis* from the lungs (Rudner et al., 2007). In systemic infection with *Candida albicans*, IL-17 receptor–deficient mice had increased tissue fungal burdens and reduced survival, which appears related to neutrophil recruitment to infected tissues (Huang et al., 2004b). In *A. fumigatus* infection, it has been demonstrated that different isolated fungal cell wall components, upon vaccination in mice, elicit different Th subsets, with glycolipids preferentially eliciting Th17 cells and secreted proteins preferentially eliciting Th2 cells (Bozza et al., 2009). Our data demonstrate that sIgM plays an important role in the production of the Th environment after fungal challenge in the pulmonary DLNs, as absence of this antibody isotype markedly impaired the generation of cells capable of producing IL-5 and IL-17. The numbers of specific precursor cells in the mediastinal DLNs producing IL-5, IL-9, and IL-17 upon restimulation with *Pneumocystis* were also

diminished in the absence of sIgM. When sIgM$^{-/-}$ mice were challenged with *Pneumocystis* inoculum preopsonized with WT serum, cells isolated from the mediastinal DLNs produced higher quantities of IL-17 and IL-5, suggesting that IgM is critically involved in generating these cytokines at sites of Th priming. The precise mechanism of decreased Th2 and Th17 priming in the absence of sIgM remains to be defined, although we hypothesize that the patterns of antigen presentation in the sIgM-sufficient environment and differential activation of APCs lead to this phenotype.

We also observed that the generation of *Pneumocystis*-specific IgG1 antibodies was significantly diminished in the absence of sIgM, whereas IgG2c was enhanced. The dramatic alteration in isotype prevalence against *Pneumocystis* is similar to what is observed in IL-4$^{-/-}$ mice challenged with *Pneumocystis* (Garvy et al., 1997), with strong inhibition of IgG1 production and enhanced production of IgG2a, the antibody isotype in BALB/c mice correlating with Th1 immunity. Interestingly, our observations contrast with those made in the mouse influenza model, in which the kinetics of virus-specific IgG2a production were inhibited in sIgM$^{-/-}$ mice, but ultimately, quantities of virus-specific IgG1 and IgG2a antibody produced were no different from WT mice (Baumgarth et al., 2000). Our data support the concept that IgM present at early stages of infection promotes Th2 effector functions while diminishing Th1 effector functions, as manifested in selective Th cells promoting class-switch recombination toward IgG1. As Th17 cells produce high quantities of IL-21, and IL-21 is implicated in the optimal production of the IgG1 antibody response upon immunization (Ozaki et al., 2002), it may be the case that higher quantities of Th17 cells producing IL-21 are primed in an IgM-sufficient environment. Additionally, we observed a net decrease in anti-*Pneumocystis* antibodies of the IgG and IgA isotype at the mucosa in the absence of sIgM. The observed decrease in mucosal IgA suggests that local B cell responses influencing the presence of antibodies at the mucosa require sIgM for optimal production, and this outcome may have implications for humoral memory responses against *Pneumocystis*. The reduced levels of anti-*Pneumocystis* IgA may be caused by the decreased frequencies of Th17 cell as these cells have been recently implicated in up-regulation of the polymeric Ig receptor and mucosal IgA responses (Aujla et al., 2008; Jaffar et al., 2009). Thus, sIgM influences both specific isotype production as well as overall presence of antibodies against *Pneumocystis* in the mucosa, and this may be initiated by sIgM-dependent antigen presentation at the very earliest stages of infection.

As individuals with X-linked hyper-IgM syndrome possess CD40L mutations, yet produce abundant IgM and are susceptible to *Pneumocystis*, it has been argued that the IgM antibody isotype is unimportant in host defense mechanisms against this infection (Mílledge et al., 2003). These studies demonstrate that functions of IgM are significant in host defense against *Pneumocystis* and in the evolving adaptive response to fungi. Additionally, individuals with HIV-AIDS, who are susceptible to *Pneumocystis* with progressing disease, suffer from polyclonal B cell activation and polyclonal hypergammaglobulinemia, with poor antibody responses to specific antigens (Moir and Fauci, 2008). Dysregulation of natural IgM could potentially play a major role in aspects of fungal antigen presentation, Th priming, adaptive antibody responses, and overall host defense influencing the phenotype of Pneumocystis pneumonia susceptibility. Investigation of how HIV infection affects the production of nAb and particular nAb specificities may provide insight into host susceptibility to opportunistic fungi. Our experiments additionally underscore the concept of sIgM as a potential adjuvant in vaccine design against fungi such as *Pneumocystis*, enhancing Th2- and Th17-type adaptive immunity.

MATERIALS AND METHODS

Mice and serum samples. 6–8-wk aged BALB/c mice and C57BL/6J mice were purchased from Jackson ImmunoResearch Laboratories and BALB/c.*scid* mice were obtained from Taconic. Mice with a deficiency in the ability to secrete IgM (sIgM$^{-/-}$), but able to express the membrane-bound form of IgM, on the C57BL/6J background were obtained from M.S. Diamond (Washington University in St. Louis, St. Louis, MO) and were previously described (Diamond et al., 2003). The parent strain was developed by J. Chen (Massachusetts Institute of Technology, Cambridge, MA) and M. Carroll (Harvard Medical School, Boston, MA; Boes et al., 1998a). Serum samples from channel catfish, *I. punctatus*, were obtained from E. Bengtén (University of Mississippi, Oxford, MS). Catfish (2 kg) were obtained from the wild by angling (Ross Barnett Reservoir, MS) or from the United States Department of Agriculture–Agricultural Research Service Stoneville, MS, and maintained in individual tanks until bleeding by caudal venipuncture. Serum samples from C57BL/6 mice reared in germ-free conditions were obtained from S. Plevy (University of North Carolina at Chapel Hill, Chapel Hill, NC) and D. Peterson and J. Gordon (Washington University in St. Louis). Mice reared in germ-free conditions were fed standard autoclavable chow and bled at 3–4 mo of age. All mice were maintained in an SPF environment in microisolator cages within the animal care facilities of the Children's Hospital of Pittsburgh under protocols reviewed and approved by the Animal Research and Care Committee. For longitudinal experiments, small volumes of blood were collected from the periorbital sinus, and at terminal sacrifice of mice, blood was obtained from caudal venipuncture under anesthesia. Pooled serum for adoptive serum transfer experiments was obtained from SPF BALB/c or BALB/c.*scid* mice aged 8 wk, via the caudal vena cava exsanguination, heated for 1 h at 56°C to inactivate complement proteins, and stored at −80°C until use. Primate cord blood was obtained from the Tulane University Primate Research Center. Human cord blood serum was collected from full-term newborn patients after informed parental consent at Magee-Women's Hospital (Pittsburgh, PA) according to the University of Pittsburgh Institutional Review Board guidelines.

Detection of antibody responses against β-glucan, chitin/chitosan, mannan, and *Pneumocystis*. *Pneumocystis* antigen was prepared as previously described (Zheng et al., 2001) and was dissolved in carbonate buffer, pH 9.5, and seeded to 96-well plates (PolySorp; Nunc) at a concentration of 1 μg/ml. Laminarin (Sigma-Aldrich), derived from the brown algae *Laminaria digitata* and composed primarily of β-1,3 linked glucan, the predominant β-glucan linkage found in fungal cell walls, was dissolved in PBS. Medium molecular weight chitosan (Sigma-Aldrich), derived from crab shells, is a polymer of chitin that is 75–85% deacetylated. Chitosan/chitin was dissolved in 2% acetic acid/PBS (vol/vol) and thereafter diluted into PBS to a final quantity of 25 μg/ml. α-1,6 linked mannan (Sigma-Aldrich) derived from *S. cerevisiae* was dissolved in PBS. Carbohydrate antigens were seeded at a concentration of 25 μg/ml to PolySorp 96-well plates and at 4°C. Plates were blocked in 10% FBS and 5% milk in PBS, and blocking buffer was used as a dilution buffer for serum and secondary antibodies. Sera were applied in

serial dilutions. Bronchoalveolar lavage fluid (BALF) was run neat. Horseradish peroxidase–conjugated secondary antibodies against mouse IgG, IgA, and IgM, human IgM, and primate IgM were obtained from Santa Cruz Biotechnology, Inc. and SouthernBiotech. Catfish IgM was detected with the mAb 9E1 (mouse IgG1), a gift from E. Bengtén, and thereafter probed with anti–mouse IgG1–horseradish peroxidase. Plates were developed with tetramethylbenzidine substrate, and the reaction was quenched with 2N sulfuric acid. Background for all ELISAs was <0.05 at an OD of 450 nM. Estimated endpoint titer was determined by assessing the lowest concentration at which signal was obtained that was two times higher than background. OD values were reported at 450 nM with background subtracted.

Flow cytometric analyses of nAb binding to fungi. Zymosan particles were obtained from Invitrogen. *A. fumigatus* isolate 13073 was obtained from the American Type Culture Collection, maintained on potato agar for 5 d, and then harvested into PBS/0.1% Tween 20 and passed over a 40-µm pore size cell strainer to remove hyphal fragments. Organisms were maintained as dormant resting conidia in PBS solution in an airtight conical at 4°C until use. *A. fumigatus* conidia were matured into growth phase by culture in RPMI at 37°C for 6 h, where they were thereafter killed by incubation at 100°C for 15 min. Additionally, a cyst-enriched preparation of *Pneumocystis* organisms was derived from the *Pneumocystis* inoculum via sucrose gradient centrifugation using methods described by Lim et al. (1973). 400,000 cells/particles were incubated with serum from BALB/c or BALB/c.*scid* mice for 3 h at a 1:4 dilution in PBS. After pelleting and washing, cells were incubated with goat anti–mouse IgG or goat anti–mouse IgM antibodies conjugated to Cy3 (Invitrogen) diluted in 2% BSA solution. Cell/particle fluorescence was assayed with FACSAria (BD).

ELISPOT for detection of antibody-secreting cells specific for β-glucan. 96-well plate nitrocellulose membranes were coated with laminarin dissolved in 80 µg/ml PBS overnight. Lung and splenic tissue were collected from naive BALB/c mice and crushed and filtered, and RBCs were lysed to obtain a single-cell suspension. Additionally, cells were collected from bone marrow and peritoneal lavage. Plates were blocked with complete tissue culture media consisting of RPMI 1640 medium, 10% FBS, 2 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2-ME at 5×10^{-5} M, and cells were seeded to plates in serial dilution. After 2 d, cells were removed, and bound IgM reactive with β-glucan was identified with goat anti–mouse IgM–alkaline phosphatase and detected with NBT/BCIP. An ELISPOT reader was used to enumerate spots (Cellular Technology). IgM^+, $CD5^+$ B cells were sorted on a FACSaria and were >98% pure.

***P. carinii* f. sp. *muris* isolate, *Pneumocystis* infection, and *Pneumocystis* cyst-enriched preparations.** The *Pneumocystis* inoculums, preparations, and infections were performed as previously described (Shellito et al., 1990; Kolls et al., 1999). Preopsonization of inoculum with complement inactivated serum was performed at 4°C at 1:4 dilution for 30 min before intratracheal administration. *Pneumocystis* cysts were isolated from trophozoites using sucrose gradient density centrifugation, using a method previously described (Lim et al., 1973). In certain experiments, organisms were unopsonized or opsonized with WT or $sIgM^{-/-}$ serum and incubated with mouse bone marrow–derived DCs as previously described (Zheng et al., 2001). Cell supernatants were collected and assayed for TNF, IL-1β, IL-6, and IL-12p40 by Luminex or IL-23 by ELISA (eBioscience). Total RNA was assayed for the expression of CCR7 transcripts using real-time PCR normalized to GAPDH expression (Assays-on-Demand; Applied Biosystems).

Adoptive serum transfer. BALB/c.*scid* mice were administered 400 µl of pooled serum from either SPF BALB/c mice or BALB/c.*scid* mice aged 7–9 wk; serum was given 100 µl i.v. and 300 µl i.p. 1 h thereafter, mice were challenged with *Pneumocystis* intratracheally, and infection was allowed to proceed until terminal sacrifice.

***Pneumocystis* burden, cytokine detection, and BALF analyses.** To assess *Pneumocystis* burden, total RNA was isolated from the right lung of infected mice by a single-step method using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Thereafter, RNA was transcribed to cDNA, and real-time PCR was performed as previously described; *Pneumocystis* ribosomal RNA copy numbers were quantified against a standard curve of known *Pneumocystis* ribosomal RNA copy numbers as previously described (Steele et al., 2003). Lung homogenate or tissue culture samples were analyzed for concentrations of 22 cytokines using a Luminex multiplex suspension cytokine array (Millipore) according to the manufacturer's instructions. Data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). Fluid from the lower respiratory tract was obtained by bronchoalveolar lavage of mice anesthetized with i.p. ketamine/xylazine as described previously (Rapaka et al., 2007). 1 ml BALF was collected from mice using sterile PBS. Lavage fluids were centrifuged at 350 *g*, and supernatant was stored at −80°C until use.

Intratracheal zymosan challenge and trafficking experiment. C57BL/6J WT or $sIgM^{-/-}$ mice were intratracheally challenged with 500 µg zymosan covalently linked to FITC (Invitrogen), in a volume of 50 µl PBS. 18 h after challenge, mice were sacrificed, mediastinal DLNs were collected and dissociated into single-cell suspensions, and RBCs were lysed. Additionally, lungs were digested with 25 U/ml DNase and 1 mg/ml collagenase A for 30 min at 37°C and then dissociated into single-cell suspensions, and RBCs were lysed. Cells were stained with CD11c-APC (BD) to identify APCs in the LNs and lungs. Cells were studied by FACSAria.

LN restimulation and IL-5, IL-17, and IFN-γ ELISPOT. Individual mediastinal DLNs were dissected from the lungs, teased apart, and placed into a 96-well plate containing 200 µl of complete media. LN cells were restimulated with 40,000 cysts from the *Pneumocystis* inoculum. 3 d after seeding, LN cells were pelleted, and supernatants were collected for analysis. For ELISPOT analyses, individual mediastinal DLNs were dissected, teased apart, and strained into single-cell suspensions, depleted of RBCs, enumerated, and placed into a 96-well nitrocellulose membrane plate containing 100 µl of complete media for 2 d. IL-5 (eBioscience), IL-9, IFN-γ (R&D Systems), and IL-17 (R&D Systems) ELISPOT kits were used, and the assays were performed according to the manufacturers' instruction. To assess intrinsic numbers of *Pneumocystis* primed cells secreting IL-5, IL-17, and IFN-γ in the DLN, without potentially expanding the population, cells were not restimulated with antigen. Spot-forming units were quantified.

Statistical analysis. Data were analyzed using GraphPad statistical software (GraphPad Software, Inc.). Comparisons between groups were made with the Mann-Whitney *U* test. Significance was accepted at a value of $P < 0.05$.

Online supplemental material. Fig. S1 shows that the specificity of anti-polysaccharide IgM is dependent on the variable chain. Fig. S2 shows the V_HDJ_H junctions of IgH genes expressed by high and low chitosan-binding B cells. Fig. S3 shows lung DC uptake of FITC-zymosan. Fig. S4 shows stimulated secreted IFN-γ, IL-4, and IL-17 in mediastinal LNs 14 d after *Pneumocystis* infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100034/DC1.

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