MECHANISMS OF ANTIBODY-BASED DEFENSE AGAINST *PNEUMOCYSTIS*

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University of Pittsburgh, 2008

Pneumocystis (PC) pneumonia is a life-threatening opportunistic fungal infection observed in individuals with severe immunodeficiencies, such as AIDS. This dissertation evaluates functions of antibodies and conserved fungal cell wall carbohydrate antigens in host defense against PC. We demonstrate that a novel recombinant protein consisting of the extracellular domain of the β-glucan receptor dectin-1 fused to the constant portion of murine IgG1, binds β-glucan and recognizes Fc-γ receptors; and functionally, impairs growth of PC in the lungs of immunocompromised mice. As an antibody-like molecule targeting a conserved fungal cell wall carbohydrate enhanced host defense against PC, we questioned whether the host produces similar antibodies. We identified natural IgM antibodies, conserved across species and not requiring microbial stimulation for production, that recognize fungal cell wall carbohydrates β-glucan and chitosan/chitin. In mice, naïve serum containing natural antibodies impairs the growth of PC organisms in the lungs at intermediate stages of infection, while at earliest stages limits pulmonary neutrophil recruitment. Mice unable to secrete IgM, sIgM(-/-), manifest similar impairments in pathogen clearance at intermediate stages of infection. Additionally, sIgM(-/-) mice demonstrate diminished trafficking of fungal cell wall carbohydrate antigen by CD11c+ cells to draining lymph nodes, impaired production of Th2 and Th17 cytokines in

lymph nodes after PC challenge, and altered adaptive antibody responses, with diminished anti-PC IgG1 (Th2 associated) while enhanced IgG2a (Th1 associated) adaptive antibody responses. Thus, sIgM, of which a significant component is natural antibody, influences PC Ag presentation at earliest stages of infection, enhancing host defense and biasing the host towards Th2 adaptive responses. Additionaly, we observe that β-glucan and chitosan/chitin are targets of induced antibody responses. PC challenge leads to the induction of specific serum IgG, and increased quantities of multiple isotypes at the lung mucosa against these carbohydrates. Mucosal responses against β-glucan and chitosan/chitin after PC challenge are regulated by CD4+ T cells, and we provide evidence that functional memory B cell responses against fungal wall carbohydrates are generated as a consequence of PC challenge. Collectively, these studies demonstrate the importance of conserved fungal cell wall carbohydrate antigens, and primitive antibody isotypes, in host defense responses against PC.

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1. INTRODUCTION

1.1 PNEUMOCYSTIS PNEUMONIA: HUMAN DISEASE & BASIC ASPECTS OF PNEUMOCYSTIS ORGANISMS

1.1.1 Epidemiology of Pneumocystis pneumonia

Pneumocystis pneumonia is a common, often fatal opportunistic infection observed in individuals with a variety of severe immunodeficiencies, either genetic or acquired. The etiologic agent underlying infection in humans, *Pneumocysis jirovecii* (previously named *Pneumocystis carinii* f. sp. *hominis*), is a fungal pathogen, with its primary mechanism of transmission and reservoir(s) poorly characterized. Multiple lines of evidence support the likelihood of person-to-person transmission, such as the observation that genotypically similar *Pneumocystis jirovecii* isolates are associated with cluster outbreaks among hospitalized immunocompromised patients, and the observation that cohousing *Pneumocystis* infected mice with uninfected mice leads to transmission of infection (1-3). However, *Pneumocystis jirovecii* nucleic acids have been detected outside of the mammalian host, such as in ambient air and in spore traps in rural locations (4, 5), suggesting that an environmental reservoir may exist for this opportunistic pathogen and may play a significant role in transmission. Regardless of the route of exposure, it is suspected that *Pneumocystis jirovecii* is encountered frequently by humans, as

most individuals possess antibodies against the fungal organism by three years of age, and rates of seropositivity continue to increase with age (6).

The incidence of *Pneumocystis* pneumonia has increased dramatically since the 1980's and the emergence of the global acquired immunodeficiency syndrome (AIDS) pandemic. In 1981, *Morbidity and Mortality Weekly Report*, Gottlieb *et al* described a case series of 5 young homosexual male patients in Los Angeles hospitals, over the span of seven months, presenting with *Pneumocystis* pneumonia, but without any known immunodeficiency (7). The authors postulated that the patients had a cellular immune dysfunction secondary to a sexually transmitted infection, which was later discovered to be caused by human immunodeficiency virus (HIV) infection, which at advanced stages of illness leads to AIDS and susceptibility to *Pneumocystis* pneumonia. The diagnosis of *Pneumocystis* pneumonia was later realized to be a strong indicator of progression to advanced stages of HIV infection and is now considered an AIDS-defining illness. The probability of the development of *Pneumocystis* pneumonia in individuals with HIV-AIDS is inversely correlated with quantities of CD4+ T cells in the blood, with a quantity of less than 200 CD4+ T cells/μL a major indicator of *Pneumocystis jirovecii* susceptibility, though dysfunction occurs within multiple cell types of the immune system, such as macrophages, CD8+ T cells, dendritic cells, and B cells (8-15). At the onset of the HIV pandemic in the United States, the incidence of *Pneumocystis jirovecii* was 20 per 100 person-years among individuals with a CD4+ T cell count below 200/μL (16). With the introduction of co-trimoxazole chemoprophylaxis to specifically prevent *Pneumocystis* pneumonia, and further with the development and widespread use of combined antiretroviral therapy (cART) to specifically inhibit HIV replication, a significant decline in the *Pneumocystis* pneumonia incidence was observed in populations where these treatments were available. As of 2001, the

incidence of *Pneumocystis* pneumonia among HIV infected individuals has declined to approximately 30 per 1000 person-years, though it still remains the most common serious opportunistic infection among individuals with HIV in Europe and North America (17, 18).

The population of HIV-infected individuals globally is estimated to range from 30 to 35 million (19), and 95% of those estimated to be infected are living in developing countries. Only an estimated 27-34% of patients who require antiretroviral therapy are actually receiving it (20), demonstrating the extraordinary limitation in accessibility of these medications and the need to develop novel strategies to combat opportunistic infections. While *Pneumocystis* pneumonia is not the most prevalent pulmonary opportunistic infection in developing countries, with tuberculosis and bacterial pneumonias often having a higher prevalence, *Pneumocystis* pneumonia is frequent. For example, in Thailand a *Pneumocystis* pneumonia prevalence of 27% to 40% was observed among HIV-infected individuals seen in a clinic setting, and it is reported to be one of the most prevalent opportunistic infections in the country (21, 22). In Kenya among acid-fast bacilli smear negative patients with chronic cough, *Pneumocystis* pneumonia was detected in the most patients, thereafter followed by bacterial pneumonia and Mycobacterial tuberculosis (23). Additionally, *Pneumocystis* pneumonia is particularly common in HIV-infected infants in developing countries, and is a frequent cause of death in these populations (24-26).

Besides HIV status, there are multiple other risk factors for *Pneumocystis* pneumonia. Primary immunodeficiencies leading to *Pneumocystis* pneumonia susceptibility include severe combined immunodeficiency, X-linked hyper-IgM syndrome, and Wiskott-Aldrich syndrome. Individuals taking chronic immunosuppressive medications for rheumatologic conditions or organ transplantation are a rapidly growing population of individuals at risk for *Pneumocystis*

pneumonia (27-30). Additionally at risk are individuals with malignancies, hematologic or otherwise, as well as patients receiving immunosuppressive chemotherapeutics for cancer treatment (31-33). The large population of individuals at risk for this illness makes critical a greater understanding of the immunologic processes underlying resistance and susceptibility.

1.1.2 Clinical features of Pneumocystis pneumonia

Symptoms of *Pneumocystis* pneumonia include shortness of breath, progressive exertional dyspnea, low-grade fever, non-productive cough, and chills. Patients often demonstrate an elevated serum lactate dehydrogenase, which is highly sensitive for infection, though of poor specificity. Early in the course of the illness the chest radiograph may be normal despite a widened alveolar-arterial O_2 gradient, however in most cases chest x-ray demonstrates diffuse bilateral infiltrates. Qualitative and quantitative differences exist in the *Pneumocystis* pneumonias experienced in the setting of HIV infection and in the absence of HIV infection, as summarized by Limper *et al.* (27). In the absence of HIV, *Pneumocystis* pneumonia is often associated with the abrupt onset of respiratory insufficiency, rather than a progressive indolent course as seen in the setting of HIV. Additionally, there are increased quantities of neutrophils and decreased quantities of *Pneumocystis* organisms in the bronchoalveolar lavage fluid (BALF) in non-HIV *Pneumocystis* patients. The mortality rate for patients with *Pneumocystis* pneumonia is higher in non-HIV patients compared to patients with AIDS and *Pneumocystis* pneumonia. Given the difference in the quality of the illness as a function of host predisposing factors, it has led many to speculate that the quality of the host's specific immunodeficiency significantly influences the course of the infection, and that prognosis is not simply a function of

the growth of *Pneumocystis* organisms within the lungs. Indeed, treatment with corticosteroids prior to initiating co-trimoxazole is aimed at inhibiting the host's inflammatory response against degraded *Pneumocystis* organisms, suggesting that the pulmonary pathology attributed to the infection is partially a function of an ineffective, overabundant host inflammatory process and that limitation of this process improves patient outcomes.

1.1.3 Limitations of therapeutic strategies against Pneumocystis pneumonia

Cotrimoxazole, pentamidine, and dapsone are preventative and therapeutic agents that inhibit the growth of *Pneumocystis* organisms. Specifically, cotrimoxazole, the primary agent used in prevention and treatment, targets the enzymes dihydropteroate synthase and dihydrofolate reductase which are key enzymes in the foliate utilization pathway in Pneumocystis. As *Pneumocystis* organisms cannot uptake folic acid from the environment, cotrimoxazole starves the opportunistic pathogen of nucleotides required for cell division. Similarly, dapsone targets dihydrofolate reductase, whereas the mechanism of pentamidine is unknown but possesses broad function against numerous protozoan parasites as well as *Candida* sp. Nonsynonymous mutations have been detected among *Pneumocystis jirovecii* isolates in the dihydrofolate reductase gene, and notably the presence of these mutations was correlated with cotrimoxazole prophylaxis failure (34). Additionally, the likelihood of dihydropteroate synthase mutations is correlated with prior use of dapsone or cotrimoxazole, and treatment of existing *Pneumocystis* pneumonia with cotrimoxazole failed more frequently in patients whose isolates had dihydropteroate synthase mutations than in those whose isolates had the wild-type gene (35, 36). These observations suggest the potential functional limitation of these medications in that the

widespread emergence of resistant organisms may occur, particularly given the global prevalence of this fungal organism, its long-term prophylactic use in many patients, and the widespread risk factor of HIV-AIDS infection. Of note, there are no immunotherapeutic strategies or vaccines in clinical use against any fungal pathogen.

1.1.4 Diversity of Pneumocystis species and limitations to their study

Pneumocystis sp. encompass a broad genus of organisms which display significant functional and genetic variation, with infection exclusive to mammals. The argument that *Pneumocystis* organisms isolated from different hosts are separate species relies solely on the observations of strong functional and genetic diversity between them. Functional diversity is exhibited at the level of host infectivity, as it has been demonstrated that *Pneumocystis* organisms isolated from one immunocompromised mammalian host are unable to establish infection in a host of a different species. For example, *Pneumocystis* isolates from immunosuppressed humans and ferrets are unable to infect immunosuppressed mice (37, 38). Additionally, functional diversity of *Pneumocystis* sp. is demonstrated at the level of antigen diversity. Mice challenged with *Pneumocystis* organisms from ferrets do not induce a protective antibody response in mice, whereas *Pneumocystis* organisms isolated from murine hosts do (39), suggesting that diversity exists at the level of immunoprotective antigens, with such diversity influencing the programming of the host adaptive immune response. Great genetic diversity between species is also observed. For example, significant sequence differences for genes encoding 5 different *Pneumocystis* isoenzymes, occurred between isolates from different hosts such as rabbits, rats, mice, and humans (40). In this study, the authors argue that population-genetic testing and

phylogenetic analysis demonstrate that *Pneumocystis* genotypes are host-specific, and that no genetic exchange occurs between isolates from different species at these loci. Additionally, the genomes of *Pneumocystis* mouse, human, rat, and ferret have been estimated to be 6.5, 7.0, 7.7, and 11 Mb of DNA, respectively, suggesting major host-species specific diversity at the genetic level (41).

A major limitation in understanding *Pneumocystis* organisms is the lack of a culture system that allows for propagation of organisms. Hence, it is only from analyzing isolated, concentrated organisms from infected lung tissue that our understanding of *Pneumocystis* organism biology, genetics, and immunology has progressed. Methods for assessing infection quantitatively, for assessing *in vitro* viability, as well as for cloning specific *Pneumocystis* genes have required more complex strategies compared to more standard methods used in the study of bacterial pathogens.

The lack of a culture system has severely curtailed our understanding of the organisms life cycle. While there are two predominant forms of Pneumocystis organisms observed in infected tissues, a large cyst with multiple intracystic bodies and a significantly smaller amorphic trophozoite, their interrelation is presumed given their appearance together in infected lungs. The current hypothesis suggests that trophic forms are generated from rupture of the cyst, with trophic forms later undergoing fusion to regenerate cyst forms. Cyst forms undergo a maturation process leading to increased numbers of intracystic bodies, leading to another cycle of cyst rupture. However, trophozoites are approximately 10 times more predominant in infected lung tissues, making it questionable as to whether the cyst form is required for continued infection. It is additionally unclear as to whether one form is infectious or both forms. However, neither of these controversies can be addressed with the existing knowledge and without a system allowing

for propagation of *Pneumocystis* organisms and the isolation of the distinct morphologic forms of the fungal pathogen.

1.1.5 Animal Models of *Pneumocystis* Pneumonia

Animal models of infection have provided significant insight into the pathogenesis of *Pneumocystis* pneumonia. Rats and mice are most often used in modeling *Pneumocystis* pneumonia, and have been useful in identifying important immune effector mechanisms in host defense. In wild type immunocompetent rodents, *Pneumocystis* pneumonia can be induced, peaking in mice around 3-4 weeks after infection, and is thereafter rapidly cleared, while in immunosuppressed rodents, infection continues to grow leading to death if allowed to continue. High quantities of *Pneumocystis* organisms can be isolated from the infected lungs of immunosuppressed rodents, such as athymic, SCID, RAG2 (-/-), or chronically corticosteroid treated hosts and used to infect other hosts, as well as used for direct study. While genetic and functional diversity between species may make questionable the extrapolation between them in the analysis of protective immunologic responses, rodent models can be made susceptible to *Pneumocystis* infection by many of the same factors that lead to susceptibility in humans, suggesting that basic host defense mechanisms are similar and when breached bear similar consequences. For example, the SCID mutation in humans, the CD40L mutation leading to the X-linked hyper-IgM syndrome, and chronic corticosteroid use leads to susceptibility to *Pneumocystis* infection in humans and in mice. Chronic CD4+ T cell depletion in mice using monoclonal antibodies targeting CD4, mirroring the CD4+ T cell depleted status of individuals in late stages of AIDS, also leads to *Pneumocystis* susceptibility in mice and rats (42-44).

Pneumocystis isolates from these different mammalian hosts also produce a qualitatively similar, slowly progressing, focal or diffuse pneumonia involving masses of *Pneumocystis* trophozoites clustered in airspace filling eosinophilic exudates, single or clustered *Pneumocystis* cysts identifiable with Gomori-methenamine silver stain, and thickened alveolar septa; without systemic dissemination of infection (45). Additionally, infection in rodent and human hosts leads to elevated serum lactate dehydrogenase, significantly decreased P_aO_2, and pulmonary neutrophilia at late stages of disease.

More recently, a primate model of *Pneumocystis* infection has been established, whereby simian immunodeficiency virus (SIV) infected macaques inoculated with *Pneumocystis* organisms experienced an often prolonged period of organism colonization followed by development of infection, coiniciding with significantly enhanced CD8+ T cells and neutrophils recruited into the lungs, while other macaques become colonized but developed resistance to infection (46). This model may be of particular benefit in studying HIV associated *Pneumocystis* disease.

1.1.6 Summary

Pneumocystis pneumonia is seen in states of immunodeficiency, and has emerged as a major medical problem as a consequence of the global AIDS epidemic and the number of individuals that are immunosuppressed secondary to treatment of cancer, autoimmune disease, and organ transplantation. While a slow-growing opportunistic fungal infection, disease is often lethal, and current chemotherapeutic strategies show evidence for the potential development of widespread resistance. There are no immune therapies or vaccines in clinical use against fungal

organisms, and little precedent in design for these strategies in the immunosuppressed. The *Pneumocystis* genus consists of multiple species with great genetic and functional diversity, with various species displaying a host-specific propensity. Rodent models are an attractive system for the study of immunity to *Pneumocystis*, as clinically and histologically the pneumonia experienced is similar, and susceptibility phenotypes in rodents, such as the CD40L mutation or chronic corticosteroid immunosuppression, also lead to disease in humans.

1.2 HOST DEFENSE AGAINST PNEUMOCYSTIS

1.2.1 Role of innate immune defenses against *Pneumocystis*

The adult human respiratory tract contains an estimated 70 m^2 of space in direct contact with external environment, and antigens are continually exposed to this surface. Host defense against fungal pathogens involves the interplay of multiple immune responses, including innate or preexisting defenses that are programmed into the host prior to antigen exposure, as well as adaptive or induced responses which are selected as a consequence of antigen (Ag) exposure. When *Pneumocystis* organisms are inhaled into the lungs, multiple innate defense mechanisms are involved in antigen recognition, degradation, and clearance. Non-specific mechanical defenses such as mucus trapping and mucocilary transport may inhibit establishment of infection by confining *Pneumocystis* organisms and eliminating them from the distal lung, as has been demonstrated for various bacterial and viral pathogens. Of note, 90% of the resident cells in the airways are macrophages, and these alveolar macrophages are critical in binding, internalizing, and degrading *Pneumocystis* organisms, likely acting at the very earliest points after

Pneumocystis inhalation. Significantly impaired clearance of *Pneumocystis* from the lungs is observed in rats depleted of alveolar macrophages, and clearance of one of the most predominant Ags at the surface of *Pneumocystis*, msgA was also dramatically decreased (47). Mannose receptors, β-glucan receptors, scavenger receptors and toll-like receptors (TLRs) are all found on alveolar macrophages and are implicated in affecting processes of recognition, internalization, and inflammatory responses against *Pneumocystis* (48-54). While superoxide dismutaion or the impairment of nitric oxide production did not affect alveolar macrophage effector function against *Pneumocystis in vitro*, catalase inhibited *Pneumocystis* killing by macrophages, suggesting that hydrogen peroxide-dependent killing mechanisms are directly involved in the degradation and killing of *Pneumocystis* organisms (55).

Dendritic cells are also resident cells of the lung, which have a central function in trafficking antigen from tissue sites towards secondary lymphoid organs, such as pulmonary lymph nodes. In the lung, myeloid DC predominate over plasmacytoid DC populations (56). It has been demonstrated that administration of bone marrow derived, CD40L expressing, DCs pulsed with *Pneumocystis* Ags leads to the induction of protective B cell responses against these Ags, underscoring the role of DC in initiating and guiding adaptive immune responses (57). It has also been shown that pulsing DC with *Pneumocystis* leads to the elicitation of IL-4, with very little IL-12 or TNF-α production (58), which could potentially influence Th1 vs Th2 polarity determination in T cell priming.

Neutropenia is a major susceptibility factor for infectious disease caused by variety of fungal pathogens including *Aspergillus fumigatus*, *Candida glabrata, Candida albicans,* and *Fusariam solani*, yet susceptibility to *Pneumocystis* is not directly correlated with neutropenia. In *Pneumocystis* infection, neutrophil influx into the airspaces appears to occur at late stages of

infection, in the setting of high pathogen burden, with timing coinciding with lung pathology (59-61). Some data partially suggest that these cells may not be required for host defense against *Pneumocystis*. For example, mice deficient in gp91(phox), required for NADPH oxidase function, mice deficient in CXCR2 which have impaired neutrophil chemotaxis to sites of infection, and mice partially depleted of neutrophils with a monoclonal antibody have similar quantities of *Pneumocystis* in the lungs relative to controls (62). Neutrophils recruited into the airways presumably bind and phagocytose *Pneumocystis* using a similar array of pattern recognition receptors, though there have been no studies evaluating neutrophil innate recognition of *Pneumocystis*.

Epithelial cells may also be involved in some of the earliest responses against *Pneumocystis*. It has been demonstrated that β-glucan isolated from the *Pneumocystis* cell wall can stimulate airway epithelial cells to secrete MIP-2, the rodent homolog of IL-8 and major neutrophil chemoattractant, by signaling at lactosylceramide receptors (63). Additionally, MCP-1 is produced by epithelial cells upon exposure to *Pneumocystis* (64). These data suggest that the epithelium is a critical sensor of infection, and may be involved in the very earliest stages of leukocyte recruitment after *Pneumocystis* exposure.

Finally, multiple proteins that are part of the normal pulmonary environment have affinity for *Pneumocystis* and influence innate recognition and defense pathways. It has been demonstrated that fibronectin and vibronectin, serum proteins but also synthesized by alveolar macrophages, bind *Pneumocystis* organisms and enhance their adhesion to the airway epithelium, with vitronectin additionally enhancing adhesion to alveolar macrophages (65-67). Through opsonization, both fibronectin and vitronectin have been demonstrated to enhance the alveolar macrophage TNF-α response against *Pneumocystis* (68). It was later demonstrated that

these proteins bind *Pneumocystis* cell wall β-glucan, and that quantities of these proteins increase in the course of infection in an IL-6 dependent manner, IL-6 in turn released by alveolar macrophages in response to *Pneumocystis* (69). Additionally, surfactant proteins A and D, secreted by type II pneumocytes of the lung and members of the collectin family of proteins, have been demonstrated to accumulate in the lungs during infection, bind *Pneumocystis* organisms and differentially affect their clearance. Mice deficient in surfactant protein A expression significantly limit *Pneumocystis* infection, whereas transgenic mice overexpressing surfactant protein D have enhanced growth of *Pneumocystis* (20, 70, 71). It has been demonstrated that surfactant protein A and D specifically bind the oligosaccharides associated with the *Pneumocystis* glycoprotein msgA, leading to enhanced interaction of *Pneumocystis* with alveolar macrophages (72, 73). Yet, the specific cellular consequences resulting from increased macrophage adhesion of these protein-*Pneumocystis* complexes, for both surfactant protein A and D, leads to impairment of alveolar macrophage phagocytosis *in vitro* through unknown mechanisms (74, 75). Additionally, it is currently unclear if surfactant coated *Pneumocystis* is recognized by other cells, such as neutrophils or dendritic cells. It is intriguing that these similarly structured proteins share a common affinity for *Pneumocystis* glycoprotein Ag, have a similar function of recruiting *Pneumocystis* to macrophages, impair phagocytosis, yet surfactant protein A has a net contribution to enhancing clearance, while surfactant protein D appears to impair clearance .

1.2.2 Role of CD4+ T cells and CD8+ T cells in host defense against Pneumocystis

T cell functions represent a central component of the adaptive immune response to a variety of fungal pathogens. Declining absolute quantities of CD4+ T cells leads to increased risk of esophageal candidiasis, and CD4+ T cells of a Th1 instead of Th2 profile are thought to have a major influence on the outcome of invasive *Aspergillus* infection (76-79). The risk of *Pneumocystis* pneumonia increases exponentially with decreasing quantities of CD4+ T cells below 200/μL, underscoring the critical functional role of CD4+ T cells in host defense against infection (80). Indeed, mice deficient in CD4+ T cells, either chronically depleted of CD4+ bearing cells with a monoclonal antibody targeting the CD4 surface protein (42, 43), or mice genetically engineered to be lacking in CD4+ T cells, as in the MHC II KO or H-2I-Aβ KO mice (81), are susceptible to the development of *Pneumocystis* pneumonia in contrast to wild-type which are not. Some data suggest that reconstitution of SCID mice with CD4+ T cells is sufficient for clearance of an existing infection, though the reaction is rigorous and is associated with excess inflammatory infiltrates in the lung, with many of the mice dying in the course of infection clearance (82). Other findings suggest that SCID reconstitution must occur with CD4+ T cells from hosts previously exposed to *Pneumocystis* or with naïve CD4+ T cells cotransferred with naive B cells for significant decreases in *Pneumocystis* burden in the lungs (83, 84). As a consequence, it is hypothesized that naïve CD4+ T cells must interact with B cells, with the B cells acting as essential antigen presenting cells in the generation of protective effector T cells as well as memory responses to *Pneumocystis*.

The functions of primed, specific, effector CD4+ T cells against *Pneumocystis* organisms, which recognize *Pneumocystis* infection in the context of MHC II recognition on professional

antigen presenting cells, are unclear. Alveolar macrophages play a central role in the clearance of a *Pneumocystis* challenge, as demonstrated by studies where these cells were depleted with liposomal clodronate in rats, and clearance of *Pneumocystis* organisms was significantly inhibited (47). It is currently hypothesized that primed effector CD4+ T cells migrate into the lungs and interact with alveolar macrophages so as to enhance their effector function against *Pneumocystis*, probably involving the functions of the central cytokines in host defense against *Pneumocystis* such as IL-1α and TNF-α released by the macrophages. IL-1R blockade leads to an inability to clear *Pneumocystis* from the lungs (85), and neutralization of TNF-α dramatically inhibits clearance of *Pneumocystis* in the murine model (86), suggesting that these cytokines play a critical role in a major effector pathway against *Pneumocystis*. IFN-γ likely also plays a role in the activation of macrophages and has been demonstrated to enhance killing of *Pneumocystis* by macrophages *in vitro* (87) but it has been established not to be required for the clearance of infection (86, 88), suggesting that it is not an essential cytokine required for CD4+ T cell effector function against *Pneumocystis*. However, as exogenous IFN-γ enhanced clearance in mice depleted of CD4+ T cells (89), it suggests that this cytokine may have a redundant yet beneficial function in enhancing host clearance of *Pneumocystis*.

CD8+ T cells are not essentially required cells in host defense against infection, as demonstrated in the murine model. SCID hosts transferred with CD8+ T cells did not influence the clearance of *Pneumocystis* organisms in the lungs (82), and β2-microglobulin (-/-) mice, deficient specifically in CD8+ T cells, are fully resistant to infection (81). Interestingly, mice depleted of CD4+ T cells alone had significantly higher physiologic and immunologic correlates of worsening pneumonia, such as increased respiratory rate and increased BALF albumin, compared to mice depleted of both CD4+ T cells and CD8 + T cells, suggesting that the CD8 + T

cell subset actively contributes to pulmonary damage (60). However, others have demonstrated that the CD8+ T cell subset has two effector populations against *Pneumocystis*, Tc1 and Tc2, the former of which is significantly induced by exogenous IFN-γ, expresses CXCR3, and has effector functions that lead to *Pnemocystis* clearance when transferred into SCID mice infected with *Pneumocystis* (90). Particularly, in the setting of CD4+ T cell depletion, but not in SCID mice, exogenous IFN-γ expression skewed production of CD8+ T cells expressing IFN-γ, and significantly enhanced their recruitment into the lungs (89). Thus, it appears that the CD8 + T cell population may be manipulated to enhance clearance of *Pneumocystis* but that it also may significantly contribute towards disease pathogenesis.

1.2.3 Role of B cells in host defense against Pneumocystis

The murine model of *Pneumocystis* infection has demonstrated that B cells are required for host defense against pneumonia. Mice with a transgenic mutation in the transmembrane exon of the IgM μ heavy chain gene arrest B cell development at the pre-B cell stage, leading to a lack of antibody and an absence of mature B cells in the periphery. Notably, these mice are highly susceptible to *Pneumocystis* pneumonia (91). While it has been argued that B cells are required for the development of gp38 expressing stromal cells of the splenic T cell areas, and that this may result in a T cell defect precluding effective clearance, this consideration has been ruled out with the demonstration that irradiated, reconstituted μMT mice transferred with bone marrow

from wt mice clear the infection whereas recipients of bone marrow from μMT mice do not (84). Hence, the absence of B cells prevents the clearance of *Pneumocystis*.

B cells have been demonstrated to play multiple roles in host defense against *Pneumocystis* infection. Hyperimmune serum from wild type mice repeatedly challenged with *Pneumocystis*, can significantly clear infection upon transfer into SCID infected hosts, notably without the hyperinflammatory response observed with transfer of CD4+ T cells (82). It has additionally been shown that vaccination by presentation of CD4+ T cell depleted mice with CD40L expressing dendritic cells loaded with *Pneumocystis* Ags produces protective antibodies, and more recently this observation has been extended to DNA based vaccination against Kexin, a specific *Pneumocystis* antigen that is a surface associated protease (57, 92). Here, adoptive transfer of B cells or serum from vaccinated hosts prevented *Pneumocystis* growth in SCID hosts challenged with *Pneumocystis*. Monoclonal antibodies, of primarily the IgG isotype, but also of the IgM isotype, derived from B cells from *Pneumocystis* challenged mice, have been shown to provide protection against infection (93, 94). More recently, it has been demonstrated that the IgG1 monoclonal must function in an environment containing an intact complement pathway and utilizes its Fc portion for optimal prophylaxis (95).

In attempting to dissect the specific requirement of B cells in host defense against *Pneumocystis*, one group using μMT mice reconstituted with chimeric bone marrow, demonstrated that antibody-secreting function is not required for host defense against *Pneumocystis* (96). However, mice deficient in FcγRI and FcγRIII had delayed kinetics of clearance, suggesting that IgG antibodies directly enhance the rate at which *Pneumocystis* infection is cleared, presumably by increasing numbers of immune complexes and enhancing *Pneumocystis* opsonization and phagocytosis by cells of the innate immune system. Of note,

serum from vaccinated mice is able to potentiate the function of alveolar macrophages against *Pneumocystis in vitro* (55).

Additionally, it is postulated that B cells, when appropriately primed can maintain host defense against *Pneumocystis* in the absence of CD4+ T cells. Mice challenged with *Pneumocystis* and thereafter depleted of CD4+ T cells are able to resist a subsequent challenge with *Pneumocystis*, and this resistance correlates with the presence of high IgG1 titers against *Pneumocystis* antigens, which actually appear to enhance through the secondary challenge (97). Mice not receiving Pneumocystis challenge prior to CD4+ T cell depletion, however, succumbed to *Pneumocystis* infection, suggesting that CD4+ T cells can program sufficient memory effector mechanisms against *Pneumocystis* that are functional in the event of future absence of CD4+ T cells. It was later additionally demonstrated that the induction of particular antibody isotypes is not a major contributor to this phenomenon, as IL-4 (-/-) mice develop a Th1-type antibody isotype response and IFN-γ (-/-) mice develop Th2-type antibody isotype response, and both are equally protective upon secondary challenge with *Pneumocystis* in the setting of CD4+ T cell depletion (98). These studies initially underscore the requirement for CD4+ T cells in the development of B cell antibody responses that are sufficient for protecting the host from *Pneumocystis.* Zheng *et al*, however demonstrate that a vaccination strategy incorporating exogenous CD40L and a gene encoding a specific *Pneumocystis* Ag also results in the production of B cells secreting Abs against protective *Pneumocystis* Ags that can be transferred to SCID hosts and result in protection (57, 92). What remains unclear is whether actual memory B cells are generated against *Pneumocystis* after exposure, whether they require CD4+ T cells for their functions, such as reactivation in the presence of Ag, and further, whether non-CD4+ T cell dependent antibodies play a role in host defense against *Pneumocystis.*

Additionally, it is unclear which B cell subsets are involved in anti-*Pneumocystis* responses, and whether these subsets have distinct roles in host defense.

Finally, B cells have been demonstrated to play a critical role in the maintenance of bone marrow hematopoeisis after *Pneumocystis* challenge, and this functionality partially involves an intact type I IFN receptor signaling pathway on B cells (99). This observation, coupled with the finding that B cells are critical APC's in priming T cell responses (84) , highlight the potentially central involvement of B cells in important host defense mechanisms besides antibody production, which for example, could additionally include cytokine and chemokine production, as elegantly demonstrated by Lund et al in other infection models (100).

1.2.4 Summary

Utilizing murine and rodent models of *Pneumocystis* infection, some essential and contributory immune defenses are characterized. These include identification of resident alveolar macrophages as essential effector cells in *Pneumocystis* recognition and clearance, the requirement for CD4+ T cells and B cells in host defense mechanisms, the utility of antibody based recognition of *Pneumocystis*, and the ability to skew CD8+ T cell subsets leading to differential function in host defense against Pneumocystis but also their implied role in lung damage and correlates of clinically severe *Pneumocystis* pneumonia. However, much remains to be unraveled, such as the specific CD4+ T cell effector pathways leading to immunity, the specificities of and the diversity of the B cell antibody responses that contribute to protective immunity, as well as the interplay between specific protein, carbohydrate, and lipid antigens of *Pneumocystis* and the multivalent recognition systems provided by the innate and adaptive

immune system. A greater understanding of these pathways will provide a better understanding of how healthy individuals are so successful in avoiding *Pneumocystis pneumonia*, how immunodeficiencies specifically affect these defense mechanisms, and how to prevent and treat *Pneumocystis* infection in the setting of immunodeficiency.

1.3 PNEUMOCYSTIS FUNGAL CELL WALL CARBOHYDRATES

1.3.1 Conserved fungal cell wall carbohydrates

Fungal organisms, of which there are an estimated 1.5 million different species, of tremendous diversity in habitat and life cycle (101), contain cell walls that are remarkably similar. The function of the extracellular fungal cell wall is multiple, it provides structure and shape to the organism, it provides rigidity, it prevents against osmotic stress, it contains hydrolytic enzymes or other proteins which assist in aspects of environmental adaptation, and notably it is continually remodels itself in the face of growth or stress. It is estimated that approximately 90% of the fungal cell wall consists of polysaccharides, and at the very core of this cell wall, for almost all fungal organisms exist two carbohydrates, β-glucan and chitin (102). β-glucan in fungal cell walls is a polymer of glucose that is β-1,3 linked predominantly, with branching β-1,6 linkages. The length of the typical β-1,3 glucan polymer ranges on the order of 1,500 units per chain, and structurally it is thought to resemble and function a coiled chain (103). β-1,6 linked glucan, with an average of 350 units per chain, is more amorphous and functionally provides adhesive and flexibility properties (103). Chitin is often linked to β-glucan chains via β-1,4 linkages. Chitin is a linear polymer of N-acetyloglucosamine, linked in a β-1,4 manner. It

forms microfibrils which orient in alternating parallel and antiparallel strands, with the typical strand length of 100-190 units (103). Chitosan, the deacetylated form of chitin, also found in fungal cell walls, is a polymer with much greater flexibility than chitin (104-108).

There is commonly variation on top of this central core of carbohydrate. For example, in the most studied fungal organism, *Saccharomyces cervesiae*, the cell wall represents 30% of the cells dry weight; β-glucan is estimated to consist 80-90%, chitin only 1-2%; and a third carbohydrate, mannan, at 10-20% (103). The bulk of the cell wall, β-glucan , plays a critical role in maintaining yeast structure, and degradation of this layer with an enzyme that hydrolyzes β-1,3 glucan linkages can lead to cell death secondary to osmotic stress. Together with chitin, β-glucan forms the inner cell wall and is termed the "electron lucent layer" as a function of its appearance under scanning electron microscopy. Chitin, while constituting a small percent of the total carbohydrate presence, plays significant role in the process of cellular reproduction. Chitin and chitosan accumulate initially where the daughter bud is forming (102, 109). These carbohydrates are then laid down linearly to generate the septum between mother and daughter cell. After cytokinesis, chitin is degraded by chitinases, though a bud scar of chitin remains at the surface of mother and daughter cell. In *S. cervesiae*, mannan is found at the very exterior portion of the cell wall, composing the "electron dense" layer. Mannan fibrils are formed in concert with protein synthesis. Cell wall proteins can be O-mannosylated with short chains of α-mannose averaging 5 units in length, containing α-1,2 and α-1,3 linkages. These short mannan fibrils bury into the β-glucan core, strengthening the cell wall (103). Additionally, cell wall proteins can be N-mannosylated, containing up to 50 α-1,6 mannan units per fibril, with multiple short α-1,2 and α-1,3 mannan branches, resulting in a mannose polymer of up to 200 units (103). Mannoproteins can be dissolved without disruption of the cell wall (110). As the *S. cervesiae*

cell wall is the best characterized among fungal cell walls, it is a useful model for comparison in the understanding of the exterior surface of the somewhat atypical fungal organism *Pneumocystis*.

1.3.2 Carbohydrates of the Pneumocystis cell wall: Organization and Function

While *Pneumocystis* sp. share many features with both protozoa and fungi, *Pneumocystis* organisms are classified within the fungal kingdom as a consequence of genetic analysis of the mitochondrial 16s rRNA subunit and noting significant sequence homology with other fungal pathogens. In line with its classification as a fungal organism, *Pneumocystis* contains the β-glucan and chitin core in its cell wall as described, as well as a layer of mannoprotein, and these are observed in all *Pneumocystis* species. An electron dense layer, as in *S. cervesiae*, is observed in both the cyst and trophozoite forms of the organism, whereas the electron lucent layer is only found in the cyst form (111). Aniline blue, a dye which selectively stains β-1,3 glucan, stains the inner cell wall of the *Pneumocystis* cyst (112). Additionally, treatment of the *Pneumocystis* cyst with zymolase, which has enzymatic function against primarily against β-1,3 linked glucans, leads to loss of the electron dense and electron lucent portions of the cell wall, as well as a loss of Aniline Blue staining. Notably, staining with the Gomori Methenamine Silver stain which is used in the diagnosis of human *Pneumocystis* pneumonia, is also lost from cysts with zymolase treatment. Using an antiserum raised against laminarin, a β-1,3 rich beta glucan isolated from brown algae, it was observed that the electron lucent layer of the *Pneumocystis* cyst stained brightly, and that staining was absent in intracystic bodies and trophozoite forms of the organism (113). Truly underscoring the relevance of this carbohydrate in *Pneumocystis* viability,

inhibitors of glucan-synthetase, the enzyme responsible for β-glucan synthesis, have been shown to prevent and to significantly inhibit *Pneumocystis* growth in rodent models (114, 115) though there is controversy in this effect.

Chitin has also been identified in the *Pneumocystis cell* wall by immunostaining and substrate specific binding studies (116). It is found in the cyst cell wall and, interestingly, surrounding intracystic bodies. Additionally, it is found in the trophozoite. Beyond identification of its presence in both life stages of the organism, the function of chitin in *Pneumocystis* biology is unclear. Of note, chitin is typically not found in the trophozoite stage of various protozoa. Chitin synthesis inhibitors have not been tested in host defense against this infection, so its requirement for the life cycle of the organism is unknown.

Mannoprotein is present at the very exterior of the cell wall of the *Pneumocystis* cyst, which is also the site of the well studied antigen gpA/Major Sruface Glycoprotein (73, 117). gpA is among the most abundant proteins at the surface of *Pneumocystis* and its association with mannan is well described. While no studies have been performed to immunolocalize mannan, given its association with the surface associated gpA, and the fact that the mannose receptor binds gpA, mediated specifically through interactions with mannan (48), and the fact that gpA is found on both cyst and trophoizoite forms of the organisms (118), these observervations collectively suggest that mannan is found at the very surface of the cell wall of cyst and trophozoite forms. Benanomicin A, a compound known to directly bind mannan in the cell walls of fungi, when used to treat *Pneumocystis* infection in nude mice, significantly reduced cysts in the lungs and improved survival (35). This data suggests that freedom of mannan moieties is essential for the persistence of *Pneumocystis* organisms in the lungs.

Collectively these studies underscore the conserved nature of fungal cell wall carbohydrates as the localization, structure, and composition between *S. cervesiae* and *Pneumocystis* sp. are similar. Additionally, the utility of targeting *Pneumocystis* carbohydrates in chemotherapeutic strategies suggest that immune-based strategies against these carbohydrates may be of benefit.

1.3.3 Beta-glucan in immune responses

β-glucan has long been noted for its anti-infective and anti-tumorigenic properties to activate leukocytes, though its mechanisms of action are poorly characterized. Notably, β-glucan is not synthesized by animals, and these carbohydrate structures are truly pathogen associated molecular patterns, found not only in fungi, but plants and certain bacteria. There is evidence that β-glucans activate macrophages, neutrophils, and NK cells, leading to the phagocytosis, and release of cytokines and anti-microbial molecules (119-121). Multiple pattern recognition receptors are implicated in the identification of β-glucans, such as complement receptor 3 (CR3), dectin-1, scavenger receptors, and lactosylceramide (122). Additionally, it has been demonstrated that β-glucans prime CR3 expressing cells to improve detection of complement coated targets such as tumor cells targeted by natural antibodies (123, 124). As well, infections are modulated by β-glucans. For example, a *Trypanosoma cruzi* vaccine had significantly enhanced efficacy when coadministered with β-1,3 glucan (125). While activation of innate cells are surely part of these responses, the pathways likely involved in enhancing intrinsic functions of the innate, and potentially adaptive, immune responses are in need of greater characterization. For example, how does activation of innate cells change their effector

functions against antigens the host was previously blind to? Are B or T cells activated by β-glucans? What are the contributions of serum components in the recognition of β-glucans?

1.3.4 Mannan in immune responses

Mannan, a sizable polymer in fungal walls and distinct from the relatively low level of mannosylation observed on proteins from mammalian cells, is also immunogenic. Multiple studies have demonstrated that mannosylated proteins have enhanced MHC I and MHC II protein Ag presentation and increased T cell activation and proliferation against particular protein Ags relative to protein Ag alone (126, 127). It has recently been demonstrated that mannosylated Ag traffics through C-type lectin recognition pathways, such as the mannose receptor and DC-SIGN, leading to differential activation of dendritic cells and stronger activation of T cells (128, 129). Additionally, soluble proteins such as surfactant A and D, as well as mannose-binding lectin, recognize mannan. TLR2 has affinity for lipomannan, but only mannan of sufficiently long chain length (130). It is unclear whether mannan is itself recognized by components of the adaptive immune system. As some mannosylation of mammalian patterns is somewhat similar to that seen in fungal organisms, it is unclear whether mannosylation is used by fungi as a host evasion strategy.

1.3.5 Chitin in immune responses

Chitin is one of the most abundant carbohydrate polymers in nature, and is found in the exoskeletons of insects and crustaceans and in the sheaths of various parasites. The biosynthetic

machinery for making chitin, and the structurally similar deacetylated form chitosan found in fungal cell walls, is absent in mammalian cells, making chitin a non-self antigen upon encounter. An ancient, preserved defense mechanism against chitin is the degradation of chitin by chitinase enzymes of the host, and in humans, the Acidic Mammalian Chitinase (AMCase) protein has been described, with such function. Of note, AMCase is induced as a consequence of Th2 responses, in an IL-13 dependent manner, and contributes to the signaling pathway of IL-13 and Th2 inflammation (131). Interestingly, it has also been shown that chitin administration into the airways of mice leads to the recruitment of arginase expressing alternatively activated macrophages, basophils, and eosinophils (132). Of note, pre-treatment of chitin with AMCase inhibited these responses, suggesting that the specific pathogen associated molecular pattern chitin activates recognition pathways leading to the recruitment of these cells. These studies underscore the observations that chitin is a potent molecular pattern with the capacity to interact with host degradation proteins such as AMCase and the balance of these molecules may influence the host Th1 vs Th2 response. From another perspective, chitin and chitosan are being evaluated as vaccine adjuvants, for their intrinsic ability to stimulate the host immune response. For example, formulation of CRM197-Diphtheria toxin with chitosan enhanced antibody responses and Th2 responses such as the production of IL-5 by splenocytes restimulated with vaccine, relative to CRM197-Diphtheria toxin as standardly prepared in formalin (133). Other groups have shown that both Th1 and Th2 responses are stimulated with chitosan-based vaccine preparations (134-136). The observation that chitosan is an effective adjuvant may be partly attributed to its physical features in enhancing antigen deposition, but its capacity to skew responses towards Th2 suggests that it is recognized by specific components of the host's immune system. Additionally, the observation that chitin inhalation leads to the accumulation of

innate cells such as eosinophils and basophils, and that alternatively activated macrophages are generated suggest that sensors, such as innate receptors, or soluble proteins, with the capacity to do more than degrade signal may be involved in chitin recognition. It is unclear how AMCase signals, besides requiring IL-13 for its induction and downstream activities, and actually functions to influence the inflammatory response. Is it the first sensor of chitin? Currently there are no membrane bound pattern recognition receptors or soluble opsonic proteins discovered with specificity for chitin or chitosan. Only uncovered thus far in chitin recognition are the enzymes chitinase and chitotriosidase, the former expressed by airway epithelial cells and macrophages, the latter expressed by professional and released by phagocytes. It is likely that other host molecules participate in identifying this abundant environmental Ag.

1.3.6 Summary

Despite the tremendous diversity of fungal organisms, almost all contain three major conserved carbohydrates in the extracellular matrix known as the cell wall. The cell wall structure is dynamic, and provides structure, organization, and essential functions to the cell. The bulk of the cell wall consists of β-1,3 glucan intermeshed with chitin which is together covered with a thin layer of mannoprotein. *Pneumocystis* organisms are classified as fungi, and fitting with this classification is the presence of the conserved fungal cell wall carbohydrates, β-glucan, chitin, and mannan in various forms of the organism. Notably, the cyst form contains β-glucan, chitin, and mannan in a similar pattern to the *S. cervesiae* cell wall, with chitin notably lining intracystic bodies, whereas the trophozoite form contains only chitin and mannan. Of note, the fungal cell wall carbohydrates found in fungi, by themselves, and in concert with protein Ag, possess great

immunostimulatory capacity. However, data regarding their innate recognition and contribution to host defense pathways, as well as allergy, are slowly emerging. Of these, it is believed that β-glucan is recognized by a wide array of membrane bound PRR as well as soluble proteins, and one mechanism whereby it contributes to anti-tumor/anti-infective responses is to prime macrophages in the recognition of Ab-coated targets. Mannoprotein has been shown to directly enhance priming of T cells relative to T cells alone, and is recognized by a variety of pattern recognition receptors. Finally, chitin has no known traditional membrane-bound pattern recognition receptors, though it appears that enzyme based signaling may contribute to its recognition, and it appears to promote Th2 and antibody responses. The immunostimulatory properties of these fungal cell wall carbohydrate antigens suggest that multiple mechanisms contribute to their recognition and how they potentially influence adaptive immune responses.

1.4 DECTIN-1 RECEPTOR IN RECOGNITION OF *PNEUMOCYSTIS* BETA-GLUCAN AND HOST DEFENSE AGAINST *PNEUMOCYSTIS*

1.4.1 β-glucan recognition

β-glucan is a major component of the cell wall of the *Pneumocystis* cyst and has come under scrutiny as a molecule recognized by innate host defenses. A crucial component in *Pneumocystis* host defense is the resident alveolar macrophage, and early on, cell wall β-glucan was implicated as a molecular pattern mediating macrophage recognition and downstream effector functions against *Pneumocystis* organisms. For example, binding of *Pneumocystis* organisms and the release of TNF-α from alveolar macrophages can be blocked by pre-

incubation with soluble β-glucans (52). Additionally, administration of isolated *Pneumocystis* β-glucan into the lungs of rats provoked the production of TNF-α and the recruitment of neutrophils into the lungs (137). Thus, there appeared to be a pattern-recognition receptor or set of recognition molecules with specificity for β-glucan that promotes inflammation, cytokine and chemokine production. It was recently determined that the dectin-1 receptor is the primary receptor on alveolar macrophages for the recognition of *Pneumocystis* β-glucan, and it is involved in recognition and the initiation of signaling cascades leading to the production of reactive oxide species involved in organism death. (49, 138).

1.4.2 Dectin-1 receptor: Molecular biology, signaling, and recognition

The dectin-1 receptor (dendritic-cell-associated C-type lectin-1) is a recently cloned, small, type II, C-type lectin receptor with a single carbohydrate recognition domain, and a single transmembrane portion, first reported by Ariizumi et al (139). It has two N-glycosylation sites on the lectin portion of the receptor, and it contains an immunoreceptor tyrosine-based activation motif (ITAM) towards its N-terminus, which is involved in signaling. It was later shown that dectin-1 based signaling additionally involves the spleen tyrosine kinase (Syk) which is specifically involved in mediating macrophage reactive oxygen production, and influencing the differential production of cytokines from DCs (140, 141). Additionally, Card9 has been identified as a key signaling transducer of dectin-1 that is involved in myeloid cell activation and cytokine production, coupling with Bcl-10 and Malt1 to influence NF-κB activation (142).

Dectin-1 is expressed by a number of cells, including dendritic cells, monocytes, and T cells, with greatest levels of expression in neutrophils and macrophages (143). It is particularly

enriched in lung tissue and the liver (144). Of note, expression of the receptor seems to be influenced by the Th environment, as upregulation of the dectin-1 receptor occurs in the presence of Th2 cytokines such as IL-4 and IL-13, while LPS and dexamethasone suppress dectin-1 expression (143).

The dectin-1 receptor has greatest affinity for β-1,3 and β-1,6 linked glucans, as these carbohydrates most significantly inhibited binding of zymosan by NIH3T3 cells exogenously expressing dectin-1 (144). Of note, dectin-1 has no apparent reactivity with any monosaccharides, demonstrating that it is the large structural polymer and the specific arrangement generated by the glycosidic bond that generates the reactive β-glucan antigen. Additionally, it was demonstrated that dectin-1 mediates non-opsonic phagocytosis, as NIH3T3 cells overexpressing dectin-1 could internalize zymosan particles and heat-killed *Candida*. Hence, the dectin-1 receptor has the capacity to preferentially recognize the predominant carbohydrate in fungal cell walls, β-1,3 glucan, and initiate signaling pathways promoting phagocytosis.

1.4.3 Dectin-1 Receptor and Host defense Against Pneumocystis

The dectin-1 receptor has an integral role in macrophage recognition of *Pneumocystis* as demonstrated utilizing *Pneumocystis* isolated from mice. Blockade of the dectin-1 receptor with a mAb targeting the dectin-1 carbohydrate recognition site (145) on murine alveolar or induced peritoneal derived macrophages, or incubation of macrophages with β-glucan, inhibited macrophage killing of *Pneumocystis* organisms (49). Binding of *Pneumocystis* organisms was enhanced by overexpression of the dectin-1 receptor on macrophages, suggesting that this pattern

recognition receptor has excellent ability to recognize *Pneumocystis* organisms and recruit them to macrophages. Additionally, internalization of *Pneumocystis* organisms is impaired in alveolar macrophages upon dectin-1 receptor blockade. Thus, binding of *Pneumocystis*, internalization, and release of hydrogen peroxide is an important pathway for the degradation of *Pneumocystis* organisms, and the dectin-1 receptor influences all of these processes. It is likely that some of these processes may be redundant with other pattern recognition receptors, such as binding of *Pneumocystis* organisms, or cytokine production, as TLRs and TLR signaling have been implicated in some of these processes (54, 138, 146). However, it was demonstrated that in the absence of MyD88, which is absolutely required for signaling by all known TLR receptors except TLR3, that reactive oxygen species production was unaffected, whereas in the dectin-1 deficient mouse reactive oxygen species production in response to *Pneumocystis* is significantly impaired (138). Hence dectin-1 appears to control a predominant killing mechanism utilized by alveolar macrophages against *Pneumocystis*. Of note, the dectin-1 knockout mouse has delayed clearance and worsened pulmonary *Pneumocystis* burden in the setting of corticosteroid treatment (138). Collectively, these studies underscore the importance of the non-TLR dectin-1 in recognition of *Pneumocystis* and the initiation and activation of killing pathways utilized by macrophages in defense against the organism. We believe that properties of the dectin-1 receptor, such as β-glucan recognition, could be utilized in the design of new therapies against *Pneumocystis*.

1.4.4 Summary

Isolated β-glucan from the cell wall *Pneumocystis* organisms leads to inflammatory responses from macrophages and leads to the recruitment of inflammatory cells such as neutrophils in the lung. The recently described C-type lectin receptor dectin-1 has affinity for β-1,3 and β-1,6 linked glucan polymer found in fungal cell walls and is significantly involved in binding *Pneumocystis* organisms to macrophages. The receptor is compact, contains one lectin domain, one transmembrane domain, and an ITAM motif and signals through multiple transducer proteins distinct from the machinery involved in TLR signal transduction. Additionally, the dectin-1 receptor is phagocytic and can internalize fungi, and controls the release of reactive oxygen species by macrophages in response to *Pneumocystis* organisms. More recently, while it has been demonstrated that the dectin-1 receptor is not absolutely required for host defense against infection, it inhibits growth of the organism in immunosuppressed rodents and affects the rate of clearance of infection in healthy rodents. Thus, host defense against *Pneumocystis* involves the dectin-1 receptor, which has the capacity to identify, internalize, and initiate pathways leading to organism degradation, suggesting that molecular therapeutics that incorporate aspects of the dectin-1 receptor in its recognition properties, or that can influence the receptor's signaling pathways, could potentially enhance host defense against *Pneumocystis*.

1.5 ROLE OF ANTIBODIES IN HOST DEFENSE AGAINST FUNGAL PATHOGENS

1.5.1 Precedent for antibodies targeting protein and carbohydrate antigens of fungi mediating host defense

While a controversial concept just one decade ago (147), there is now ample evidence that antibodies are critical effector molecules that could be of benefit in the design of therapies against opportunistic fungal infection (148). Many models have demonstrated that protein and carbohydrate antigens are important targets of protective antibodies against fungi. For example, antibodies against the protein Hsp60 of *Candida albicans* (149) are protective against infection, as are antibodies against the histone-H2B like surface protein of *Histoplasma capsulatum* (150). As multiple carbohydrate antigens are present at the fungal cell wall surface, it has been demonstrated that these too, are useful targets of antibody-based defense against fungi. Among the most definitive examples of antibodies recognizing carbohydrates in anti-fungal host defense include antibodies directed against the polysaccharide glucuronoxylmannan, the carbohydrate comprising the capsule of *Cryptococcus neoformans.* When this carbohydrate is conjugated to the protein carrier tetanus toxoid and administered to rodents, it results in the production of antibodies reactive with the fungal capsule that prevent infection (151, 152). Additionally monoclonal antibodies specific for the glucuronoxylmannan antigen, such as the IgG1 mAb first described by Dromer *et al* induce dose-dependent inhibition of infection (153). More recently, it has been demonstrated that a vaccine consisting of laminarin, primarily a β-1,3 linked glucan, linked to the carrier protein diphtheria toxoid, produced antibodies that could significantly prevent against systemic and vaginal candidiasis; and significantly increased survival in systemic

Aspergillus fumigatus infection (154). It was additionally demonstrated that passive transfer of the induced antibodies could transfer the phenotype of enhanced immunity, and further, the antibodies when incubated with *Aspergillus fumigatus* and *Candida albicans* directly inhibited the growth of the organisms. Thus, defense against these pathogens was partly attributed to binding cell wall β-glucan, perhaps interfering with cell wall structure and/or remodeling, and inhibiting the life cycle of the organism. However, it is possible, if not likely, that these antibodies have multiple functions in host defense, in shaping the strategy of the host to diminish infection.

1.5.2 Mechanisms of antibody-mediated defense against fungal organisms

There are multiple mechanisms whereby antibodies influence host defense against fungal infections. Antibody neutralization, or the prevention of virulent pathogen components from interaction with host components secondary to antibody binding, is one of the earliest described functions of antibodies. While most commonly described in the setting of viral infection or in the interaction with bacterial toxins, antibody neutralization also occurs against fungi. For example, a monoclonal antibody targeting *C. albicans* mannoprotein demonstrated an ability to inhibit binding of organisms to human epithelial cells, which is required for the establishment of mucosal infection (155) Direct opsonization of pathogen with antibody, priming immunologic cells for recognition of antigen through the identification of Fc portions of antibodies, is another common strategy whereby antibodies enhance anti-fungal host defense. Many reports are described of antibodies with the capacity to enhance macrophage recognition of fungi. For example, antibodies from humans vaccinated with the glucuronoxylomannan conjugate

significantly enhance phagocytosis of *C. neoformans* by peripheral blood mononuclear cells (156). Antibodies also have the capacity to enhance binding of complement proteins to their surface, mediating recognition of pathogen by interaction of complement proteins/split products with complement receptors at the surface of phagocytic cells. Antibody-Antigen interaction initiates the classical pathway of complement activation, and the IgM isotype complexed with antigen is considered the most potent isotype in initiating this process. With complement cascade activation, complement protein 3 and its cleavage products C3b, iC3b, and C4b bind the surface of pathogens, facilitating recognition by complement receptors 1-4 (CR1-4) on phagocytic cells. It has been demonstrated that complement plays an important role in antibody-mediated host defense against fungi, as for example, monoclonal antibodies targeting *C. albicans* β-mannan have protective function only in the setting whereby the complement cascade is intact (156). Additionally, antibodies can be directly fungicidal, as demonstrated with the laminarin conjugate vaccine and been seen in the study of antibodies targeting *Candida* sp., with antibodies directly capable of inhibiting growth and suppressing respiration (155, 157).

More recently, antibodies have been demonstrated to have a role in the shaping the broader host response to fungal infection. For example, B cell deficient mice transferred with an antibody that normally enhances immunity against *Cryptococcus* have a significantly higher pathogen and diminished survival, along with enhanced IFN-γ and MCP-1 relative to wild type controls (158). While the mechanism of action is unclear, it suggests that antibody effector function specifically requires B cells, which in turn guides the cytokine response against infection and influences pathogen clearance. It has also been demonstrated, in this infection, that transfer of a normally protective IgG1 against cryptococcal infection is only functional in the presence of CD4+ T cells, and additionally requires IFN-γ, suggesting that somehow the IgG1-

Ag complexes enhance CD4+ T cell effector function leading to clearance (159). In the study of the monoclonal antibody targeting a histone-like protein of *H. capsulatum* that enhances host defense against this pathogen, it was observed that the antibody elevates quantities of IL-6, IL-4, and IFN-γ in the lungs (150), suggesting that antibody may enhance antigen presentation, and perhaps adaptive immune responses such as T cell priming given the increases in IL-4 and IFN-γ production. Thus antibodies may influence aspects of inflammation and the functions of adaptive immune cells such as B and T cells. Net consequences of this, may be for example, enhanced recognition of fungal antigen, or the avoidance of detrimental inflammatory responses.

1.5.3 Complexities of Antibody Interactions with Fungi

Antibodies against multiple fungal antigens are observed in the setting of health and infection, hence dissection of their functions in host defense is complicated. Are the antibodies observed against fungi, in the serum of healthy individuals, merely bystanders in immune responses, do they enhance host defense, or still, can they actually impair recognition? It is most likely that these responses cannot be considered in the bulk, but rather according to exact specificities and cross-reactivities, strength of antigen binding, isotype, and concentration.

In the murine model of *Cryptococcus* infection, it has been demonstrated that antibody isotype critically influences host defense against fungi. Monoclonal antibodies administered at the same dosage and having the exact same antigen recognition portion but differ in isotype only (160, 161), have differing capacities to clear infection , and it was later determined that part of this issue is secondary to differences in isotype influencing the ability of this identical

recognition site to bind antigen, perhaps due to steric aspects of interaction, suggesting that isotype influences Ag:Ab interaction in this model of protein:polysaccharide interaction (162).

Studies with monoclonal antibodies against glucuronoxylmannan have demonstrated that protective antibodies administered at particularly high doses may actually impair host defense against *Cryptococcus*. This phenomenon is known as a "prozone-like effect." The prozone-like effect for protective monoclonal antibodies was first observed for the IgM isotype but is extended to IgG subclasses as well (163, 164). It is unclear why this effect occurs, but perhaps high doses of protective antibodies impairs recognition of pathogen by multiple alternate defense pathways that additionally are required for defense against infection.

The concept of non-neutralizing antibodies facilitating infection and pathology has emerged as a pathologic mechanism in viral diseases such as dengue (165, 166). This phenomenon has not been evaluated in the setting of fungal infection, but could potentially contribute to pathophysiology of fungal diseases that contain an intracellular growth phase, such as *H. capsulatum.* The factors described add complexity to the analysis of the relevance of antibodies in host defense against fungi, and in the design of antibody-based immunotherapies.

1.5.4 Operative, identified antibody-mediated defense mechanisms against *Pneumocystis*

In the setting of CD4+ T cell or B cell deficiency, macrophage effector function against *Pneumocystis* is insufficient in limiting growth of the organism in the lungs. However, exogenous administration of antibodies significantly enhances clearance of *Pneumocystis*, through mechanisms that are only partially defined. One operative mechanism involved in this defense is the activity of IgG at FcγRs on macrophages, neutrophils, and dendritic cells.

Preopsonization of *Pneumocystis* organisms with serum from mice immunized with *Pneumocystis* specifically enhanced macrophage effector function against the organism, leading to decreased pathogen viability (49). Mice deficient in FcγRI, FcγRIII, and the FcεR (via FcγR deletion) have a delayed rate of clearance of *Pneumocystis* infection suggesting that signaling of antibody:antigen immune complexes at these receptors facilitates more efficient *Pneumocystis* clearance (96). It was additionally demonstrated that absence of the C3 protein, central to the complement cascade, does not influence the rate of clearance of *Pneumocystis*, suggesting that antibodies may not necessarily require complement function for effective signaling at FcγR leading to the enhanced rate of clearance. However, a study evaluating the efficacy of a monoclonal IgG1 antibody in prophylaxis against *Pneumocystis* demonstrated that an intact complement pathway was needed for host defense against the organism, as depletion of complement impaired efficacy (95). This study additionally underscored the importance of FcγR signaling, as the Fab portion of this protective IgG1 was also not as efficacious as the intact protein in preventing infection. However, this could be attributed to the fact that the IgG1 longevity is influenced by the presence of the Fc portion of the antibody, which was not addressed by the authors.

As described, transfer of hyperimmune serum obtained from *Pneumocystis* challenged mice was associated with pathogen clearance, but without excessive inflammation, as seen with transfer of CD4+ T cells (82, 167). Efficacy was observed both in prevention of infection, as well as significant inhibition of growth at early, intermediate, and late stages of pneumonia. The observation that clearance mediated by antibodies does not lead to deleterious inflammatory responses suggests that antibody-dependent clearance mechanisms likely involve different signaling and degradation pathways. In a model of passive immunization, where *Pneumocystis*

clearance was assessed in neonatal mice, it was observed that mice born to mothers who had been immunized against *Pneumocystis* had enhanced clearance of pathogen from the lungs, with significantly increased quantities of *Pneumocystis* specific Ig in the blood and BALF, and without differences in recruitment of CD4+ or CD8+ T cells, or B cells, or quantities of inflammatory cytokines/chemokines such as TNF-α, IFNγ, MCP-1, and IL-6 (168). It is unclear whether the cytokines that appear to be suppressed occurred merely as a function of reducing quantities of pathogen in the lungs. However, in contrast to T cell mediated clearance strategies, non-pathologic inflammatory responses are utilized in antibody-dependent clearance. This is an attractive aspect of antibody based defense against *Pneumocystis* as a potential therapy in settings such as AIDS, where the immune system is in a chronic state of activation and the limitation of inflammatory response to an infection may result in a better outcome for the host.

1.5.5 Summary

Fungal pathogens can be targeted by specific antibodies against protein and carbohydrate antigens. High affinity antibodies generated from conjugation of polysaccharide to protein carriers resulted in protective antibodies targeting Cryptococcus glucuronoxylmannan and as well as the common conserved fungal cell wall β-glucan. Antibodies function through multiple mechanisms, and in the case of the β-glucan conjugate vaccine, one function was simply inhibiting the life cycle of target pathogens such as *Candida* and *Aspergillus*. Other mechanisms employed by antibodies include neutralization, opsonophagocytosis, and enhanced complement fixation. Additionally, antibodies may shape inflammatory responses, or may require the presence of certain adaptive immune system cells, such as B and T cells, for function,

suggesting a coordinate interaction. Besides these beneficial functions, antibodies seem to potentially have detrimental functions in host defense, as in *Cryptococcus* infection certain specific isotypes were demonstrated to be ineffectual if not capable of worsening infection, whereas antibodies with the same Fab portion but of a different isotype were protective.

In *Pneumocystis* host defense, antibodies have been demonstrated to enhance clearance of infection. It has been demonstrated that they can enhance alveolar macrophage effector function against *Pneumocystis* organisms. Additionally, they appear to operate in a manner, which relative to CD4+ T cell based clearance, is less inflammatory, presumably by utilizing degradation pathways that are very different.

1.6 NATURAL ANTIBODIES IN HOST DEFENSE AGAINST MICROBES

1.6.1 Definition of natural antibodies and cellular origins

Natural antibodies are defined as antibodies generated in the absence of exogenous antigenic stimulation. Practically, they could be considered to be antibodies found in germ-free mice, in cord blood, or more loosely, antibodies found in individuals in the absence of apparent immunization (169). Mice reared under germ-free or axenic conditions, free of all demonstrable associations with fungi, viruses, protozoa and bacteria, and maintained on a chemically defined "antigen-free" diet, have nearly identical quantities of IgM in the serum, but significantly reduced quantities of IgG in the serum and IgA at the mucosa, compared to mice reared under specific pathogen free conditions (170). The lack of difference in quantities of IgM in the absence of antigenic stimulation is suggestive of the likelihood that the majority of IgM isotype

found in the serum is made in a different manner than by B cells secreting the majority of the IgG and IgA isotypes, and is likely the representative isotype comprising natural antibodies. Indeed, it has been demonstrated that a particular subset of B cells, B-1 cells, localized to the pleural and peritoneal spaces in rodents, are responsible for the majority of the production of serum IgM (as well as natural IgA found in the gut, with both natural and "induced" i.e. bacterial exposure dependent IgA derived from B-1 cells) (171-174). Additionally, IgG natural antibodies have been described (175).

B-1 cells are the first B cell lineage produced in the neonate, in contrast to traditional B-2 cells which are generated later in development from the bone marrow. B-1 cells are produced from progenitors in the fetal omentum and liver and are notably absent from the bone marrow, and are a self-replenishing population in the periphery thereafter (176). It is assumed that selection or maintenance of B-1 cells occurs against self-antigen, as B cells require a positive selection signal through the IgM receptor to survive in the periphery (177). As such, natural antibodies have reactivity against a variety of self-Ags, including to Thy-1, rheumatoid factor, T cell receptors, keratin, and single stranded DNA (169, 178, 179). It is speculated that these antibodies may have function in aiding the clearance of apoptotic cells (180) or function in other physiologic processes. However, it is also clear that natural antibodies react strongly with conserved pathogen associated molecular patterns, such as α-1,3 linked dextran, phosphorylcholine, and hemagglutinin, suggesting that the selection of natural antibody specifities may be linked to their functional utility in host defense. It has been demonstrated that perturbation or absence of the B-1 subset leads to impaired production of natural antibodies targeting *Streptococcus pneumoniae* (181) or *Borrelia hermesiae* (182), which has significant consequences for host defense against these infections. Furthermore, these studies have

demonstrated that the B-1 subset is further partitioned based on functionality, with the B-1a subset (CD5+) demonstrated to have more of a role in the production of natural antibody, while the B-1b (CD5-) subset demonstrated to have more of a role in the induction of antibodies targeting carbohydrate antigen (183).

Natural antibodies may play multiple roles in host homeostasis. Antibody specificities may be present or absent depending on the species and the potential for autoimmune reactivity. For example, hyperacute rejection of xenotransplants occurs in humans receiving transplants of porcine origin, and this is mediated by the host possessing natural IgG and IgM targeting the carbohydrate Galα1–3Galβ1–4GlcNAc (abbreviated as α1,3Gal), expressed on porcine epithelium. Of note, these natural antibodies are entirely absent in mice and in pigs, as these animals have the enzyme, α1,3-galactosyltransferase to make this antigen, whereas humans do not. When the enzyme is deleted from the mouse, the mice spontaneously make high quantities of this natural IgM specificity (184), and it appears that reintroduction of the enzyme via mixed bone marrow chimerism inhibits production of this particular antibody (185). Hence, the natural antibody repertoire is exquisitely regulated, and particularly deleterious natural antibody reactivities are selected against. Of note, natural α1,3Gal IgG antibodies observed in humans were found to be cross-reactive with LPS, and with bacteria such as *Escherichia*, *Klebsiella*, and *Salmonella*, as well as with *Trypanosoma* and *Leishmania* parasites (186, 187). The authors of these papers argue that it is likely these natural antibodies are continually induced by the normal flora of the host. Thus, their definition of natural antibody partially implicates exogenous antigenic stimulation in the production and maintenance of B cells with these specificities. Indeed, it is likely that if natural antibodies are selected partially as a function of their potential to interface with pathogen associated molecular patterns, that exogenous antigens could

influence the functions of these B cells. For example LPS or TLR stimulation of peritoneal B cells *in vivo* induces migration of B-1 cells to secondary lymphoid organs such as the spleen, where they differentiate into antibody-secreting cells (188, 189). Hence, a B cell secreting a natural antibody may have its baseline activity and functions affected by exogenous antigenic stimulation.

Specificities of natural antibodies are described as polyreactive and encoded by germline variable regions without somatic hypermutation; it is also reported that there might be some preferred variable genes utilized in the production of these antibodies. Natural antibodies are reported to have a wide range of binding avidities, from as low as 5*10^-3 to as high as 5*10^-11 M (190). While the natural antibody repertoire is described as broad and cross-reactive, it is clearly limited in its reactivity, and it is likely that specificities of low utility in host defense and homeostatic function are less represented, just as harmful specificities are deleted.

1.6.2 Natural antibodies and host defense against infection

There are multiple lines of evidence supporting a role for natural antibodies in the very earliest aspects of host recognition and defense against pathogens. Ochsenbein *et al* demonstrated that low titers of IgM are present in the serum of specific pathogen free mice against specific viruses such as LCMV, VSV, and Vaccinia, as well as the bacteria *Listeria monocytogenes* (191). The IgM isotype against these pathogens significantly predominated in the serum relative to the IgG isotype and natural IgM against VSV was capable of neutralizing the virus, preventing against lytic infection of target cells. Additionally, to assess the functional relevance of these IgM, they compared the ability of μMT, RAG (-/-), and wild type mice to handle dissemination of viral

infection. They showed that wild type mice could restrict dissemination of infection with enhanced pathogen burden in the spleen relative to other tissue sites such as the kidney, blood, and liver where burden was enhanced in the μMT mice. To prove that this phenomenon was mediated by preexisting specific components of the serum, natural antibodies, they observed that with transfer of high quantities serum from wildtype mice into μMT mice they could restrict viral dissemination and improve survival. Of note, since these specificities were present in specific pathogen free mice and predominantly of the IgM isotype they were considered natural antibodies, however, it was not confirmed that their presence occurs in the absolute absence of exogenous stimulation, criteria proposed by other authors to characterize the natural antibody repertoire.

Natural antibodies also appear to play a role in a murine model of burn injury and defense against bacterial infection. Serum IgM antibodies decline after burn injury leading to impaired defense against *Pseudomonas* infection; this effect however can be reversed by treatment with IL-18 prior to infection, which significantly enhanced quantities of natural IgM leading to improved resistance to *Pseudomonas* upon exposure (192) . Splenectomized mice are more susceptible to *Streptococcus* infection, and transfer of IgM from specific pathogen free mice or pretreatment of mice with IL-18 which increases baseline IgM, and enhances host defense against infection (193). Serum from humans that are non-immune to *Leishmania* contain IgMs able to agglutinate promastigotes, enhance complement deposition, and increase macrophage ingestion by monocytes (194). Interestingly, mice and humans not previously exposed to HIV gp120 possess IgM antibodies with the capacity to proteolytically cleave gp120 *in vitro*, suggesting that some natural IgM molecules may also have enzymatic effector function (195).

As natural antibodies are predominantly of the IgM isotype, another model to assess the functions of innate secreted IgM in host defense is the sIgM (-/-) mouse. The sIgM (-/-) mouse contains a deletion in the exon driving secretion of IgM but retains the capacity to make membrane bound IgM receptor allowing for positive selection of B cells in the periphery, and the production of antibodies of other isotypes. These mice have significantly worsened survival in a model of septic peritonitis which can be corrected by prior treatment with purified IgM from specific pathogen free wild type mice (196). Also noted in this model, proinflammatory cytokines TNF-α, IL-6, and neutrophils were decreased in the peritoneum relative to wild type controls, suggesting that lack of IgM impaired aspects of local antigen presentation. Systemic quantities of LPS and chemokines were, however, enhanced relative to wild type controls, arguing that secreted IgM (sIgM) plays an important role in preventing dissemination of antigen. In a model of filarial infection, it was demonstrated that lack of IgM impaired clearance in primary and secondary challenges (197). Another well described role for sIgM in host defense emerged in studies of the murine model of influenza virus. Mice lacking in sIgM had impaired survival and reduced clearance of influenza virus (198). Mice additionally had impaired kinetics of induced IgG responses against influenza, suggesting that natural and induced IgM antibodies are required for an optimal adaptive antibody response. It was further demonstrated, using allotype chimeras, that IgM antibodies derived from both the B-1 subset and the traditional B-2 subset are each independently required for the optimal induction of adaptive IgG responses and are each needed for survival. This data suggests that the natural antibodies of the IgM isotype derived from the B-1 and B-2 source have unique and required effector functions against influenza, and that the IgM isotype plays some functional role in antigen deposition or antigen presentation that is critical for B cells undergoing germinal center reactions, and further, that an

infection of the pulmonary mucosa relies on natural and induced IgM for specific host defenses. This observation is somewhat unexpected, as IgA is the predominant mucosal Ab, and the role of IgM in host defense against pulmonary infections is poorly explored.

The relevance of natural antibodies and their potential mechanisms of action in host defense are just emerging. From what is currently known, it is reasonable to assume that natural antibodies have a significant role in the very earliest aspects of host recognition and antigen presentation, and can influence infections to the point of host protection vs. host death.

1.6.3 Natural antibodies and fungal disease

Of note, the role of natural antibodies has been understudied in defense against fungal infections. It has been demonstrated that a monoclonal IgM that targets keratin has cross-reactivity with *C. albicans* germ tubes, and mice transgenically overexpressing this natural antibody can impair the growth of infection and inhibit the actual growth of *C. albicans* organisms. Thus, it is suggested that at least a surface epitope on *C. albicans* has similar structure to keratin, and that natural antibodies targeting this structure have a function in host defense. Additionally, using a looser definition of natural antibody, "natural" IgG antibodies targeting the fungal cell wall carbohydrate mannan have been described in healthy individuals (i.e. in the absence of any overt infection), with wide variation in titers when comparing individuals (199). In contrast, it was noted that anti-mannan IgG was not detected in rabbits unless directly infected with *Candida*. In a separate study by Kozel and colleagues, IgG against mannan was observed to exist at higher titers than the mannan specific IgM in the general population. Additionally, these authors

demonstrated that increased anti-mannan IgG levels correlated with an increased rate of complement protein 3 (C3) binding to *Candida* (200).

The potential contribution of the entire natural antibody repertoire and the IgM isotype, and the particular mechanisms of action of natural antibodies in influencing the evolving innate and adaptive response to fungal infection, have not been dissected for any fungal infection at the time of this writing.

In a disease such as *Pneumocystis*, it has been assumed that IgM is of very little importance in host defense. As mice deficient in CD40 overproduce PC-specific IgM (96) and are still susceptible to infection, it is clear that natural IgM are not sufficient for host defense against infection. However, it does not exclude their relevance in the very earliest aspects of recognition and innate defense and their contributions to an evolving adaptive immune response.

1.6.4 Summary

Natural antibodies are commonly defined as antibodies present in the serum in the absence of exogenous antigenic stimulation. Traditionally of the IgM isotype, present at relatively low titers, and with a wide range in binding affinities for various antigens, they consist a broadly reactive repertoire. Many natural antibodies are cross-reactive with host antigens, suggesting they may play some role in normal physiologic processes of the host, such as aiding in the identification and removal of apoptotic or dying cells. However, natural antibodies also possess specificities against non-self antigens, including a variety of conserved pathogen associated molecular patterns, suggesting that the repertoire may be shaped by selection pressures that influence the host's response to infection. Indeed, natural antibodies have been demonstrated to

bind pathogens, neutralize pathogen, enhance complement deposition and enhance survival against infection. They are able to restrict dissemination of infections. Natural antibodies are further implicated in shaping aspects of the host's adaptive immune response, as they participate in some of the earliest recognition events, and are likely very much involved in enhancing antigen deposition in secondary lymphoid organs, and hence antigen presentation. Of note, the repertoire is restricted, as some natural antibody specificities are selected against. Interestingly, natural IgM has been demonstrated to play a critical role in defense against mucosal infections of the lung, such as influenza.

Studies in humans have utilized a broader definition of natural antibody, defining natural antibodies as those that are present in individuals in the absence of specific infection with pathogen. It has been demonstrated that IgG antibodies targeting *Candida* mannan, found in healthy "uninfected" individuals, can enhance complement opsonization of *Candida*. Additionally, in fungal infection, it has been demonstrated that a natural IgM cross-reactive with keratin is also highly reactive with *Candida,* and that upon overexpression prevents infection by directly inhibiting germ tube growth (179). The role of natural antibodies in shaping host defense responses against *Pneumocystis* remains to be studied, and could provide very useful information in the design of new therapies against infection.

1.7 ADAPTIVE ANTIBODY RESPONSES AGAINST CARBOHYDRATE ANTIGENS IN HOST DEFENSE

1.7.1. Carbohydrate Antigens and the induction of antibody responses against mucosal infections: Implications for *Pneumocystis*

The production of immunoglobulins targeting carbohydrate antigen are among the most protective immune responses generated in humans. Additionally, some of the most essential vaccines in clinical use induce protective antibodies against carbohydrate antigens in the cell walls of bacteria such as *Streptococcus pneumoniae*, *Hemophilus influenzae*, or *Neisseria meningitidis*, preventing against human diseases ranging from pneumonia to meningitis (201). Despite the importance of these responses in host defense against infection, the actual mechanisms regulating the induction of antibody responses against carbohydrate antigens are poorly characterized.

Antigens are classified according to their requirement for T cell involvement in the production of antibodies. Thymus-dependent, or T-D, antigens require MHC class II restricted T cell help for the induction of antibody, whereas Thymus-independent, or T-I antigens do not. T-I antigens are further subdivided based on the mechanism whereby they activate B cells. TI-1 antigens are polyclonal activators of B cells, such as LPS, which signal through receptors besides the B cell receptor in order to induce B cell activation and antibody production. TI-2 antigens are described as repetitive carbohydrates, such as the capsular polysaccharide used in the *Streptococcus* vaccine, consisting of multiple repeating epitopes mediating significant cross-linking of B cell antigen receptors. TI-2 antigens are additionally characterized by their high

molecular weight given their polymeric nature, and their resistance to degradation. With receptor stimulation by TI-2 antigen, the enzyme Btk is activated, which is the critically involved in the TI-2 signaling cascade (202). Of note, *xid* mice, deficient in Btk and noted to lack B-1 B cells, have significant impairment of the TI-2 response, but relatively normal TI-1 responses (203). TI-2 responses are impaired in splenectomized mice and humans (204, 205), suggesting that B cells producing these antibodies require this environment potentially for activities such as activation, antigen presentation, or differentiation into antibody secreting cells. Collectively, given the specific localization requirement for some activities of B cells involved in TI-2 responses, their specific signaling requirements, and the unique antigenic stimuli leading to the production of these antibody responses, it suggests that the rules driving these B cells differ from those of traditional B-2 cells.

B-1 and the marginal zone (MZ) B cell subset of the spleen are implicated in responding to TI-2 antigens, as both subsets are implicated in the recognition of carbohydrate antigen, both subsets possess overlapping antibody idiotypes, for example reactivity against phosphorylcholine, as demonstrated by Martin *et al* (206), and both subsets can produce IgM antibody without CD4+ T cells. When the MZ B cell subset is absent, as seen in pyk-2 deficient mice, the early wave of antibody responses to TI-2 antigens to dextran and TNP-Ficoll is impaired (207), and as noted, xid mice are lacking in B-1 cells and have difficulty mounting TI-2 antibody responses.

Of note, very few studies have been performed to assess antibody responses to carbohydrate antigens on intact pathogens. As intact pathogens contain carbohydrates and proteins often covalently linked together, or in tight association, it is unclear whether antibody responses against carbohydrate antigens might be influenced or regulated by CD4+ T cells. One

might hypothesize that proteins associated with carbohydrates might provide peptide that could be presented on MHC II proteins, allowing for CD4+ T cell help in the activation of B cells secreting antibodies against carbohydrates, operating much like a conjugate vaccine. Studies from the laboratory of Snapper *et al* have demonstrated that antibody responses to phosphorylcholine, a component of the capsular polysaccharide of *Streptococcus,* in mice intraperitoneally challenged with *Streptococcus pneumoniae*, are indeed regulated by TCR-αß$^{+}$ T cells, CD40L, and B7-CD28/CTLA4 costimulation, in that IgG titers are significantly diminished in the absence of these molecules or when they were disrupted (208). This group went on to show that capsular polysaccharide of *Streptococcus*, another TI-2 antigen, also requires CD4+ T cells for the IgG response, and even more specifically requires an intact T cell receptor in order to produce IgG, in contrast to the anti-phosphorylcholine IgG response which does not (209). These studies truly underscore the complexity of the nature of antibody responses against thymus independent antigens, and that while perhaps the IgM response against TI-2 antigen may not require CD4+ T cells, aspects of these responses may be influenced by CD4+ T cells, such as the process of isotype class-switch, the specific idiotypes generated, and the actual quantities of antibodies produced (210-212). There may be further variation depending on the type of TI-2 antigen.

As *Pneumocystis* pneumonia is a disease most commonly seen in the setting of chronic CD4+ T cell deficiency, and antibodies have been identified to protect against infection, it is important to evaluate the role of CD4+ T cells in the production of antibodies targeting different protein and carbohydrate antigens. Thus far, there are no studies on the induction of antibody responses against *Pneumocystis* carbohydrate antigens, and it is unclear how a deficiency in CD4+ T cells might influence the production antibodies targeting carbohydrates. While there is

abundant evidence that antibodies sufficient for host defense against *Pneumocystis* are generated in a CD4+ T cell sufficient environment, it is unclear whether antibodies generated in a CD4+ T cell insufficient environment also have some protective efficacy, or modulate aspects of the host immune response.

Additionally, in the study of a mucosal disease such as *Pneumocystis*, where the a significant portion of the infection consists of trophozoites tightly adhering to the apical surface of epithelial cells, it is unclear what role CD4+ T cells have in guiding aspects of potential TI-2 antibody responses in lungs. The pulmonary compartment, for example, has significantly higher quantities of IgA than the serum, suggesting that aspects of immune responses at this distinct tissue site may influence host defense. Indeed, mice unable to transport IgA and IgM across the epithelium have greater susceptibility to pulmonary challenges with *Mycobacteria*, and *Streptococcus*, and mice vaccinated against *Vibrio cholerae* are protected only if pIgR is intact (213). Yet, in all of these studies, the specifities of these protective antibodies are unknown, as well as their reliance on CD4+ T cells for their production. An evaluation of the antibody responses produced at the mucosa against *Pneumocystis* carbohydrate antigens, and the role of CD4+ T cells in these processes may inform on other epitopes identified by antibodies and the role of CD4+ T cells in the generation of these molecules. Identification of antigens that produce antibodies in a CD4+ T cell independent manner, and additionally positively affect the immune response against *Pneumocystis* may allow for the design of new therapies against infection that enhance protective strategies already employed by the host.

1.7.2 B cell memory against carbohydrate antigens: Implications for *Pneumocystis*

The ability of B cells to produce specific antibodies for many years after an exposure to a given antigen, and the significant enhancement of antibody responses after secondary exposure to antigen underscores the ability of the immune system to learn and to improve its functional utility over the course of an individual's lifetime. Traditionally, antibody-based immunologic memory has been considered in the context of the generation of at least two types of functional memory B cells (214). The first of these effector memory B cells is the long-lived terminally differentiated antigen specific plasma cell, which localizes predominantly to the bone marrow in mouse and human after differentiation, where it receives growth factor support (215, 216). The second effector memory B cell population, known as the quiescent memory B cells, do not secrete antibody at steady state, but upon reexposure to secondary antigen rapidly divide and differentiate into plasma cells, dramatically enhancing antibody responses within a short period of time. Multiple lines of evidence suggest that quiescent memory B cells home to secondary lymphoid organs such as the spleen for their long-term survival (217, 218). The production of B cell memory subsets secreting antibody against protein antigen requires CD4+ T cells, and the migration of antigen activated B cells to germinal centers, where, they undergo isotype class-switching and affinity maturation (214). While this model is well accepted for protein antigens, it is unclear if such memory responses against carbohydrate antigens are generated. As individuals vaccinated with capsular polysaccharide make high quantities of specific antibodies, presumably via the generation of memory plasma cells, with the capacity to secrete antibody for many years, it is unclear if the quiescent memory B cell compartment forms against TI-2 antigens. A recent study by Obukhanych *et al* suggests that quiescent memory B cells are

generated in response to TI-2 antigen challenge, and that the regulation of antibody production by these quiescent memory B cells, upon rechallenge, is inhibited by high quantities of antigen specific IgG in the serum (219).

In the setting of *Pneumocystis* susceptibility, where CD4+ T cell antibody-dependent responses are not reliable, it is important to understand if antibodies targeting carbohydrates antigens are generated, whether CD4+ T cells influence their production, as well as whether functional memory responses against carbohydrate antigens generated. Also, it would be very informative to understand whether these responses can be reactivated in a CD4+ T cell insufficient environment, as this might be the manner in which one might rely on any carbohydrate vaccine to operate in the setting of HIV-AIDS.

1.7.3 Summary

The parameters guiding antibody responses against fungal cell wall carbohydrates in a model of mucosal infection are poorly characterized. As *Pneumocystis* pneumonia is a disease whereby the most obvious cellular change results in a dramatic loss of CD4+ T cell numbers, it is important to characterize antibody responses that may be operative in a CD4+ T cell insufficient environment. B-1 and MZ B cell subsets are implicated in the generation of TI-2 responses. The conserved fungal cell wall carbohydrates of *Pneumocystis* resemble TI-2 antigens, and there is some evidence that T cells influence aspects of these responses, such as quantity of antibody production and isotype switching. While CD4+ T cells are required for the production of B cell memory, it is unclear whether they are required for functional B cell memory, elicited by either

plasma cells or antibody secreting cells. A greater understanding of TI-2 and B cell reponses antigens in host defense against *Pneumocystis* may identify new targets in the design of vaccines against this infection.

1.8 SPECIFIC AIMS

Specific Aim I: To evaluate whether a Dectin-1: IgG1 Fc fusion protein enhances host clearance of Pneumocystis. We hypothesize that a molecule capable of recognizing the conserved inner cell wall carbohydrate, β-1,3 glucan, as well as FcγRs, will function as an antibody and promote host defense against *Pneumocystis*.

Specific Aim II: To determine the presence, role, and regulation of *Pneumocystis* cellular wall carbohydrate directed antibodies, natural antibodies, and the IgM isotype in host defense against *Pneumocystis*. We hypothesize that contributory antibodies in host defense against *Pneumocystis* involve CD4+ T cell independent natural antibodies targeting conserved fungal cell wall carbohydrates found in the cell wall of *Pneumocystis*. We hypothesize that natural antibodies influence host defense against *Pneumocystis,* and influence aspects of antigen presentation, and that the IgM isotype, which is the primary isotype constituting the natural antibody repertoire, has an important role in host defense and in guiding adaptive immune responses. Additionally, we hypothesize *Pneumocystis* challenge leads to the actual induction of antibody responses against these fungal cell wall carbohydrates, and may lead to functional immunologic memory responses against them.

2.0 ENHANCED DEFENSE AGAINST PNEUMOCYSTIS CARINII MEDIATED BY A NOVEL DECTIN-1 RECEPTOR: IGG1 FC FUSION PROTEIN

2.1 INTRODUCTION

Pneumocystis carinii (PC) pneumonia is a major cause of illness and death among the immunocompromised, most often observed in individuals infected with HIV. Although incidence of PC pneumonia has declined following the introduction of highly active antiretroviral therapy (HAART), it remains the most prevalent of opportunistic infections in industrialized countries (17). Further, the vast majority of the world's 45 million infected with HIV are without access to HAART and at high risk for PC pneumonia (26, 220-223). Individuals receiving chronic immunosuppressive therapy as a consequence of organ transplantation or autoimmune disease represent another growing, at-risk population for this opportunistic infection. The identification of dihydropteroate synthase mutations in PC isolates from humans (224) suggests the potential for resistance of PC organisms to existing chemotherapeutic strategies and additionally underscores the need for alternate, immune-based strategies against PC infection.

Murine models of PC infection, using the host specific pathogen *P. carinii* f. sp. *muris*, have demonstrated the potential utility of Ab-based immunity in host defense against PC. $CD4^+$ T cell deficiency or blockade of CD40L renders mice susceptible to PC and is coincident with

diminished IgG responses against PC (42, 225). μMT knockout mice, deficient in B cells, are also highly susceptible to PC pneumonia and signify the critical nature of B cells and their effectors in PC resistance (91). Adoptive transfer of serum from PC-vaccinated mice or mAbs directed against PC Ags can prevent the establishment of infection upon challenge (57, 93) or inhibit progression of existing PC pneumonia in early through late stages of disease (167). Interestingly, mice challenged with PC and thereafter depleted of $CD4^+$ T cells have persistent anti-PC IgG and are able to resist infection given a subsequent exposure, suggesting that Ab-based immunity may be sufficient for preservation of anti-PC resistance in the setting of $CD4^+$ T cell deficiency (97). PC-directed Abs likely function through multiple mechanisms to increase host resistance to infection and have been shown to significantly potentiate alveolar macrophage anti-PC effector function in vitro (49, 92, 226). Though recent studies suggest that PC-specific IgG is not absolutely required for host resistance, FcγR-deficient mice clear PC organisms with delayed kinetics (96). These observations suggest that PC-specific Abs may couple with FcγR-bearing cells to modulate aspects of host recognition and clearance of PC and illustrate the potential of Ab-based strategies against PC in the setting of immunodeficiency.

The specific Ags involved in Ab-based recognition and degradation of PC, identifying either cystic and/or trophozoite forms of the organism, are unclear. PC protein Ags under investigation as vaccine targets, such as the major surface glycoprotein complex and the surface-associated protease Kexin, exhibit interspecies- and intraspecies-specific variation (92, 118, 227), which may complicate potential vaccination strategies. Conserved pathogen-associated molecular patterns (PAMPs) of PC identified by innate immune cells include the major carbohydrates of the fungal cellular wall, β-glucan, and mannan. It is unknown whether these carbohydrate Ags could be effective targets of Ab-based responses. We have previously shown that the β-glucan receptor

Dectin-1 is involved in alveolar macrophage recognition, nonopsonic phagocytosis, and killing of PC in vitro (49). RAW 264.7 macrophages overexpressing Dectin-1 bound PC organisms at higher levels than control cells, suggesting a critical, and possibly independent, role of this receptor in PC identification. In this study, we report on the potential of a recombinant protein, consisting of the extracellular domain of Dectin-1 fused to the murine IgG1 hinge through constant heavy (CH)2 and CH3 domains, as a novel strategy for the identification, specific targeting, and degradation of PC organisms.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Male C57BL/6 mice, 6–8 wk of age, were purchased from the National Cancer Institute, National Institutes of Health. Male B6.*scid* mice, 6–8 wk of age, were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free environment in microisolator cages within an American Association for Laboratory Animal Science-certified animal facility in the Rangos Research Center at the Children's Hospital of Pittsburgh. Animal studies were reviewed and approved by the Children's Hospital of Pittsburgh Animal Research and Care Committee.

2.2.2 DNA constructs and adenoviral vectors

The design of the Dectin-Fc recombinant gene construct was modeled after the TNFR-IgG H chain, described by Peppel et al. (228). A PCR 3.1 plasmid containing the full-length murine Dectin-1 receptor, provided by G. Brown (University of Cape Town, Cape Town, South Africa), and the pACCKP2 plasmid containing the TNFR extracellular domain linked to murine IgG1 H chain (229) served as PCR templates. The cDNA encoding the extracellular domain of the Dectin-1 receptor, consisting of amino acids 69–244 (139), was amplified by PCR with oligonucleotide primers ggtaccga CGACACAATTCAGGG (corresponding to an engineered *Kpn*I site, a frame spacer, and the 5' end of the Dectin-1 receptor extracellular domain) and ggatccacgcggaaccag CAGTTCCTTCTCACAG (corresponding to the hexapeptide linker with thrombin cleavage site and the 3' end of the dectin-1 receptor). The cDNA-encoding CH2-CH3 was amplified with primers ctggttccgcgtggatcc GTGCCCAGGGATTGTGGT (corresponding to the hexapeptide linker with thrombin cleavage site and the 5' end of the IgG moiety) and gaattc TCATTTACCAGGAGAGTG (corresponding to an engineered *Eco*RI site, an antisense stop codon, and the 3' end of the IgG moiety). cDNA products were isolated on an agarose gel and purified via extraction (Qiagen). The products were combined at a 1:1 ratio and subjected to five cycles of denaturation and reannealing to promote recombination of homologous regions. The 5' primer for the dectin-1 receptor extracellular domain and the 3' primer for the IgG moiety were used to amplify the recombinant product Dectin-Fc. The chimeric PCR product was isolated and purified via gel extraction and subcloned into the TOPO-TA Vector (Invitrogen Life Technologies). Using M13 primers, the Dectin-Fc DNA was amplified via PCR, digested with *Kpn*I and *Eco*RI, and inserted in-frame into the multiple cloning site of pSecTag 2C mammalian

expression vector (Invitrogen Life Technologies), containing the IgK leader sequence facilitating protein secretion. To confirm the fusion gene product, dideoxy sequencing was performed.

Ad-Dectin-Fc is an E1-E3 replication-deficient rAd5-based vector containing the entire pSecTag 2C-Dectin-Fc expression cassette. To generate Ad-Dectin-Fc, the Dectin-Fc expression cassette was amplified and inserted into the Invitrogen Gateway entry vector pENTR/D-TOPO, then shuttled in an exchange reaction using LR Clonase into pAd/CMV/V5 DEST (Invitrogen Life Technologies). The resultant plasmid was purified, digested with *Pac*I to expose adenoviral ITR sequences, and then transfected into 911 cells supplying the E proteins required for adenovirus propagation. Viruses were purified and concentrated by either CsCl density gradient centrifugation or by membrane adsorption (Vivapure Adenopack; Sartorius), and titered by a plaque assay on 911 cells, as previously described (89, 229). The control AdLuciferase vector is identical but instead encodes the firefly luciferase gene and was obtained from the Pittsburgh Pre-Clinical Vector Core. The particle:PFU ratio was ~100:1 and virus stocks contained <0.01 ng/ml endotoxin as determined by the QCL-1000 *Limulus* lysate assay (BioWhittaker).

2.2.3 Production of Dectin-Fc and Western blot

For Dectin-Fc protein generation, 293 cells cultured in DMEM plus 10% FCS were transiently transfected with the pSecTag 2C-Dectin-Fc vector with Lipofectamine 2000 (Invitrogen Life Technologies). Supernatants were collected and cells were removed by centrifugation and passage of supernatant over a 0.2 μM low protein-binding filter. Supernatants were preserved at –80°C. For some experiments, Dectin-Fc was affinity purified over a column consisting of Sepharose beads conjugated to goat-anti-mouse H chain IgG (Sigma-Aldrich), and SDS-PAGE followed by Coomassie staining was used to confirm purity. Protein reactivity was studied by

Western analysis. To evaluate protein structure, Dectin-Fc-conditioned supernatant was pretreated with 2-ME. Conditioned supernatant or purified protein was isolated on SDS-PAGE, transferred to nitrocellulose membranes, and blocked overnight with BSA (2% for tissue culture supernatant, 4–8% for mouse BAL, serum, or lung homogenate). Rat anti- murine-Dectin-1 (R&D Systems) followed by goat anti-rat IgG-AP (Santa Cruz Biotechnology), or goat anti-mouse IgG-AP (Bio-Rad), all at 1:2000 in blocking buffer, were used for immunodetection, and blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT reagent (Bio-Rad).

2.2.4 β-glucan ELISA

Laminarin and mannan (Sigma-Aldrich) were each dissolved in PBS and seeded to a Nunc Polysorp-treated 96-well plates at 0.025 mg/ml at 4°C for 48 h. As a negative control, β-1,3-glucan linkages of laminarin were hydrolyzed by pretreatment with laminarinase (β-1,3 endoglucosidase) isolated from *Trichoderma* sp. as per manufacturer's protocol (Sigma- Aldrich) and similarly seeded at 0.025 mg/ml. The wells were washed, blocked for 2 h in PBS, 10% FBS, and 2.5% milk, then washed again. Dectin-Fc-conditioned supernatant or purified Dectin-Fc was dissolved in blocking buffer, serially diluted 1:2, and administered to wells in triplicate. After 2 h of incubation, wells were washed, and a 1/1000 dilution of goat-anti-mouse IgG-HRP was applied for 2 h. Wells were washed and developed with tetramethylbenzidine (TMB; BD Biosciences-BD Pharmingen) and the absorbance at 450 nm was measured and subtracted from baseline absorbance.

2.2.5 BIAcore analysis

Real-time surface plasmon resonance experiments were performed on a BIAcore 3000 Instrument with CM5 sensor chips (BIAcore) and all interactions were studied at 25°C. *N*-hydroxy succinimide/*N*,*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodimide hydrochloride amine coupling was used to immobilize goat anti-mouse Abs (Molecular Probes) diluted 1/50 in 10 mM sodium acetate (pH 5) and empty reactive sites were quenched with 1 M ethanolamine. Affinity purified Dectin-Fc (100 µg/ml) was captured by injection at a flow rate of 5 µl/min (total 35 µl). Laminarin was dissolved in PBS and diluted to 100, 2, or 0.2 µM 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 goat anti-mouse Abs served as a control surface and nonspecific binding of laminarin was subtracted from the surface plasmon resonance signal in the active flow cell. The association rate constant (k_a) and dissociation rate constant (k_d) were calculated and the dissociation constant was determined (K_D) using the BIAevaluation 3.1 software.

2.2.6 P. carinii f. sp. muris isolate

The PC inoculum was prepared as previously described (42, 89). Briefly, B6.*scid* mice with PC pneumonia were injected with a lethal dose of pentobarbital and the lungs were aseptically removed and frozen in 1 ml of PBS at –80°C. Lungs were mechanically dissociated in sterile PBS, filtered through sterile gauze, and pelleted at 500 x *g* for 10 min at 4°C. The pellet was resuspended in sterile PBS and a 1/5 dilution was stained by a modified Giemsa stain (Diff-Quik; Baxter). The number of PC cysts was quantified microscopically and the inoculum concentration was adjusted to 2×10^6 cysts/ml. Gram stains were performed on the inoculum preparations to

exclude contamination with bacteria. For in vitro studies, aliquots of the inoculum were stored at concentrations of 10^6 cysts/ml at –80°C until use.

2.2.7 Peritoneal and alveolar macrophage isolation

For peritoneal macrophage isolation, male C57BL/6 mice were administered 3% sterile thioglycolate i.p. After 3–5 days, mice were sacrificed and a peritoneal lavage was performed using 5 ml of PBS. The lavage fluid was centrifuged at 300 x *g* for 10 min, resuspended in RBC lysis buffer (Sigma-Aldrich) for 5 min, then washed, pelleted, and resuspended in RPMI 1640. Cell pellets were studied and enumerated using a hemacytometer. To isolate alveolar macrophages, male C57BL/6 mice were anesthetized with isoflurane and sacrificed via terminal exsanguination. With an intratracheal catheter, calcium and magnesium-free PBS was used to lavage lungs. A total of 10 ml was used per mouse in 0.5-ml increments with a 30 s dwell time. The lavage fluids were pooled and centrifuged at 300 x *g* for 10 min, and the cells were collected for the coculture assay. Cell preparations were generally >98% enriched for peritoneal or alveolar macrophages.

2.2.8 Zymosan association assay

To assess macrophage surface association with zymosan, 10^5 RAW 264.7 cells were suspended in RPMI 1640 plus 10% FCS in 5-ml polystyrene tubes. Some groups were pretreated for 30 min with 5 µg of 2a11 (provided by G. Brown, as described in Ref. (145)), a mAb with specific blocking activity against the murine Dectin-1 receptor, and/or 2.5 µg of FcγRII/III blocking Ab 2.4G2 (eBioscience), and maintained at 37°C. FITC-zymosan (Molecular Probes) was suspended in culture medium, sonicated, enumerated, and preopsonized with conditioned supernatant

containing Dectin-Fc, control medium consisting of DMEM plus 10% FCS, or neat normal mouse serum, for 30 min at 4°C. Ten particles of preopsonized zymosan were administered per macrophage and allowed to incubate with macrophages at 37°C for 90 min. A total of 10,000 events were analyzed with a FACSAria flow cytometer for relative FITC intensity (BD Biosciences). Macrophages were distinguished from free zymosan by forward and side scatter profiles. Mean fluorescence intensity (MFI) was calculated by averaging all events across the macrophage live cell gate; fold changes were calculated by normalizing the observed MFI to the baseline MFI obtained from RAW cells incubated with zymosan preopsonized with control medium.

2.2.9 Staining of Pneumocystis organisms with Dectin-Fc

PC organisms were heat-fixed to glass slides and incubated with Dectin-Fc-conditioned medium or control medium. After primary incubation, organisms were extensively washed; bound Dectin-Fc was detected by incubating with Cy3-conjugated goat anti-mouse-IgG. Following additional washes, ProLong-mounting medium and coverslips were applied. Slides were examined under a Zeiss Axioplan 2 upright fluorescent deconvolution microscope and images were captured using 3I Slidebook version 4.0 software.

2.2.10 Pneumocystis viability assay

Macrophages (10^6/ml) suspended in a volume of 100 µl of RPMI 1640 medium containing FCS were cocultured in round-bottom 96-well plates with PC (2×10^4 cysts/ml, 50 µl), yielding an effector to total PC organism ratio of 1:1 (estimated 1:10 cyst to trophozoite ratio). Before addition of PC, organisms were preopsonized with 50 µl of Dectin-Fc-conditioned

supernatant/purified Dectin-Fc or 50 µl of DMEM plus 10% FCS. A viability control of PC incubated with control medium, consisting of DMEM plus 10% FCS, was included. The plates were spun at 2500 rpm to pellet PC organisms. The supernatants and cell pellets were collected and total RNA was isolated using TRIzol-LS reagent (Invitrogen Life Technologies). Viability of PC was analyzed with real-time PCR measurement of PC large subunit rRNA copy number (GenBank accession number AF257179) and quantified against a standard curve of known copy number of PC rRNA as previously described (49). This method detects viable PC organisms as evidenced by loss of detectable PC rRNA in heat-killed organisms or those exposed to trimethoprim/sulfamethoxazole.

2.2.11 Adenoviral gene transfer and Pneumocystis challenge

SCID mice (6–8 wk) received adenovirus at 1 x 10^9 PFU periorbitally (total volume 100 µl) under isoflurane analgesia. Three days later, mice were anesthetized and challenged with PC at 2 x 10^5 cysts delivered intratracheally. At days 1, 5, 14, and 28 after PC challenge, mice were sacrificed by terminal exsanguination under isoflurane analgesia; serum and bronchoalveolar lavage fluid (BALF) was collected. Right lungs were preserved for quantification of PC lung burden. A portion of the left lung from individual mice was homogenized, normalized to a total protein content of 1 mg/ml, and MIP-2 concentrations were determined by ELISA (R&D Systems).

2.2.12 Real-time PCR analysis of Pneumocystis infection

Total RNA was isolated from the right lung of infected mice by a single-step method using TRIzol reagent (Invitrogen Life Technologies) as per manufacturer's instructions. Thereafter,

RNA was transcribed to cDNA and real-time PCR was performed as previously described; copy numbers were quantified against a standard curve of known PC rRNA copy numbers (57). This assay has a correlation coefficient of 0.98 with PC rRNA copy number.

2.2.13 BALF analyses

Fluid from the lower respiratory tract was obtained by bronchoalveolar lavage of mice anesthetized with i.p. pentobarbital as described previously (89). The first milliliter of BALF from SCID mice infected with PC was collected at 5, 14, and 28 days was processed at 500 x *g* and supernatant was stored at –80°C until use. BALF samples were studied for Dectin-Fc expression or for relative lactate dehydrogenase (LDH) activity as determined with an LDH colorimetric assay kit (Sigma-Aldrich) detecting the reduction of substrate NAD →NADH. Absorbance was determined at 530 nM. The remaining cell pellet and an additional 9 ml of BALF was pooled and centrifuged at 800 x *g* for 10 min to collect cells and samples were cytospun onto slides, stained by modified Giemsa, and analyzed for leukocyte differential.

2.2.14 Statistical analysis

Data were analyzed using GraphPad statistical software. Comparisons between groups when data were normally distributed were made with the Student *t* test; comparisons among nonparametric data were made with the Mann-Whitney *U* test. The Wilcoxon rank-sum test was used when comparing experimental groups to a theoretical value. Significance was accepted at a value of $p < 0.05$.

2.3 RESULTS

2.3.1 Characterization of Dectin-Fc protein reactivity and structure

A recombinant fusion gene consisting of the extracellular domain of the murine dectin-1 receptor (amino acids 69–244), a thrombin sensitive hexapeptide linker, and the murine IgG1 hinge and CH_2 through CH_3 domains, was constructed and inserted into the eukaryotic expression vector, pSecTag 2C, directing protein secretion. The cloned portion of dectin-1 contains the entire, relatively compact, carbohydrate recognition domain (CRD) of the receptor, which includes two putative *N*-glycosylation sites. Western analysis of the supernatant generated from 293 transfectants revealed a 116-kDa product, reactive with both goat-anti-mouse IgG Abs (Fig. 1*A*) and anti-murine dectin-1 Ab (Fig. 1*B*). Under reducing conditions, the molecular mass of the protein product was halved, demonstrating that the recombinant protein adopts a disulfide-linked bivalent structure (Fig. 1*B*). A schematic portrayal of the fusion protein itself is presented in Fig. 1*C*. As the murine hinge plus Fc domain is 60 kDa, the extracellular domain of dectin-1 is roughly 28 kDa, in accordance with a previous report describing the molecular structure of the receptor (139).

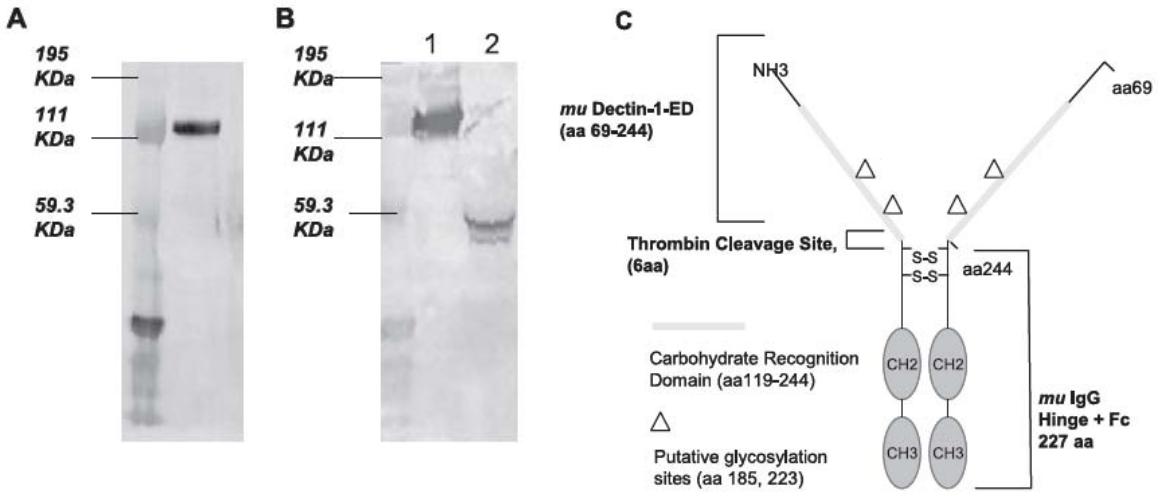


Figure 1. Analysis of Dectin-Fc protein reactivity and structure. 293 cells were transiently transfected with a pSecTag 2C vector containing the Dectin-Fc fusion gene cassette and culture supernatant was analyzed by Western blot. *A*, Reactivity of culture supernatant with goat-anti-mouse Abs. *B*, Culture supernatant reacted with mDectin-1 Abs followed by goat anti-rat IgG-HRP under nonreducing (*lane 1*) and strong reducing conditions (*lane 2*). *C*,

2.3.2 Analysis of Dectin-Fc Ag-binding site

To define the properties of the carbohydrate recognition domain of Dectin-Fc, we assessed its ability to recognize laminarin, a ~7.7-kDa water soluble carbohydrate β-glucan polymer isolated from *Laminaria digitata*. Laminarin contains primarily β-1,3 glucan linkages with some β-1,6 glucan linkages, similar in ratio to the β-glucan linkages found in the cellular wall of *Saccharomyces cerevisiae* and PC (110, 112, 230). In a study where dectin-1 was expressed exogenously in the nonphagocytic NIH3T3 cell line, laminarin was identified as the most potent competitive antagonist of zymosan recognition among a panel of carbohydrates, underscoring the molecular affinity of this carbohydrate for the dectin-1 receptor (144). By ELISA, we observed that Dectin-Fc bound laminarin in a dose-dependent manner (Fig. 2). This effect was entirely lost upon specific hydrolysis of β-1,3 linkages with laminarinase. Thus, the Ag recognition site of Dectin-Fc displays high specificity toward the β-1,3 linkages of the β-glucan laminarin.

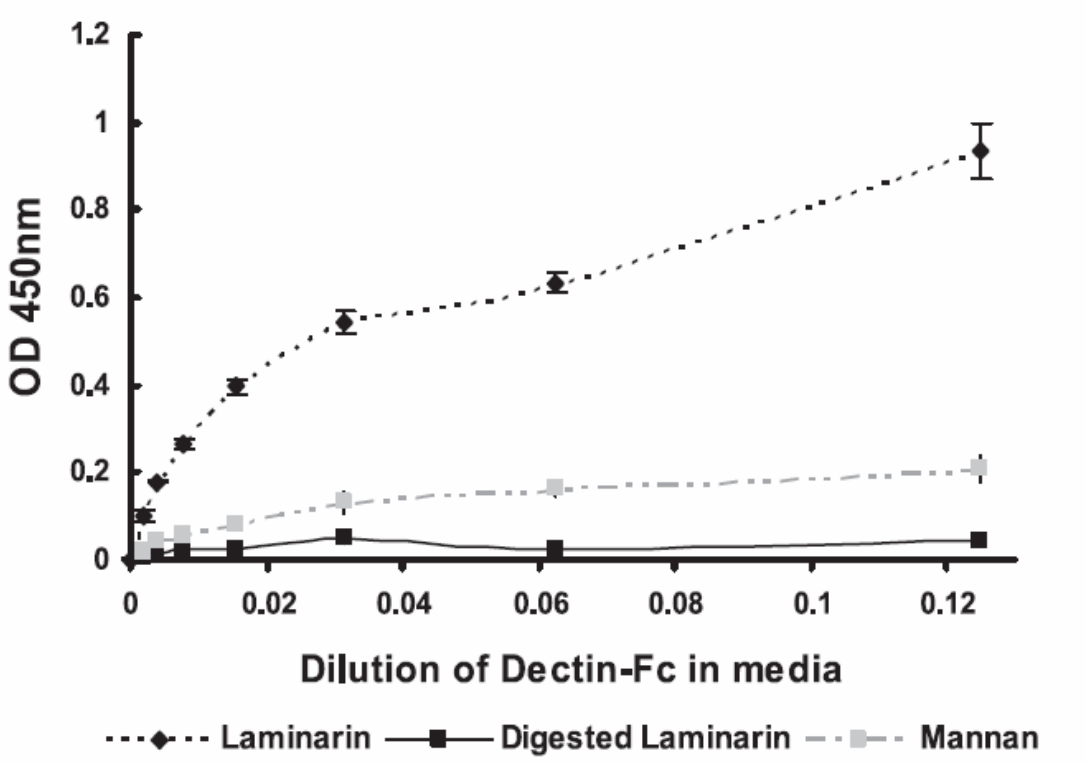


Figure 2. The Dectin-Fc carbohydrate recognition domain identifies the β-glucan laminarin. Laminarin, mannan, or β-1,3 endoglucanase laminarinase-treated laminarin were coated to individual ELISA plate at 25 μg/ml. Dectin-Fc supernatant was applied in a 2-fold dilution series, identified with HRP-conjugated goat anti-mouse IgG1 Abs, and developed with TMB.

We next determined the affinity of the Dectin-Fc carbohydrate recognition domain for β-glucan linkages with real-time surface plasmon resonance measurements (BIAcore). Affinity purified Dectin-Fc was captured with immobilized goat-anti-mouse Abs; laminarin was applied at various doses (0.2, 2, and 100 μM) and studied for interactions with the Dectin-Fc Ag-binding site (Fig. 3). As expected, laminarin bound Dectin-Fc and binding was dose dependent. The rate of association (k_a) was 5.6×10^3 $M^{-1}s^{-1}$ and the rate of dissociation (k_d) was 1.14×10^{-3}/s, signifying that the stability of the complex slightly exceeds its capacity to form. An overall equilibrium dissociation constant (K_D) of 2.03×10^{-7} M was calculated, demonstrating high-affinity binding of Dectin-Fc to laminarin, similar in quality to various carbohydrate directed Abs or lectin receptors for their ligands (231-235).

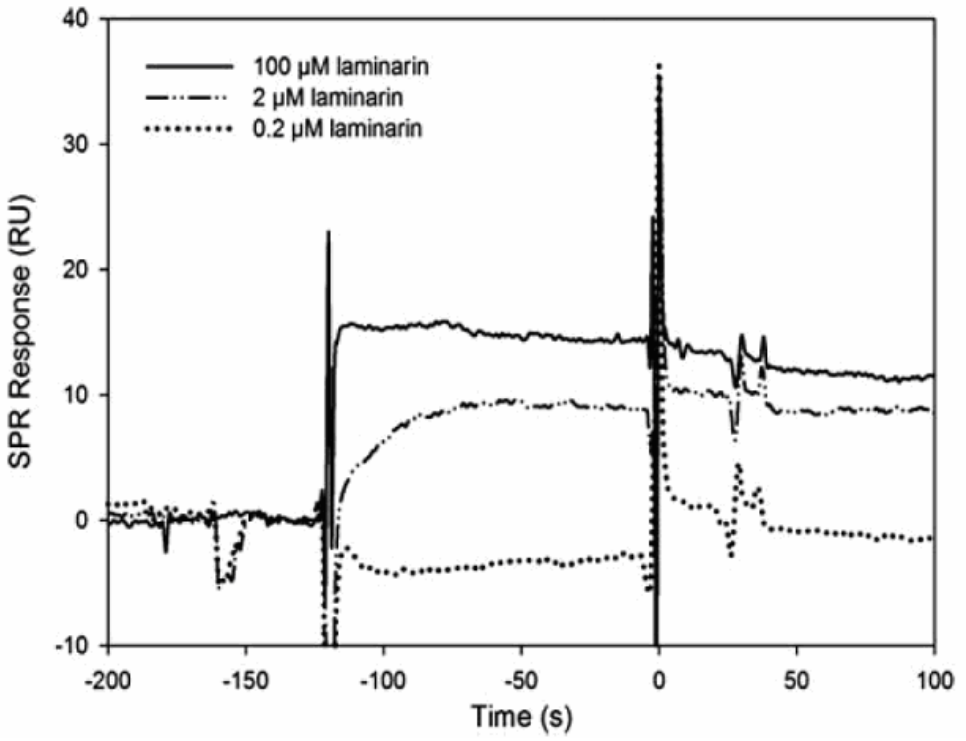


Figure 3. Surface plasmon resonance analysis of the interaction of Dectin-Fc with the beta-1,3 linked glucan laminarin. Binding between Dectin-Fc and laminarin was studied in real-time using a BIAcore 3000 instrument. Goat anti-mouse Abs were chemically immobilized to CM5 chips and used as a capture surface for Dectin-Fc. Three doses of laminarin were injected at time –120 s (association) and replaced with buffer at time 0 s (dissociation). Nonspecific binding, which was minimal, was measured using a control flow cell consisting of immobilized goat anti-mouse Abs and was subtracted from each laminarin binding curve. The interaction between Dectin-Fc and laminarin is characterized by a K_D of 2.03×10^{-7} M.

2.3.3 Dectin-Fc enhances macrophage recognition of zymosan

To address the potential of Dectin-Fc to enhance macrophage recognition of particulate β-glucan, we evaluated whether Dectin-Fc could enhance RAW264.7 macrophage recognition of fungal PAMP-containing particulate zymosan. Zymosan is an insoluble cellular wall polysaccharide derived from *S. cerevisiae*, composed mostly of β-glucan and mannan (236). FITC-labeled zymosan was preopsonized with either Dectin-Fc-conditioned medium, control medium, or normal mouse serum and added to macrophages for 90 min, and macrophage-associated FITC fluorescence was determined by flow cytometry as a correlate of cellular association with zymosan. As demonstrated in Fig. 4*A*, we observed enhanced binding by macrophages of zymosan opsonized with normal mouse serum, which increases baseline binding of zymosan predominantly via CR3-based recognition (145). However, we observed even further enhancement of macrophage recognition when zymosan was instead preopsonized with Dectin-Fc (Fig. 4*A*). Analysis of the average macrophage MFI showed that recognition of zymosan by RAW cells was significantly inhibited by Dectin-1 receptor blocking Ab 2a11, as previously reported (145), and that Ab-mediated blockade of FcγRII and FcγRIII did not perturb FITC-zymosan association from baseline levels (Fig. 4*B*). Yet, Dectin-Fc enhanced the average cellular MFI by 27% from baseline levels (Fig. 4*B*), and the observed increased recognition of Dectin-Fc preopsonized zymosan was entirely lost upon blockade of macrophage FcγRII and FcγRIII (Fig. 4*B*). As murine IgG1 immune complexes signal primarily through FcγRII and FcγRIII (237), we conclude that Dectin-Fc-opsonized zymosan was recognized through binding at these receptors.

Interestingly, when the Dectin-1 receptor on macrophages was blocked, recognition of Dectin-Fc preopsonized zymosan was further enhanced (Fig. 4*B*). Although the mechanism through which this occurs is unclear, it raises the possibility that immune-complex associated, multimerized FcRs generate stronger or more prolonged macrophage associations with zymosan relative to that which occurs under competitive binding with the Dectin-1 receptor. Thus, Dectin-Fc enhances recognition of zymosan by macrophages, and in the absence of cellular Dectin-1 receptor recognition, by promoting binding through FcγRII and FcγRIII.

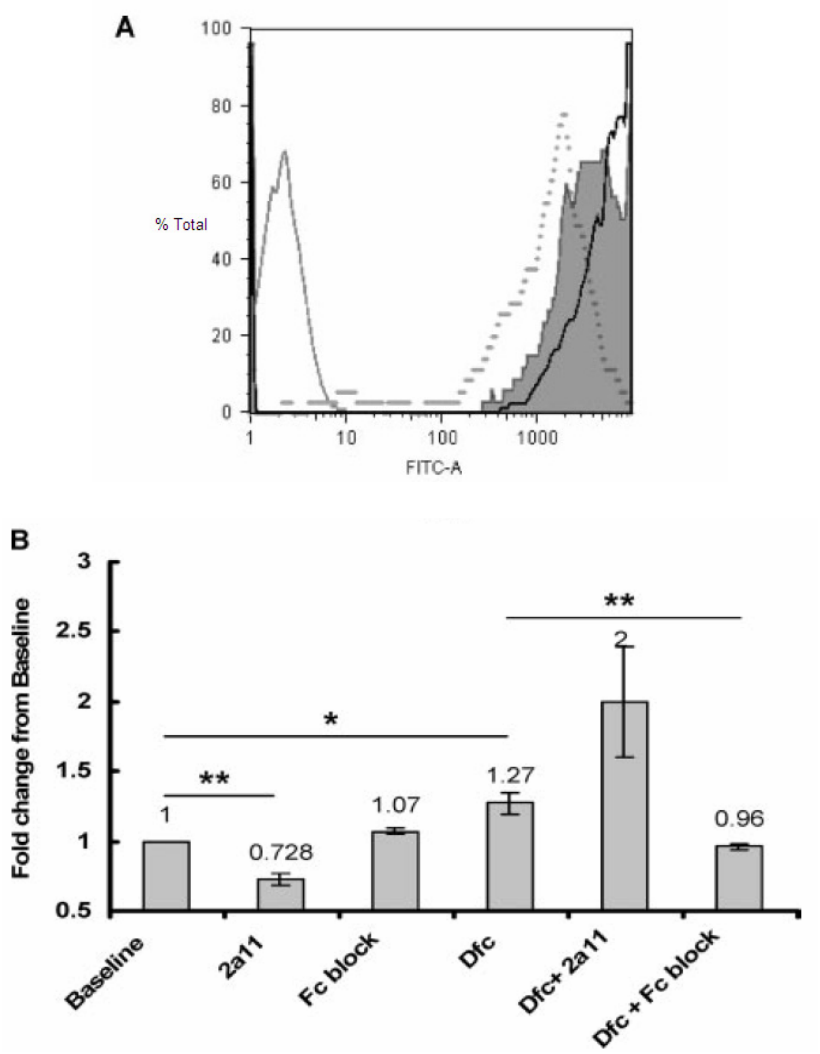


Figure 4. Dectin-Fc enhances macrophage recognition of zymosan. FITC zymosan was preopsonized with either control medium, serum, or Dectin-Fc-conditioned culture medium and administered to RAW 264.7 macrophages for 90 min. 2a11 and anti-CD16/32 Abs were preadministered to macrophages to block the dectin-1 receptor or FcγII/FcγIII, respectively. A total of 10,000 cells were collected and analyzed by flow cytometry for association with FITC particles. *A*, Histogram plot from zymosan association assay showing baseline fluorescence of macrophages (gray line), macrophages plus FITC-zymosan opsonized with control medium (dotted line), macrophages plus FITC-zymosan opsonized with serum (gray line, filled), and macrophages plus FITC-zymosan opsonized with Dectin-Fc (black line). *B*, Cumulative results from five separate experiments investigating the effects of Dectin-Fc on FITC-zymosan binding to RAW 264.7 macrophages, normalized to baseline MFI. For comparisons to the baseline condition, $*$ represents a $p < 0.05$ and $**$ represents a $p < 0.01$ as calculated with the Wilcoxon rank-sum test after setting the baseline at a theoretical value of 1. For comparisons between Dectin-Fc and Dectin-Fc plus Fc block, $**$ represents a $p < 0.05$ by Student's *t* test.

2.3.4 Dectin-Fc binds *Pneumocystis* organisms and enhances macrophage-dependent killing

The cellular wall of the PC cyst consists of a thick electron-lucent layer composed predominantly of β-glucan, buried below a thin, electron-dense surface layer consisting mainly of mannan and glycoprotein (111, 112). Despite a seemingly shielded location, it is believed that PC β-glucan is sufficiently exposed to permit recognition by the innate immune system via Dectin-1 (49) and other pattern recognition receptors, such as the lactosylceramide-associated β-glucan receptor (63). Indeed, the specific interaction of PC β-glucan with these innate receptors has been demonstrated to influence patterns of cytokine and chemokine expression, such as the elicitation of the neutrophil chemoattractant MIP-2. By fluorescent deconvolution microscopy, we observed that Dectin-Fc bound to the surface of PC cysts (Fig. 5*A*). This observation demonstrates the accessibility of Dectin-Fc to β-glucan ligands on PC organisms.

As we observed specific binding of Dectin-Fc to PC organisms, we evaluated whether Dectin-Fc could enhance the recognition and degradation of *Pneumocystis* organisms by FcγRII/III-bearing cells. We studied decreases in absolute quantities of PC large rRNA copy numbers in the presence or absence of macrophages as a correlate of in vitro PC killing, a methodology validated by previous work (49, 87). PC organisms were isolated from infected murine lung homogenates and were preopsonized with Dectin-Fc, then administered to macrophages at a 1:1 PC organism to effector cell ratio for 24 h. In studies with thioglycolate-elicited peritoneal macrophages, preopsonization of PC organisms with Dectin-Fc diminished overall copy numbers by 3-fold relative to organisms preopsonized with control medium (Fig.

5*B*). When macrophages were pretreated with an Ab blocking activity at the Dectin-1 receptor, killing of Dectin-Fc preopsonized PC occurred to a similar degree. This observation suggested that Dectin-Fc promoted PC killing independent of native Dectin-1 recognition. Additional blockade of FcγRII and FcγRIII abrogated the killing effect, indicating that Dectin-Fc-dependent targeting of PC toward FcγRII and FcγRIII was responsible for the diminished PC rRNA signal. Resident alveolar macrophages appeared to have a moderately higher level of effector activity against Dectin-Fc preopsonized PC compared with recruited peritoneal macrophages, decreasing PC rRNA copy numbers by nearly 10-fold relative to medium opsonized PC (Fig. 5, *C* and *D*). We hypothesize that differences between alveolar and elicited peritoneal macrophage populations in responses such as phagocytosis, reactive oxide species generation, and Ab-dependent killing may be responsible for the enhanced effector function against Dectin-Fc preopsonized PC by alveolar macrophages. We conclude from these studies that primary murine macrophages have enhanced recognition and killing of Dectin-Fc preopsonized PC that is mediated via FcγRII and FcγRIII and this occurs independently of dectin-1-based recognition.

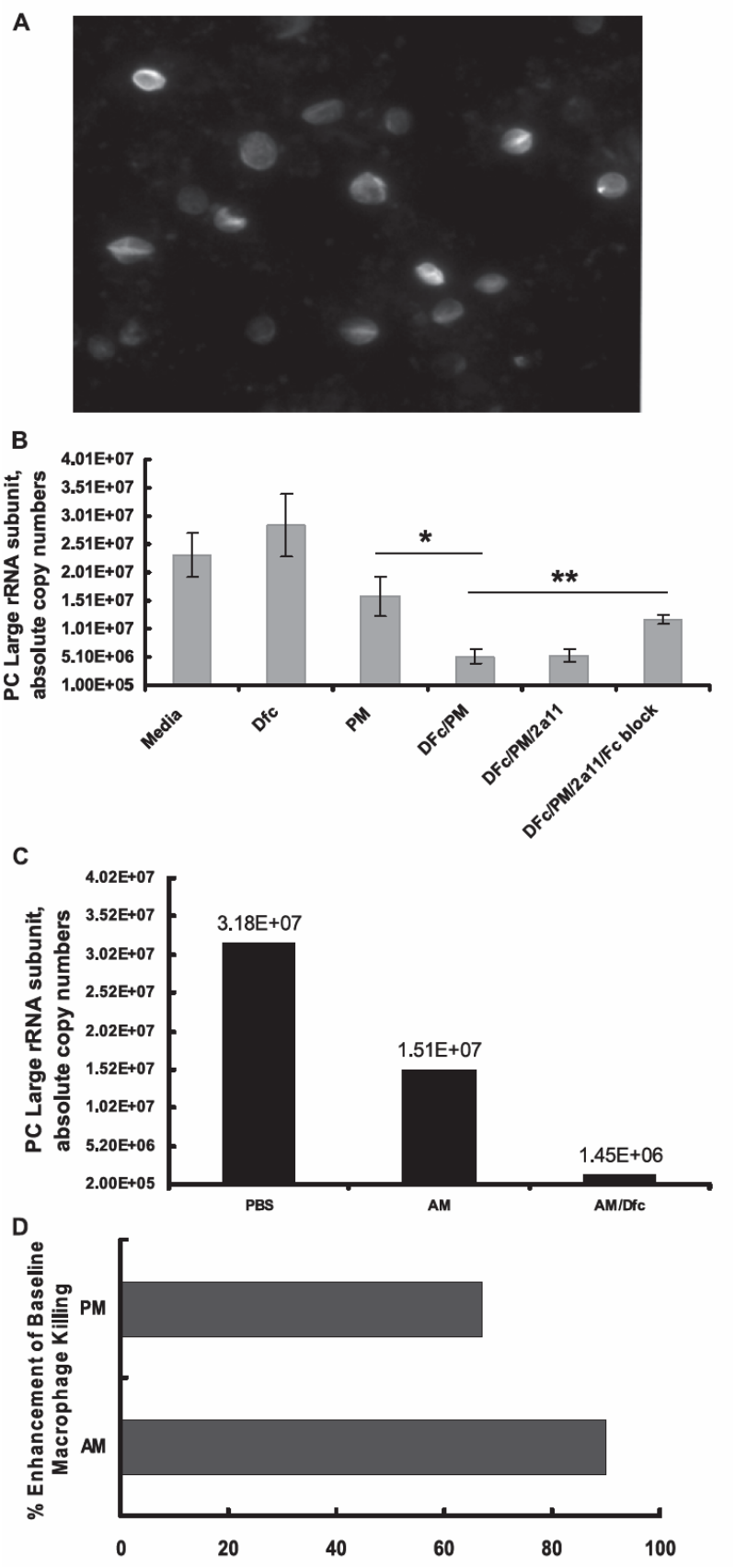


Figure 5. Dectin-Fc enhances macrophage killing of PC. *A*, Immunofluorescence patterns of Dectin-Fc-conditioned medium followed by goat anti-mouse Cy3 on heat-fixed preparations of PC cysts. No specific fluorescence was detected with control medium (data not shown). Original magnification, x630 oil emersion. *B*, PC was preopsonized with Dectin-Fc or control medium and added at a 1:1 PC organism to effector cell target ratio to elicited peritoneal macrophages from male BL/6 mice. PC alone (medium) and PC in the presence of Dectin-Fc (Dfc) was used as a control for PC viability. Total RNA was harvested, cDNA was synthesized, and viability was assessed through real-time PCR measurement of PC large subunit rRNA copy number. Data are expressed as mean copy number ± SEM from three experiments. *, A p value of <0.05; **, a p value of <0.01 by Student's t test. *C*, A similar assay was performed with alveolar macrophages and affinity purified Dectin-Fc at 10 µg. The figure shows representative data from three experiments. Data are expressed as PC large subunit rRNA copy number. *D*, Effect of Dectin-Fc on macrophage killing summarized as a percent of baseline macrophage effector function, calculated as 1 – (Dectin-Fc + macrophage killing/macrophage killing) x 100%.

2.3.5 Dectin-Fc enhances clearance of *Pneumocystis* in SCID mice

To study the effect of Dectin-Fc on PC infection in vivo, we developed a replication incompetent type 5 adenovirus, Ad-Dectin-Fc, containing the entire Dectin-Fc expression cassette, including an Ig$_{\kappa}$ leader sequence facilitating protein secretion upon cellular transduction. As IgG protein administered directly into the lungs is rapidly degraded (238), we considered adenoviral-based transgene delivery of Dectin-Fc as a more appropriate system to evaluate the effect of Dectin-Fc on this chronic, slow-growing infection. Systemic administration of adenoviral vectors encoding transgenes leads to high and prolonged levels of transgene protein in the lungs in the absence of virus (239) and mice deficient in $CD4^+$ T cells do not mount significant neutralizing Ab responses against adenoviruses (240), underscoring the suitability of gene transfer as an effective means for long-term expression of Dectin-Fc in the setting of immunodeficiency. SCID mice were treated with either Ad-Dectin-Fc or an identical adenovirus expressing firefly luciferase (Ad-luciferase), intravenously, at 1×10^9 PFU. Serum and lung homogenate were analyzed for Dectin-Fc protein expression at various points after adenoviral treatment (Fig. 6*A*). The fusion protein was detected in mice at high levels in both compartments by 4 days after treatment through at least 31 days after treatment. It is well-established that IgG crosses mucosal barriers, presumably through transcytosis or interactions with FcRn (241), to function in secretions. Dectin-Fc was also observed in the BALF, demonstrating the presence of the fusion protein within the mucosal surfaces and within compartments typical of murine IgG.

To assess the ability of Dectin-Fc to enhance host clearance of PC, SCID mice were treated with Ad-Dectin-Fc or Ad-luciferase i.v. and rested for 3 days, followed by intratracheal

challenge with 2 x 10^5 PC cysts. Mice were then sacrificed at specific time points after challenge and studied for PC burden within the lungs by assessment of total copy numbers of PC large rRNA subunit (Fig. 6*B*). Despite equivalent cyst counts for the inoculum between studies, PC rRNA counts were significantly different and this may be due to the challenge of quantifying noncystic PC forms. In a first experiment, 14 days after PC challenge, an average of 25% of the original inoculum is present in the Ad-luciferase-treated mice while the Ad-Dectin-Fc-treated mice contained only an average of 6.9% of the original inoculum. In a second experiment, 28 days after PC infection, mice treated with Ad-luciferase contained a substantially higher quantity of PC in the lungs at an average of 549% of original inoculum relative to the Ad-Dectin-Fc-treated mice (averaging at 139% of the inoculum). A third study, performed where a single inoculum was administered and mice were analyzed at serial time points, confirmed the inhibition of the growth kinetics of the PC organism within the lungs of mice receiving a control vector compared with mice receiving Dectin-Fc (Fig. 6*C*). Hence, treatment of mice with Dectin-Fc before PC infection considerably diminished the growth of the organism within the lungs, contributing to the ability of the SCID host to prevent PC multiplication within the lung environment. The efficacy of Dectin-Fc to inhibit PC growth is most apparent at later stages of PC infection, as innate host clearance mechanisms lose their capacity to control PC replication within the lungs.

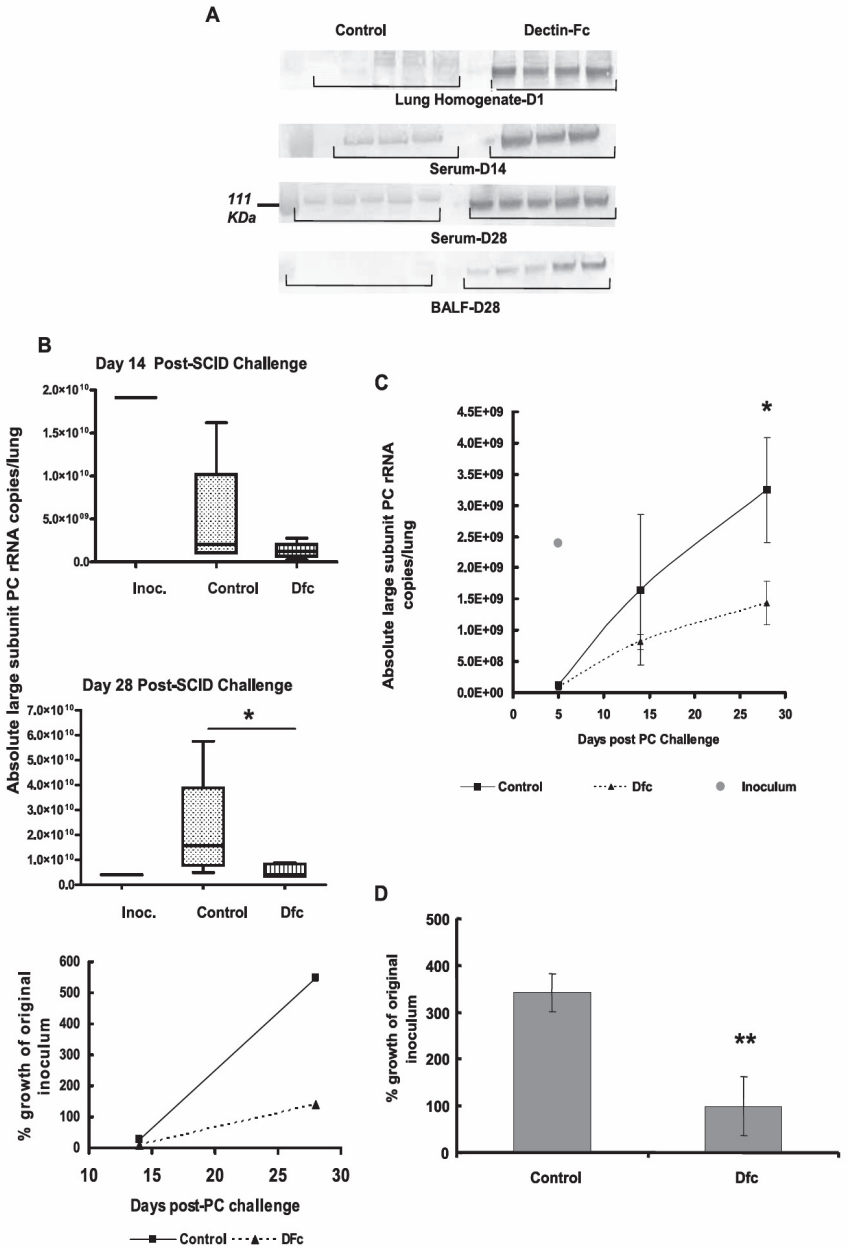


Figure 6. In vivo activity of Dectin-Fc in SCID mice challenged with PC. *A*, A replication incompetent Ad5 vector containing the entire pSecTag2 C Dectin-Fc expression cassette was generated. SCID mice were i.v. treated with 1 x 10^9 PFU Ad-Dectin-Fc or control vector Ad-Luciferase; Dectin-Fc expression was assessed in various compartments at given time points after treatment. Individual mouse samples were studied for reactivity with mDectin-1 Abs at 116 kDa. *B*, Three days post-gene transfer (n = 5/condition/time point), mice received 2 x 10^5 cysts i.t. and at specific time points thereafter, lungs were analyzed for total PC large rRNA subunit copy numbers. Days 14 (*top*) and 28 (*middle*) are displayed. Boxes represent the interquartile range of data between the 25th and 75th percentiles and whiskers represent the upper and lower limits of the data. The median is represented by the dividing line within the box. *Bottom*, Average copy numbers of PC organisms within the lungs of individual SCID mice normalized to the original inoculum per time point, plotted over time. *C*, To confirm inferences on kinetic growth a single inoculum was administered to mice (n = 5/condition/time point), lungs were harvested at various time points, and total PC large rRNA copy numbers are displayed. *D*, Data pooled from both SCID mice studies, day 28 after PC challenge (n = 10/time point/group), with individual PC lung burdens expressed as a percentage of total inoculum. In all frames, * represents a p value <0.05, and ** represents a p value of <0.01 between mice receiving Ad-Luciferase (control) and Ad-Dectin-Fc (Dfc) by Mann-Whitney U test.

2.3.6 Effect of Dectin-Fc on correlative markers of lung injury in Pneumocystis infected SCID mice

Although hyperinflammatory responses mediated by $CD8^+$ T cells generate significant lung damage and dysfunction in the murine response to PC (242), SCID mice suffer lung damage at late stages of pneumonia as a consequence of uncontrolled PC replication and immunodysregulation (60, 243). We assessed the capacity of Dectin-Fc to limit correlates of pulmonary damage associated with PC infection by assessing the presence of intracellular enzymes in the alveolar fluid. BALF collected from the study described in Fig. 6*C* was analyzed over the time course of infection for LDH activity. We observed significantly decreased levels of BALF LDH in mice treated with Dectin-Fc, compared with control mice, even as early as 5 days after PC challenge, when there is low PC burden (Fig. 7*A*). As a significant portion of the PC inoculum is cleared soon after intratracheal instillation, as we (Fig. 6*C*) and others previously observed (97), it is possible that targeting of PC to myeloid cells via FcγR-dependent host recognition contributed to this phenotype. Twenty-eight days after PC challenge, when there is a significantly higher PC burden in the lungs of control mice relative to Dectin-Fc-treated mice, BALF LDH was nearly twice as high in control mice relative to mice receiving Dectin-Fc, suggesting that Dectin-Fc limited both PC burden and cellular damage associated with infection. Also by day 28 postchallenge, there were significant differences in the quantities of neutrophils observed in the BALF, with decreased levels in mice receiving Dectin-Fc (Fig. 7*B*). It has been shown that SCID mice recruit high numbers of neutrophils into the lungs at late stages of PC infection which is correlative with lung damage (60) and in humans with PC pneumonia, increased BALF neutrophil counts are associated with poor prognosis (27, 244). MIP-2, a

neutrophil chemoattractant secreted by alveolar macrophages and airway epithelial cells in response to PC 3-glucan (49, 63), and TNF-α, a proinflammatory cytokine also secreted by alveolar macrophages in response to PC 3-glucan (52), were also measured in the lung homogenate. Though the differences were not statistically significant, we observed a trend toward higher levels of both cytokines in the lungs of control mice relative to Dectin-Fc-treated mice (e.g., MIP-2 averaging 155 ± 57 pg in the control group, 60 ± 21 pg in the Dectin-Fc group; TNF-α levels were similar). Taken together, these data suggest that correlates of pulmonary damage associated with PC infection are limited by Dectin-Fc and this may be due to enhanced clearance of PC and/or direction of PC organisms toward FcγR-dependent recognition and degradation within the lungs.

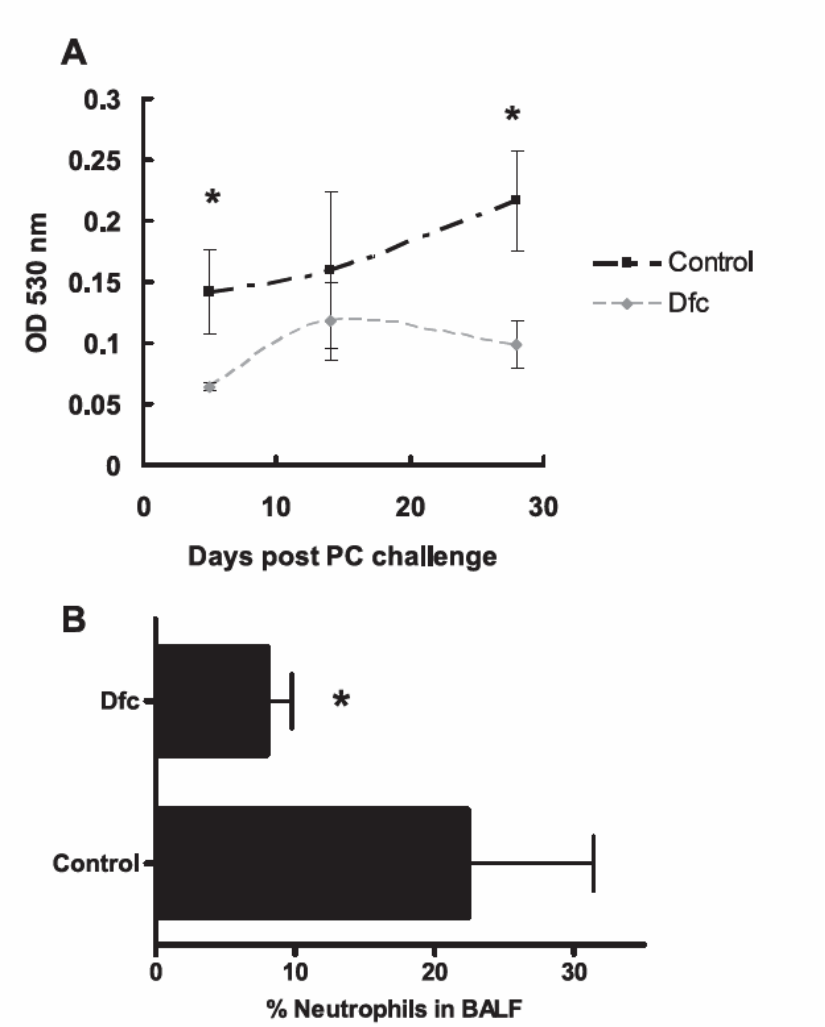


Figure 7. Correlates of PC-related lung damage after Dectin-Fc treatment. SCID mice were i.v. treated with 1 x 10^9 PFU Ad-Dectin-Fc or control vector Ad-Luciferase. Three days post-gene transfer, mice received 2 x 10^5 PC cysts i.t., and at specific time points thereafter, protein and cellular components of the BALF was studied. *A*, BALF from mice was analyzed for LDH activity over the time course of PC infection. Data are expressed as mean OD ± SEM from five mice in each group per time point. *, A p value <0.05 between mice receiving Ad-Luciferase (control) and Ad-Dectin-Fc (Dfc) by Student's t test. *B*, Alveolar cells from BALF were analyzed on day 28 of PC infection for the percent of neutrophils by cytospins. Data are expressed as mean neutrophil percentage ± SEM from five mice in each group. *, A p value <0.05 between mice receiving Ad-Luciferase (control) and Ad-Dectin-Fc (Dfc) by Mann-Whitney U test.

2.4 DISCUSSION

The β-glucan receptor Dectin-1 is critically involved in innate immune responses against PC (49), as well as *Candida albicans* (245, 246) *Aspergillus fumigatus* (55, 247), and *Coccidioides posadasii* (248). Two recent reports show that Dectin-1-deficient mice have compromised clearance of PC (138) and *C. albicans* (249) as well as attenuated macrophage responses to these fungal organisms. In these fungal pathogens, β-glucan is the major component of the inner cellular wall, contributing to cellular rigidity and structure, and eliciting host proinflammatory responses upon exposure to the innate immune system (250). Notably, the PC cyst contains high amounts of cellular wall β-glucan while the trophozoite form does not (112, 251, 252). Yet, the echinocandins, a class of synthetically modified lipopeptides that inhibit the fungal-specific enzyme β-1,3-glucan synthase, rapidly diminish PC organisms in rodent models of infection (114, 252). Given the abundance and apparent accessibility of PC β-glucan, as well as its potentially critical role in the fungal life cycle, we considered immune-based strategies targeting this defined cell wall component as a novel therapy for PC pneumonia.

As macrophages overexpressing Dectin-1 have enhanced recognition and uptake of PC organisms relative to normal macrophages, we hypothesized that the CRD of Dectin-1 might be a sufficient targeting structure for PC identification. Immunoprotective Abs against PC enhance effector function at the level of the myeloid cell and, as production of PC-specific IgG1 is most perturbed by murine $CD4^+$ T cell dysfunction (253), we chose to couple the Dectin-1 CRD with the murine IgG1 Fc fragment to promote recognition and signaling through FcγRs. We expected

that Dectin-Fc would function similarly to an Ab and would promote host recognition and clearance of PC organisms by enhancing the effector function of Fc7R-bearing cells.

The recombinant protein, Dectin-Fc, demonstrated specific binding to laminarin, a 3-glucan-containing glycosidic linkages analogous in stoichiometry to that observed in the PC cyst wall (112). Hydrolysis of 3-1,3 linkages of laminarin entirely blocked Dectin-Fc recognition, indicating its specificity for these glycosidic bonds. Characterization of the Dectin-Fc CRD with surface plasmon resonance measurements revealed a K_D of 2.03 x 10^{-7} M for laminarin, suggestive of high-affinity interaction. The observed affinity of Dectin-Fc for 3-glucan is similar, if not somewhat higher, than that observed for lectin receptors; this may be attributable to the dimeric nature of the fusion protein. For example, the interaction of SIGN-R, a major mannose receptor on peritoneal macrophages, with terminal mannose epitopes is characterized by a K_D of 9 x 10^{-6} M (231) while E-selectin, a receptor mediating tethering of granulocytes to the vascular membrane via recognition of E-selectin-ligand-1, possesses a K_D of 6.2 x 10^{-5} M (232). The Ag-binding sites of anti-carbohydrate Abs, such as those directed against either *Chlamydia* or *Shigella* LPS O Ags, possess K_D values ranging from 10^{-5} to 10^{-6} M (233) while murine anti-carbohydrate Ab responses from conjugate vaccines against meningococcemia yielded K_D estimates ranging from 10^{-6} to 10^{-9} for Ag (235) The affinity of Dectin-Fc for 3-glucan, thus, falls within a range typical for anti-carbohydrate Abs and lectin receptors for their ligands.

Preopsonization of zymosan with Dectin-Fc enhanced baseline macrophage recognition of these particles and this phenotype was lost upon blockade of the primary receptors for murine IgG1, Fc7RII, and Fc7RIII. Our study demonstrates the sufficiency of the Dectin-1 CRD as a targeting epitope for particulate 3-glucan in vitro and is in accordance with evidence suggesting that TLR2 and TLR6, receptors that cooperate with dectin-1 and regulate MyD88- and NF-B-

dependent signaling in response to zymosan, are not required for the actual β-glucan recognition event mediated by Dectin-1 (254). As anticipated, Dectin-Fc bound to PC cysts, demonstrating that the PC cell wall β-glucan is accessible and recognized by this fusion protein. Our observation is in line with studies demonstrating an abundance of PC β-glucan in the inner cyst wall (112) and previous observations that overexpression of the Dectin-1 receptor in RAW macrophages enhanced binding and recognition of PC cysts (49). We have previously shown that Dectin-Fc binds *A. fumigatus* swollen conidia (55) and our current study now extends the immunolocalization of Dectin-Fc to PC cysts.

Dectin-Fc increased killing of PC by alveolar and elicited peritoneal macrophages and such levels were sustained when macrophage Dectin-1 receptors and were blocked. Apparent efficacy was lost when FcγRII and FcγRIII were additionally blocked, demonstrating that Dectin-Fc targets PC for degradation through these receptors. The importance of alveolar macrophages in PC degradation and clearance is well-documented (47, 55, 255), yet optimal macrophage-dependent clearance requires coordination with B and T cell-dependent responses against PC, as macrophages and other innate cells are ultimately insufficient in the control of infection. The development of a fusion protein capable of recognizing PC β-glucan coupled with the capacity for FcγR-dependent targeting represents a novel mechanism for the enhancement of baseline macrophage effector function against PC, in the absence of B and T cell-dependent immune responses against the pathogen.

Adenoviral delivery of Dectin-Fc in SCID mice significantly reduced the kinetics of PC growth and overall PC burden within the lungs by 28 days postchallenge and led to decreased pulmonary LDH and BALF neutrophils, correlates of PC-related lung damage. These findings demonstrate that Dectin-Fc promotes PC recognition and clearance in vivo and increases the

specificity of the immune response, presumably through enhancement of FcγR-dependent clearance mechanisms directed by macrophages. Because the ability of Dectin-Fc to inhibit PC growth is most apparent at late stages of PC infection, as innate host clearance mechanisms lose their capacity to control PC replication within the lungs, our studies suggest that Dectin-Fc may improve the clearance of existing PC lung infections.

Though there was significantly altered growth and an overall reduction of PC burden in SCID mice treated with Dectin-Fc, the establishment of infection could not be prevented. This observation may be a consequence of the expression pattern and relative exposure of β-glucan by PC in the context of the lung environment. Although we have shown that PC β-glucan can be targeted in vitro by macrophages both in this study and in previous work (49), β-glucan may not be present or sufficiently exposed by the as-yet uncharacterized infectious form(s) of the organism, leading to early escape from Dectin-Fc-dependent recognition. In the course of PC infection, soluble β-glucan is shed into the blood and BALF (256)and this may additionally contribute to evasion mechanisms inhibiting β-glucan-dependent recognition of PC organisms. Further, pulmonary collectins target fungal cellular wall carbohydrates and these may compete with Dectin-Fc for binding to PC β-glucan. In particular, surfactant protein D (SP-D) targets β-1,6-linked glucan (257) and recognition of these PC cell wall epitopes by SP-D may inhibit binding of Dectin-Fc to its primary target, β-1,3-linked glucan. Notably, SP-D-deficient mice clear PC more rapidly than wild-type mice (74) and while SP-D is capable of binding PC, it appears to inhibit macrophage internalization (258), underscoring the advantages of harnessing macrophage effector function in β-glucan targeting strategies.

Abs and IgG Fc-based targeting molecules directed at fungal cellular wall carbohydrates are emerging as important mediators of host defense against a various fungal species. Natural

IgG Abs directed against mannan have been shown to enhance complement protein 3 binding to *C. albicans*, through both direct and alternative pathways (259, 260). Others have demonstrated that a mannose receptor human IgG1 fusion protein increased internalization of PC organisms by human polymorphonuclear cells (261), underscoring the ability of carbohydrate-directed Fc-fusion proteins to enhance myeloid cell function. In a recent study, a novel vaccine, consisting of laminarin conjugated to a diphtheria toxoid carrier, generated β-glucan specific IgG that protected against vaginal candidiasis as well as lethal hemogenous challenges of *C. albicans* and *A. fumigatus* in mice (154). Interestingly, as β-glucan-specific IgG diminished growth of these fungal species in the absence of effector cells in vitro, one mechanism through which these Abs may function is via direct antimicrobial effects. This study demonstrated that high-affinity β-glucan directed IgG, presumably requiring $CD4^+$ T cell-dependent B cell costimulation, targeted antigenically conserved β-glucan to enhance host clearance of two very different fungal pathogens. Our studies demonstrate that an additional fungal pathogen, PC, can be effectively targeted via β-glucan recognition with an IgG1 Fc-fusion protein, increasing macrophage killing of PC organisms and significantly reducing PC burden within the lungs of SCID mice.

Models of vaccination against PC have demonstrated that immunity carried by Abs can protect $CD4^+$ T cell-deficient hosts (97) and strategies to develop Ab responses against PC within the actual setting of $CD4^+$ T cell deficiency are emerging (57, 92). Targeting PC β-glucan with Dectin-Fc by passive vaccination, as demonstrated here through adenoviral delivery, may complement these evolving approaches. By targeting PC specifically to FcRs on myeloid cells such as dendritic cells, Dectin-Fc could potentially improve aspects of PC Ag presentation to residual $CD4^+$ T cells. Also, as Dectin-Fc recognizes what is thought to be the most inflammatory component of fungal cell wall (250) and recognition of PC by dectin-1 leads to the elicitation of

neutrophil chemotactic factors such as MIP-2 (49), this fusion protein could function to limit potentially detrimental host responses to PC pneumonia, such as excessive neutrophil recruitment into the lungs (244).

Our work supports the concept that Ab-dependent targeting of β-glucan may be a promising strategy against PC infection in the immunocompromised host. Continued effort to understand the potential of fungal carbohydrates as targets of protective immunity, or as Ags in the generation of protective immunity, may inform on optimal vaccination strategies against PC pneumonia.

3.0 IDENTIFICATION OF NATURAL IGM ANTIBODIES TARGETING CONSERVED FUNGAL CELL WALL CARBOHYDRATES AND FUNCTIONS OF NATURAL ANTIBODIES AND IGM IN HOST DEFENSE AGAINST *PNEUMOCYSTIS*

3.1 INTRODUCTION

Opportunisitic fungal pathogens, such as *Pneumocystis jirovecii*, cause severe, often fatal diseases among immunosuppressed individuals. As host defense against fungi is significantly influenced by the functions of CD4+ T cells, and susceptibility to fungal pathogens largely correlates with deficiencies in cell-mediated immunity, the functions of B cells and antibodies in host defense against fungi have been considered to be of less relevance. However, host defense against *Pneumocystis* pneumonia requires the concerted function of CD4+ T cells and B cells in murine models of infection (43, 91). While antibody production is not absolutely required for host defense against *Pneumocystis* pneumonia (96), antibodies produced in vaccinated murine hosts are capable of significantly inhibiting the growth of infection (82, 97, 167), notably without hyperinflammatory responses associated with CD4+ T cell-dependent clearance (82), and can enhance alveolar macrophage effector function against *Pneumocystis* organisms (49, 92). However, the specificites of protective antibodies, the relevance and function of particular

isotypes, and their mechanisms of enhancing *Pneumocystis* degradation or inhibiting *Pneumocystis* growth are not well characterized.

Fungal pathogens contain conserved cell wall carbohydrates β-glucan, chitin, and mannan that may be targets of multiple host defense pathways. *Pneumocystis* sp., while atypical fungi in a number of structural and biochemical aspects, contain these conserved fungal cell wall carbohydrates within their cell walls as demonstrated by immunohistochemical and biochemical studies (111-113, 117, 137, 262). Additionally, some of these carbohydrates have been investigated for their importance in innate recognition by pattern recognition receptors such as the mannose receptor (48), and the β-glucan receptors dectin-1 (49, 138) and lactosylceramide CDw17 (63). 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 *Pneumocystis jirovecii* and other opportunistic fungal pathogens (256, 263, 264). However, the possibility that the fungal cell wall carbohydrates of *Pneumocystis* are targeted by antibodies has not been investigated.

Natural antibodies are predominantly of the IgM isotype, generated without the requirement for exogenous antigenic stimulation, and are primarily produced by the B-1 B cell subset. Here, we present evidence for the existence of natural antibodies targeting fungal cell wall carbohydrates, and examine the role of natural antibodies and the IgM isotype in host defense against the pulmonary mucosal infection *Pneumocystis*.

3.2 MATERIALS AND METHODS

3.2.1 Mice

BALB/c mice, C57BL/6J mice, and BL/6.*scid* mice, 6-9 weeks in age, were purchased from Jackson Immunoresearch Laboratories, and BALB/c.*scid* mice 6-9 weeks of age were obtained from Taconic Farms. Mice with a deficiency in the ability to secrete IgM (sIgM (-/-)), but able to express the membrane bound form of IgM, on the C57/BL6J background were obtained from M. Diamond (Washington University, St. Louis) and were previously described (265). The parent strain was originally developed by Jianzhu Chen (MIT, Cambridge) and Michael Carroll (Harvard Medical School, Boston), and are described (196, 266). Mice were age and sex matched within individual studies. All mice were maintained in a specific pathogen-free environment in microisolator cages within the animal care facilities of Children's Hospital of Pittsburgh under protocols reviewed and approved by the Animal Research and Care Committee. Mice were provided with water and food ad libitum and received 12-h light/dark cycles.

3.2.2 Serum Samples

Serum samples from channel catfish, *Ictalurus punctatus,* were obtained from Eva Bengten (University of Mississippi). Catfish (2kg) were obtained from the wild by angling (Ross Barnett reservoir, MS) or from USDA-ARS Stoneville, MS, and maintained in individual tanks until bleeding by caudal venipuncture. Catfish, among the earliest evolved of jawed vertebrates and hence among the first animals with adaptive immune systems, are telosts (bony fish) and contain a tetrameric homolog of IgM as well as an IgD homolog in the serum.

Serum samples from C57BL/6 mice reared in germ-free conditions were obtained from Scott Plevy (University of North Carolina); and Daniel Peterson and Jeffrey Gordon (Washington University). Mice reared in germ-free conditions were fed standard autoclavable chow and bled at 3-4 months of age. For longitudinal studies with mice, small volumes of blood were collected from the periorbital sinus. At terminal sacrifice of mice, blood was obtained from caudal venipuncture under anesthesia. For all studies, serum was isolated from collected blood by use of serum separator tubes.

Human cord blood serum was collected from full-term newborn patients after informed parental consent at Magee-Women's Hospital, Pittsburgh, according to University of Pittsburgh Institutional Review Board guidelines.

Pooled serum for adoptive serum transfer studies was obtained from specific-pathogen free mice BALB/c or BALB/c.*scid* mice aged 6-8 weeks, via the caudal vena cava exsanguination, heated for 1 hr at 56 degrees C° to inactivate complement proteins, and stored at -80 until use.

3.2.3 Detection of antibody responses against Beta-glucan, chitin/chitosan, mannan, and *Pneumocystis*

Pneumocystis (PC) antigen was prepared as previously described (57) and derived from the lungs of infected BALB/c.*scid* and C57BL/6.*scid* mice by differential centrifugation to derive organisms, which were then sonicated. PC antigen was normalized to protein concentration after sonication of organisms. PC antigen was dissolved in carbonate buffer, pH 9.5, and seeded to Nunc-Polysorp 96 well plates at a concentration of 1 μg/mL. Laminarin (Sigma), derived from

the brown algae *Laminaria digitata*, is composed primarily of β-1,3 linked glucan, the predominant β-glucan linkage found in fungal cell walls (102). Medium molecular weight chitosan (Sigma), derived from crab shells, is polymer of chitin that is 75-85% deacetylated. Chitosan/chitin was dissolved in 2% acetic acid/PBS (v/v) at a concentration of 0.5-1mg/mL and thereafter diluted into PBS to final quantity of 25 μg/mL. α-1,6 linked mannan (Sigma) derived from *Saccharomyces cerevesiae* was dissolved in PBS. Carbohydrate antigens were seeded at a concentration of 25 μg/mL to Nunc-Polysorp 96 well plates and kept overnight at 4 degrees. 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 was applied in serial dilutions. BALF was run neat. HRP-conjugated secondary antibodies against murine IgG, IgA, and IgM; and human IgM were obtained from Santa Cruz Biotechnology and Southern Biotech. Catfish IgM was detected with the monoclonal antibody 9E1 (murine IgG1), a kind gift from Eva Bengten, and thereafter probed with anti-mouse IgG1-HRP. Plates were developed with TMB and the reaction was quenched with 2N sulfuric acid. Background for all ELISAs was typically less than .05 at an OD of 450 nM. Estimated endpoint titer was determined by assessing the lowest concentration at which signal was obtained that was 2 times higher than background. If this value fell between 2 concentrations, then the titer was approximated relative to the numerical distance between the reading of the upper and lower concentration. Optical density values were reported at 450 nM, absolute or with background subtracted, as indicated per graph.

3.2.4 ELISPOT for detection of antibody-secreting cells specific for beta-glucan

96-well plate (Millipore, MSIPS4W10) nitrocellulose membranes were coated with laminarin dissolved in PBS (80 μg/mL) overnight. Lung and splenic tissue were collected from naïve male BALB/c mice were crushed with a syringe plunger over a 70-μm pore size cell strainer to obtain a single-cell suspension. Additionally cells were collected from bone marrow and peritoneal lavage. For all tissues, red blood cells were lysed with NH_4Cl/Tris solution for 5 min on ice and remaining cells were enumerated. Plates were blocked with complete tissue culture media consisting of RPMI 1640 medium, 10% fetal bovine serum, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin, and 2-ME at 5 x 10^{-5} M, and cells were seeded to plates in serial dilution. After two days, cells were removed, and bound IgM reactive with beta-glucan was identified with goat anti-mouse IgM-AP and detected with NBT/BCIP. An ELISPOT reader was used to enumerate spots (Cellular Technology).

3.2.5 P. carinii f. sp. muris isolate

The *Pneumocystis* inoculum was prepared as previously described (42, 89). Briefly, B6.*scid* and BALB/c.*scid* mice with *Pneumocystis* pneumonia were injected with a lethal dose of ketamine/xylazine and the lungs were aseptically removed and frozen in 1 ml of PBS at –80°C. Lungs were mechanically dissociated in sterile PBS, filtered through sterile gauze, and pelleted at 500 x *g* for 10 min at 4°C. The pellet was resuspended in sterile PBS and a 1/5 dilution was stained by a modified Giemsa stain (Diff-Quik; Baxter). The number of *Pneumocystis* cysts was quantified microscopically and the inoculum concentration was adjusted to 2 x 10^6 cysts/ml. Gram stains were performed on the inoculum preparations to exclude contamination with bacteria.

3.2.6 Flow cytometric analyses of natural antibody binding to fungi

Zymosan particles were obtained from Molecular Probes. *Aspergillus fumigatus* isolate 13073 was obtained from ATCC, was maintained on potato agar for 5 days, then harvested into PBS/.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. *Aspergillus fumigatus* conidia were matured into growth phase by culture in RPMI at 37° C for 6 hours, where they were thereafter killed by incubation at 100° C for 15 minutes to arrest growth. Additionally a cyst enriched preparation was derived from the *Pneumocystis* inoculum via sucrose gradient centrifugation, as described by Lim et al ((267). 400,000 Cells/particles were incubated with serum from BALB/c or BALB/c.*scid* mice for 3 hours, 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 Biosciences).

3.2.7 Pneumocystis infection

Mice were anesthetized and intratracheally challenged with the cyst enumerated preparation of 2 x 10^5 *Pneumocystis* cysts in 100 µL PBS. At various timepoints after challenge, mice were sacrificed and right lung was homogenized in 1 mL TRIzol, and saved at –80°C for later analysis of *Pneumocystis* organism burden via real-time PCR. The left lung was homogenized in 1 mL of PBS containing 0.05% Triton X and complete protease inhibitor (Roche). Lung

homogenates were centrifuged at 13,000 x *g* for 10 min, and supernatant was stored at –80°C for later analysis of pulmonary cytokines and chemokines.

3.2.8 Adoptive serum transfer

BALB/c.*scid* mice were administered 400 µL of pooled serum from either specific pathogen free BALB/c mice or BALB/c.*scid* mice aged 7-9 weeks; serum was given 100µL intravenously and 300 µL intraperitoneally. One hour thereafter, mice were challenged with *Pneumocystis* intratracheally and infection was allowed to proceed until sacrifice.

3.2.9 BALF analyses

Fluid from the lower respiratory tract was obtained by bronchoalveolar lavage of mice anesthetized with i.p. ketamine/xylazine as described previously (89). Only 1 mL of BALF was collected from mice using sterile PBS. Lavage fluids were centrifuged at 350 x *g* and supernatant was stored at –80°C until use. The cell pellet derived from centrifugation was cytospun onto slides, stained by modified Giemsa, and analyzed for leukocyte differential.

3.2.10 Real-time PCR analysis of Pneumocystis infection

Total RNA was isolated from the right lung of infected mice by a single-step method using TRIzol reagent (Invitrogen Life Technologies) as per manufacturer's instructions. Thereafter, RNA was transcribed to cDNA and real-time PCR was performed as previously described; copy numbers were quantified against a standard curve of known PC rRNA copy numbers (57). This assay has a correlation coefficient of 0.98 with PC rRNA copy numbers.

3.2.11 Cytokine analysis

Lung homogenate samples were analyzed for protein levels of 22 cytokines, including IL-1α, IL-1β, KC, MCP-1, TNF-α, G-CSF, IL-2, IL-6, IL-17, IFN-γ, IL-9, IL-5, MIP-1α, IL-10 using a Luminex multiplex suspension cytokine array according to the manufacturer's instructions (Linco/Millipore). The data were analyzed using Bio-Plex Manager software.

3.2.12 Intratracheal Zymosan challenge and trafficking experiment

sIgM (-/-) or C57/BL/6 mice were intratracheally challenged with 500 μg zymosan covalently linked to FITC, in a volume of 50 μL (Molecular Probes). 18 hours after challenge, mice were sacrificed, and mediastinal lymph nodes were collected and dissociated into single cell suspensions by dispersing through a 70-μm pore size cell strainer. Additionally, lungs were collected, digested with 25 U/ml DNase and 1 mg/ml collagenase A for 30 minutes at 37° C. Cells were then passed through 70-μm pore size cell strainer, and RBC's were lysed with NH_4Cl/Tris solution for 5 min on ice. Lymph nodes and lungs were studied individually. Cells were stained with CD11c-APC (BD-Pharmingen) to identify antigen presenting cells in the lymph node and lungs. Cells were studied with FACSAria (BD Biosciences).

3.2.13 In Vitro Lymph Node Restimulation

Fourteen days after *Pneumocystis* challenge, individual mediastinal lymph nodes from sIgM (-/-) mice and control C57BL/6 mice were dissected from the lungs, teased apart, and placed into 96 well plate containing 200 μL of complete media. Lymph node cells were restimulated with an

enumerated 40,000 cysts from the *Pneumocystis* inoculum. Three days after seeding, lymph node cells were pelleted, and supernatants were collected and stored at -80° C until later luminex analysis.

3.2.14 IL-5, IL-17, and IFN-γ ELISPOT

Fourteen days after *Pneumocystis* challenge, individual mediastinal lymph nodes from sIgM (-/-) mice and control C57BL/6 mice were dissected, teased apart, and strained into single cell suspensions by pressing over a 70 -μm pore size cell strainer and enumerated, and placed into 96 well nitrocellulose membrane plate containing 100 μL complete media for 2 days. Additionally 28 days after infection, lungs from mice sIgM (-/-) and wt mice were collected, and digested with 25 U/ml DNase and 1 mg/ml collagenase A for 30 minutes at 37° C. Cells were then passed through 70-μm pore size cell strainer, RBC's were lysed, and cells enumerated and placed into culture under the same conditions for lymph node cells. IL-5 (eBiosciences), IFN-γ (R&D), and IL-17 (R&D) ELISPOT kits were utilized and the assays were performed according to the manufacturer's instructions. To assess intrinsic numbers of *Pneumocystis* primed cells secreting IL-5, IL-17, and IFN-γ in the draining lymph node, without potentially expanding the population, cells were not restimulated with antigen. An ELISPOT reader was used to determine spot forming units (Cellular Technology).

3.2.15 Statistical Analysis

Data were analyzed using GraphPad statistical software. Comparisons between groups were made with the Student *t* test or Mann-Whitney *U* test. Significance was accepted at a value of $p < 0.05$.

3.3 RESULTS

3.3.1 High quantities of IgM antibodies targeting fungal cell wall carbohydrates observed in mice prior to fungal pathogen challenge

To assess antibody responses against fungal cell wall carbohydrates induced as a consequence of *Pneumocystis* exposure, BALB/c mice were challenged with *Pneumocystis* intratracheally, and immunoglobulin responses were monitored prior to challenge and followed over time. Unexpectedly, we noticed high quantities of IgM, reactive with laminarin, a primarily β-1,3 linked glucan, and chitosan/chitin, a polymer of 75-85% deacetylated chitin, prior to *Pneumocystis* challenge (Figure 8a). Of note, IgM in the serum was not reactive with α-1,6 linked mannan. Titers of IgM against β-glucan and chitosan/chitin rapidly enhanced through two days after challenge, and subsided to approach baseline levels one week after exposure to *Pneumocystis*. These observations demonstrate that a resident population of B cells is continually, spontaneously secreting IgM reactive with beta-glucan and chitosan/chitin.

3.3.2 IgM targeting β-glucan and chitosan/chitin are found in diverse species and do not require microbial stimulation for their production

As IgM specificities targeting β-glucan and chitosan/chitin are present in the serum of mice reared in specific pathogen free conditions, we questioned whether adventitious exposure to environmental pathogens, or colonization of the gut by bacteria or fungal organisms, for example, might be inducing the differentiation of B cells producing these antibodies. Serum from age-matched C57BL/6 mice reared in germ-free conditions was compared to serum from mice reared in specific pathogen free conditions for quantities of IgM targeting beta-glucan and chitosan/chitin (Figure 8b). The particular specificities were present at essentially equivalent concentrations in the germ-free and specific pathogen free mice, suggesting that these antibodies might be natural antibodies, produced in the absence of exogenous microbial stimulation. Since natural antibodies have been demonstrated to play a critical role in host defense against infection, likely acting at the very earliest moments after pathogen exposure (191), it is hypothesized that natural antibody specificities may have been selected over evolutionary time partially as a function of their ability to defend against infection. Hence, we investigated whether these antibody specificities might be present in some of the earliest evolved animals with adaptive immune systems. We probed the serum of channel catfish, *Ictalurus punctatus*, which contains a tetrameric homolog of IgM, for IgM reactivity against beta-glucan and chitosan/chitin. Antibodies with these reactivities were also present in catfish serum, with quantities of β-glucan specific IgM higher than chitosan/chitin specific IgM (Figure 8c). Finally, to explore whether these IgM specificities are present in the human preimmune repertoire we assessed human cord blood for reactivity against these carbohydrates. Since IgM does not cross the placenta, IgM present in human cord blood represents specificities generated in the preimmune repertoire by the fetus. We observed IgM targeting β-glucan in three of four patients, and IgM targeting chitosan/chitin in four of four patients; none of the patients had IgM antibodies reactive with

mannan (data not shown). Of responders, IgM against β-glucan and chitosan/chitin was present though at relatively low concentrations, with β-glucan specific IgM demonstrating greater average reactivity than chitosan specific IgM (Figure 8d). Hence, IgM specifities exist that target important structural carbohydrates present in most all fungal cell walls, and these specificities are conserved across multiple species, and apparently do not require exogenous microbial stimulation for their production.

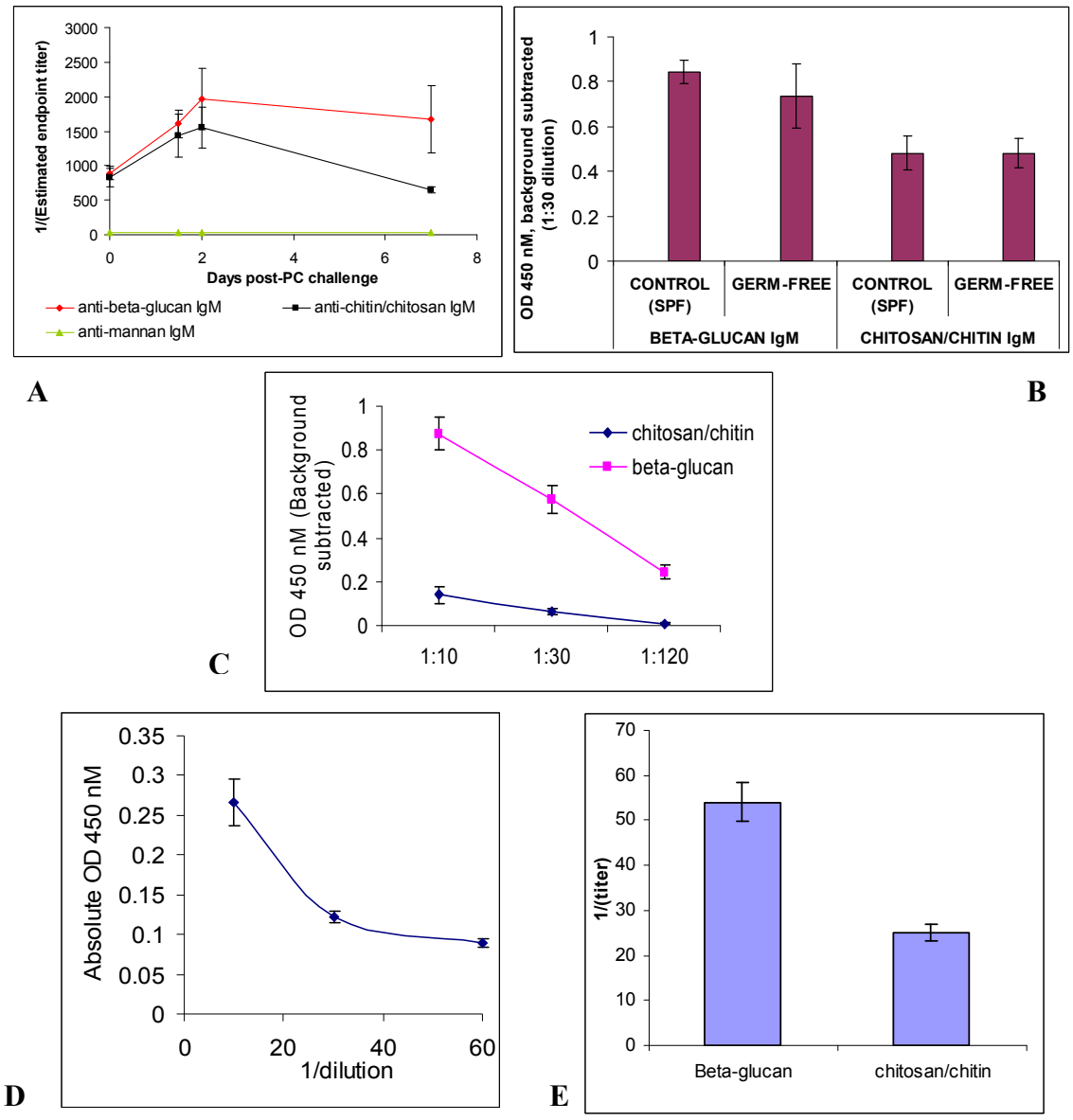


Figure 8: IgM antibodies targeting conserved carbohydrates of fungal cell walls among diverse species, and in the absence of exogenous microbial stimulation. Serum samples were used in an ELISA against laminarin (a primarily β-1,3 linked glucan) and chitosan/chitin seeded at 25 µg/mL. Detection antibodies were HRP conjugates and plates were developed with TMB and reactions quenched with 2N H_2SO_4. Error bars represent standard error of the mean. (A) Specific pathogen free BALB/c mice were challenged with *Pneumocystis* intratracheally and serum was assessed by ELISA for quantities of IgM reactive with beta-glucan and chitosan/chitin over time. Bound antibody was detected with anti-mouse IgM-HRP. Estimated endpoint titers are presented. (B) Serum from age-matched C57BL/6 mice reared in germ-free conditions or specific pathogen free (SPF, control) conditions was assessed for ELISA reactivity against beta-glucan and chitosan/chitin. Serum was diluted 1:30 and bound IgM was detected with anti-mouse IgM-HRP. Optical densities were determined at 450 nM, after subtraction of background. (C) Serum from channel catfish (*Ictalurus punctatus*) was studied for ELISA reactivity against beta-glucan and chitosan/chitin. Bound IgM was detected with a mouse anti-catifish IgM, which was then identified with anti-mouse IgG1-HRP. Optical densities were determined at 450 nM, after subtraction of background. (D) Among responders, human cord blood IgM was studied for reactivity against beta-glucan. Bound IgM was detected with anti-human IgM HRP and average OD is presented. Background OD at 450 nM was .049. (E) Estimated endpoint titer of concentrations of human cord blood IgM targeting conserved carbohydrates of fungal cell walls

3.3.3 B cells spontaneously producing IgM targeting β-glucan predominate in the spleen

Given the observation of natural IgMs targeting conserved fungal cell wall carbohydrates in the serum of specific pathogen free mice, we assessed the localization of B cells spontaneously producing IgM targeting β-glucan. B-1 B cells are considered the predominant B cell subset involved in the production of natural IgM antibodies (169), and in mice are present in peritoneal and pleural spaces. Hence, using specific pathogen free BALB/c mice, we compared cells derived from peritoneal lavage, to cells from the spleen, bone marrow and the lung, for the ability to produce IgM reactive with β-glucan by ELISPOT. We observed that the highest quantities of cells producing IgM reactive with β-glucan are present in the spleen, followed thereafter by the bone marrow (Figure 9). It has been identified that peritoneal B-1 cells do not spontaneously secrete natural antibodies directly from the peritoneum (184), but rather migrate into the spleen and secondary lymphoid organs upon stimulation by antigen or TLR ligands, (188, 189, 268), where they differentiate into antibody secreting cells. Our data support studies suggesting that the spleen is a common site for natural antibody secreting cells, such as the natural antibodies targeting conserved fungal cell wall carbohydrates.

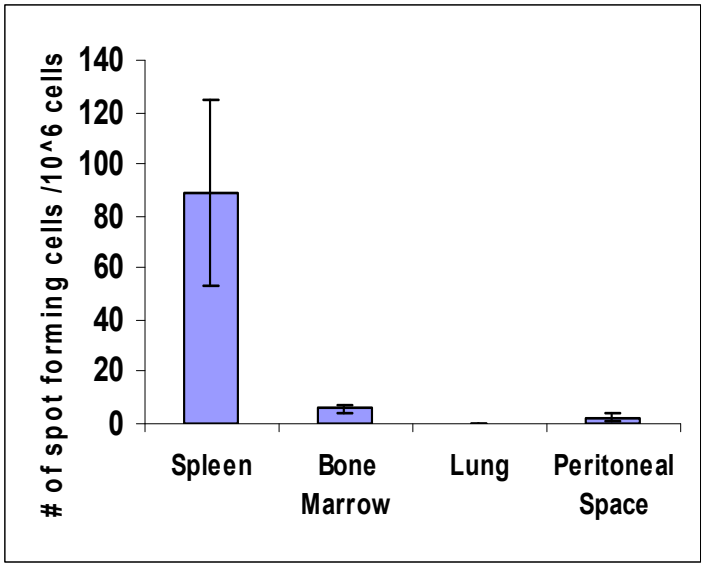


Figure 9: Site of antibody secreting cells spontaneously producing IgM specific for β-glucan. ELISPOT was performed to detect individual IgM secreting cells secreting antibody reactive with β-glucan in various tissues of individual mice. 3 mice per were evaluated per tissue. The results are representative of two similar experiments. Mean +/- SEM are presented.

3.3.4 Natural antibodies bind fungal cell walls

Given the observation that natural IgM antibodies exist in the serum of multiple host species with specificity for beta-glucan and chitosan/chitin, we investigated whether natural antibodies, present in specific pathogen free mice, are reactive with diverse fungal species cell walls. We incubated *Pneumocystis* organisms, derived from a cyst enriched preparation, with diluted serum from either BALB/c.*scid* mice or SPF naïve BALB/c mice, and assessed for binding of natural IgM. We observed high quantities of natural IgM binding to *Pneumocystis* (Figure 10a) relative to natural IgG binding (Figure 11a). We then compared binding affinity of natural IgM for zymosan, a particulate derived from the cell wall of *Saccharomyces cervesiae*, primarly consisting of polysaccharides, of which β-1,3 glucan predominates. Zymosan was also highly reactive with IgM present in specific pathogen free mice (Figure 10b). Finally, we compared the ability of natural IgM to bind another opportunistic pulmonary fungal pathogen, *Aspergillus fumigatus*. Resting conidia were notably poorly reactive with natural IgM, while conidia that were matured to a swollen, prehyphal state by incubation at 37° C for 6h, bound significant quantities of IgM (Figure 10c). Additionally, swollen conidia had significantly higher reactivity with natural IgM compared to natural IgG (Figure 11b). These data suggest natural IgM antibodies are a common opsonin for diverse species of fungi. As TLR2, TLR4, and dectin-1 mediate non-opsonic recognition of zymosan by macrophages, natural IgM are also reactive with zymosan, natural IgM may recognize ligands similar to those recognized by pattern recognition receptors. Additionally, specific ligands targeted by natural antibodies may be more abundant or accessible during different stages of the fungal life cycle.

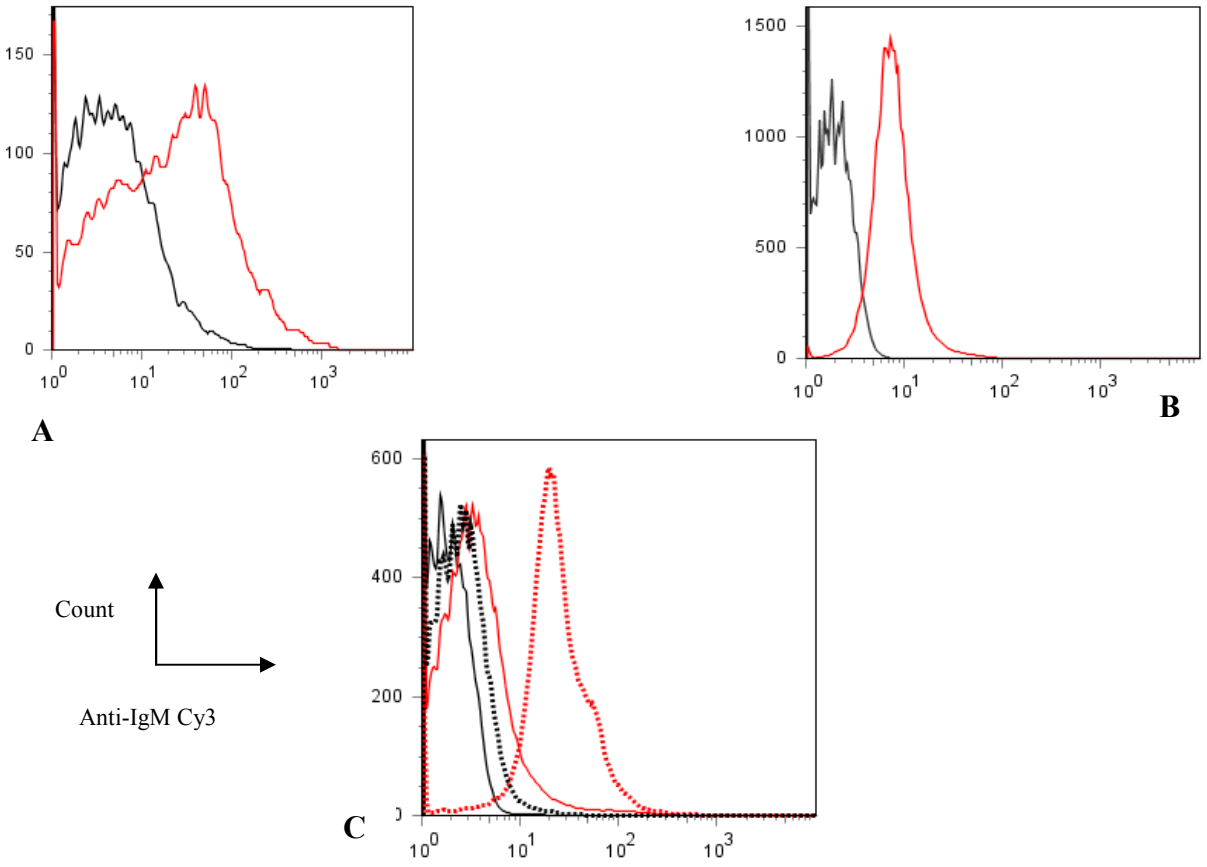


Figure 10: Assessment of natural IgM binding to fungal cell walls. (A) *Pneumocystis* organisms from a cyst enriched preparation, (B) Zymosan particles, (C) and *Aspergillus fumigatus* resting (solid lines) and swollen conidia (dotted lines) were incubated with serum from naïve BALB/c.*scid* or BALB/c mice (Dilution 1:4). Cells/particles were washed, and incubated with anti mouse-IgM Cy3 and analyzed by flow cytometry. Red lines denote incubation with BALB/c serum, black lines denote incubation with BALB/c.*scid* serum.

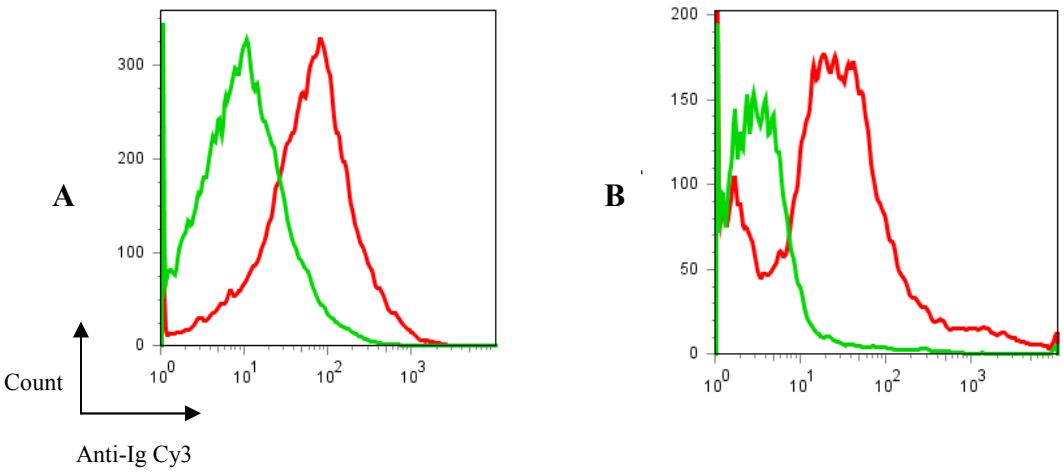


Figure 11: **Assessment of natural Ig isotype specificity in fungal cell wall recognition**. *Pneumocystis* organisms from a cyst enriched preparation (A) and *Aspergillus fumigatus* swollen conidia (B) were incubated with serum from naïve BALB.*scid* or BALB/c mice (Dilution 1:4). Cells/particles were washed, and incubated with anti-mouse IgG or anti mouse-IgM Cy3 and analyzed by flow cytometry. Red lines denote IgM reactivity, green lines denote IgG reactivity.

3.3.5 Impaired growth of Pneumocystis infection in the lung, and enhancement of pulmonary IL-1a and IL-6 as a consequence of natural antibodies

To assess the relevance of natural antibodies in host defense against infection, we compared serum derived from wt naïve BALB/c mice, containing innate IgM reactive with fungi such as *Pneumocystis*, to immunoglobulin-free serum from BALB/c.*scid* mice, in the ability to affect the growth of *Pneumocystis* organisms in the lungs of susceptible mice. BALB/c.*scid* mice were transferred with complement-inactivated serum from host source, administered in a combination of intravenous and intraperitoneal routes, and one hour later challenged with *Pneumocystis* intratracheally. Both intravenous and intraperitoneal routes were utilized for administration of high quantities of serum, as such a combination was observed to be efficiacious in enhancing survival in murine VSV infection by Ochsenbein et al (190). We observed that serum containing natural antibodies suppressed the growth of *Pneumocystis* infection, as observed at 5 and 14 days after infection (Figure 12a, Figure 12b). But rather than simply neutralizing pathogen to facilitate clearance, as one might expect as a primary effector function of IgM, the presence of wt naïve serum enhanced inflammatory responses in the lungs of infected mice, suggesting that immune complex formation led to enhancement of antigen presentation. Quantities of IL-1α, IL-6, G-CSF, and IL-2 were elevated in the lung homogenates of mice receiving wt naïve serum compared to mice receiving SCID serum (Figure 13). IL-6 and IL-1α, in particular, had impaired kinetics of production in mice receiving SCID serum. Of note, signaling at the IL-1 receptor has been demonstrated to be required for host defense against

Pneumocystis pneumonia (85) and IL-6 is implicated in diminishing numbers of neutrophils and CD8+ T cells recruited into the lungs after *Pneumocystis* challenge (269), hence natural antibody containing serum enhanced the production of these cytokines important in the evolving host response to infection. We hypothesize that natural antibodies enhance antigen presentation, leading to increased clearance and increased production of host defense related cytokines. We demonstrate that host defense against a mucosal fungal infection is significantly influenced by the presence of naïve serum containing natural antibodies.

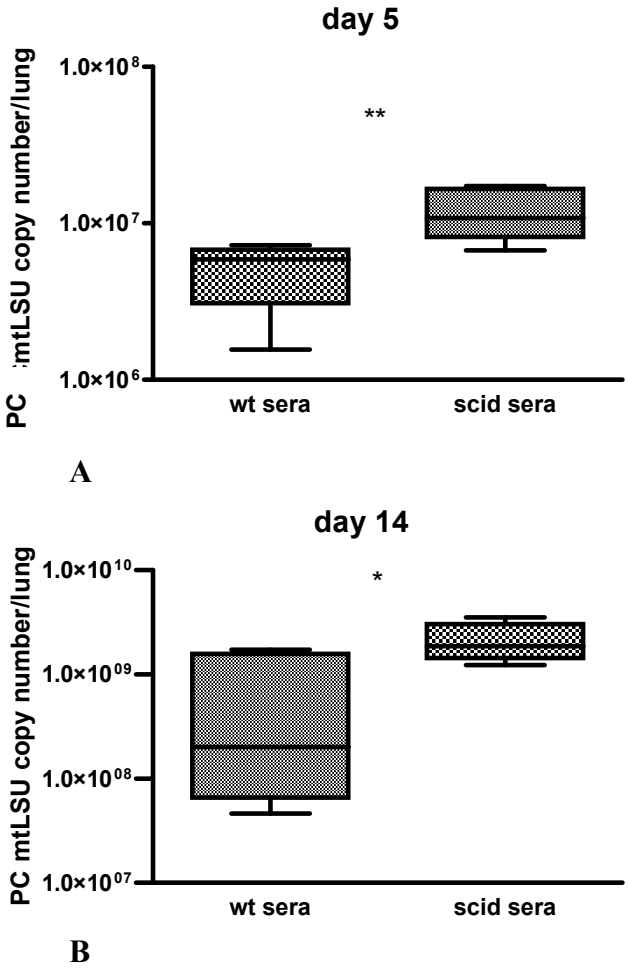


Figure 12: **Pathogen burden in the lungs of SCID mice transferred with naïve serum and challenged with *Pneumocystis***. Cohorts of 4-5 SCID mice received a total of 400 µL serum (100µL of serum intravenously and 300µL of serum intraperitoneally), from either specific pathogen free wt mice or SCID mice. Mice were then challenged with *Pneumocystis* intratracheally. (A) Pathogen burden was determined at 5 and (B) 14 days thereafter, by assessing lungs for total PC mtLSU subunit copy numbers. Boxes represent the interquartile range of data between the 25th and 75th percentiles and whiskers represent the upper and lower limits of the data. The median is represented by the dividing line within the box. * denotes p<. 05, ** denotes p<.01. Day 5 study was independently repeated 3 times with similar results.

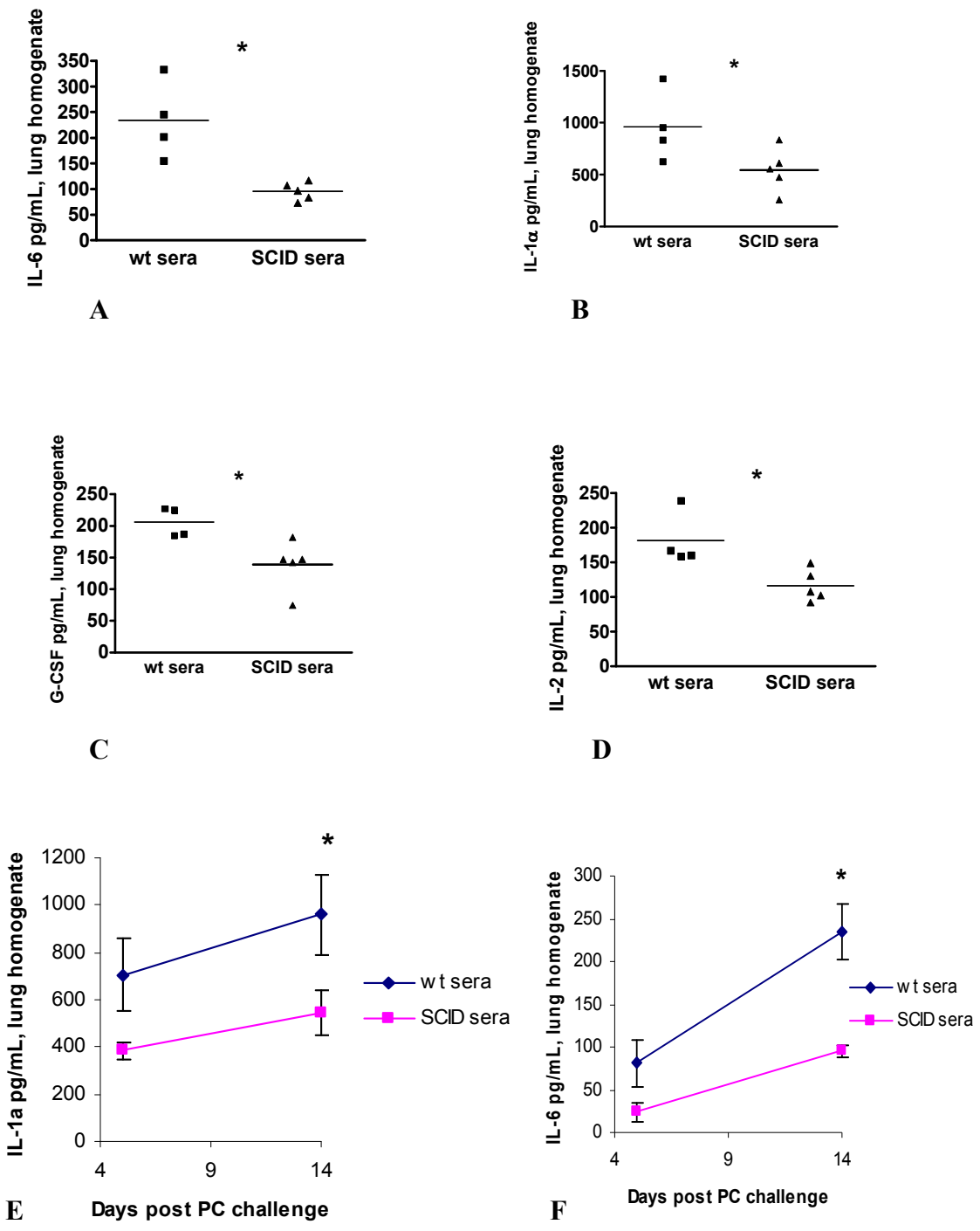


Figure 13: **Inflammatory responses in the lungs of SCID mice transferred with specific pathogen free serum and challenged with *Pneumocystis*.** 4-5 mice per group were evaluated per timepoint. Lung homogenates from mice described in FIGURE 5 were evaluated for quantities of (A) IL-6 , (B) IL-1a (C) G-CSF and (D) IL-2 as determined by luminex assay. (E) Kinetics of IL-1a induction (F) Kinetics of IL-6 induction. Error bars represent SEM. * indicates P<.05

3.3.6 Natural antibodies limit pulmonary neutrophil recruitment in the setting of acute *Pneumocystis* challenge

Our group and others have demonstrated that intratracheal challenge of SCID or wild type mice with *Pneumocystis* leads to rapid clearance of the inoculum (47, 97, 270), thereafter followed by growth of infection, which wild type mice ultimately clear but SCID mice ultimately succumb to. We sought to assess whether natural antibodies influence the process of rapid clearance of the majority of the inoculum in the host response to acute *Pneumocystis* challenge. SCID mice were transferred with either naïve wt or SCID sera, then challenged with Pneumocystis intratracheally, and assessed for pathogen burden, neutrophil recruitment into the airspaces, and the production of inflammatory cytokines and chemokines in the lungs. We observed no difference in pathogen burden 20 hours after challenge, yet by 3 days mice receiving wt naïve serum begin to show some level of protection against *Pneumocystis*, and by 5 days the growth of the infection in the lungs is impaired (Figure 14a), which continues into 14 days (Figure 12b). Interestingly, while there was no difference in pathogen burden at 20 hours, there was a significant decrease in the frequencies of neutrophils recruited into the lungs at 20 hours and 3 days, in mice receiving naïve wt serum (Figure 14b). In relation to the diminished frequencies of neutrophils observed in the mice receiving wt sera, levels of IL-1β (Figure 14c), TNF-α (Figure 14d), KC/CXCL1 (Figure 14e), and MCP-1/CCL2 (Figure 14f) in lung homogenates were decreased. The presence of high quantities of neutrophils recruited into the airways does not positively influence the rapid clearance of Pneumocystis, underscoring the observation that this function is primarily driven by alveolar macrophages (47). We conclude that while naïve serum

containing natural antibodies does not appear to enhance the rapid clearance of *Pneumocystis* organisms after acute intratracheal challenge, it significantly diminishes relative neutrophil recruitment into the airways, hence acts at the very earliest timepoints after *Pneumocystis* challenge. We hypothesize that binding of natural antibodies to the surface of *Pneumocystis* organisms limits inflammation guided by the resident innate effector cells in the lung, alveolar macrophages and the alveolar epithelium, which produce chemokines and cytokines driving neutrophil recruitment. As natural antibody specificities target β-glucan, and isolated β-glucan is inflammatory in the lungs (137), natural antibodies may also bind shed β-glucan from degraded organisms, shielding it from recognition by innate inflammatory cells. Thus, it appears that in the setting of acute challenge, natural antibodies act at the very earliest aspects of antigen presentation, thereby limiting neutrophil recruitment into the lungs, while at intermediate stages of infection enhance *Pneumocystis* clearance and promote the production of cytokines involved in host defense processes.

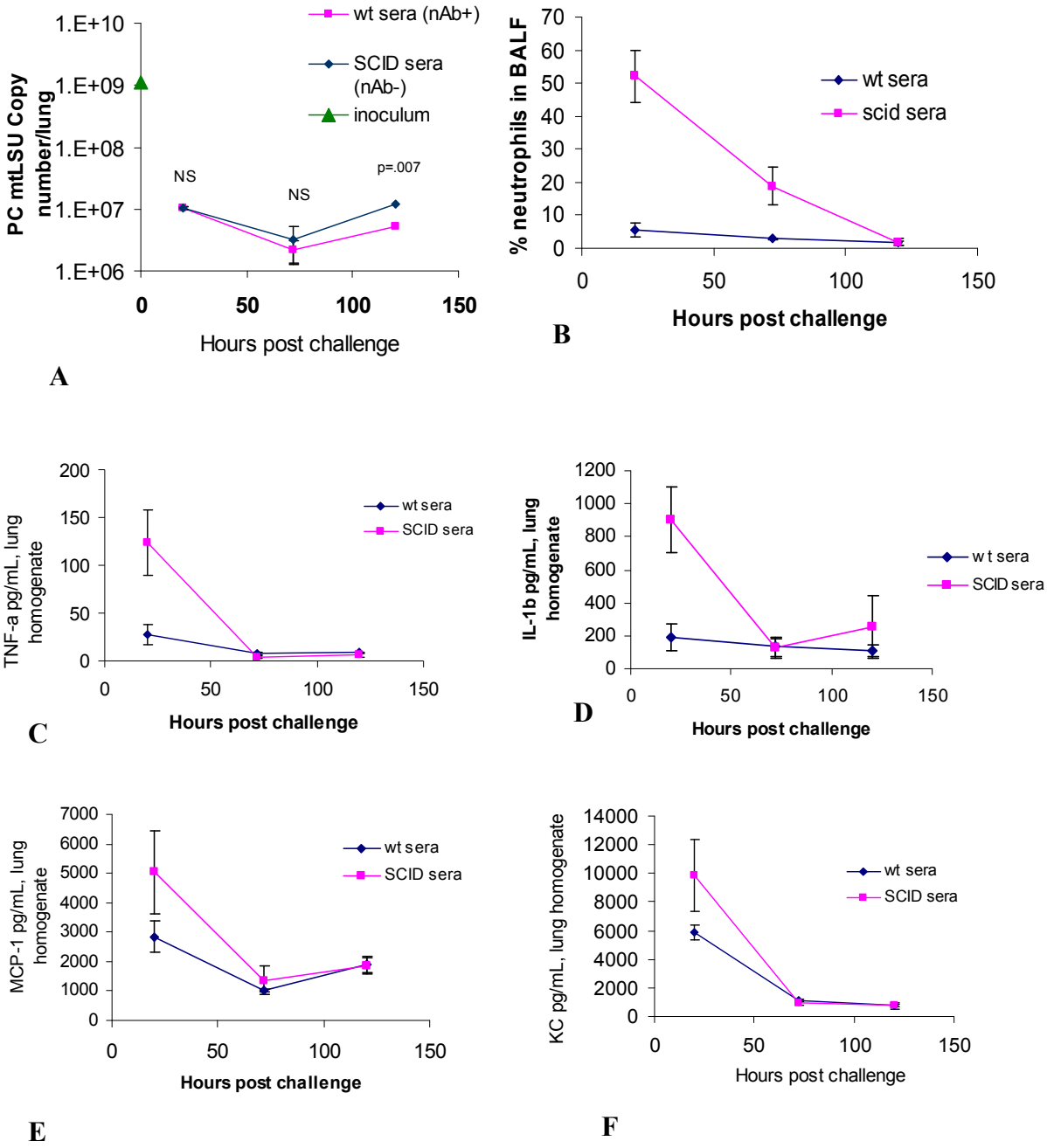


Figure 14: **Influence of serum containing natural antibodies on early clearance of Pneumocystis and early inflammatory responses**. SCID mice were transferred with naïve specific pathogen free BALB/c or SCID serum, then challenged with Pneumocystis intratracheally. 4-5 mice per group were evaluated per timepoint. (A) The presence of Pneumocystis organisms during stages of early clearance was determined by assessing PC mtLSU copy numbers over time. p value or NS indicated. (B) Percent neutrophils in cells obtained from BALF (C) Quantities of TNF-a, (D) Quantities of IL-1b, (E) Quantities of MCP-1, (F) Quantities of KC observed in lung homogenate as determined by luminex assay. Error bars represent SEM.

3.3.7 Mice with a deficiency in the ability to secrete IgM have increased burden of *Pneumocystis* in the lungs and diminished IL-1a and IL-6 levels in the lungs

As we have demonstrated the presence of natural IgM antibodies targeting conserved carbohydrates of fungal cell walls, and as natural antibodies, which are predominantly of the IgM isotype, inhibit the growth of *Pneumocystis* in the lungs, we sought to assess aspects of host defense against *Pneumocystis* in mice unable to secrete IgM. Mutant mice that are unable to secrete IgM but normally express B cell surface IgM and IgD, and isotype class switch normally to secrete other immunoglobulin isotypes, have been previously reported and characterized (196, 265, 266). Using C57/BL6 mice with this mutation, sIgM (-/-), we compared their relative ability to clear *Pneumocystis* infection to wt mice at an intermediate timepoint, fourteen days after intratracheal challenge. We observed that sIgM (-/-) mice have impaired clearance of *Pneumocystis* relative to wt mice (Figure 15). Additionally, these mice have diminished production of IL-1a and IL-6 (Figure 16) in the lungs, cytokines that were notably enhanced by the transfer of naïve serum containing natural antibodies (Figure 13) in SCID mice challenged with *Pneumocystis*. Interestingly, the sIgM (-/-) mice additionally promoted production of IL-10 in the lungs, a cytokine which inhibits the kinetics of *Pneumocystis* clearance (271). Hence, we conclude that IgM antibodies have an important role in host defense at early stages of *Pneumocystis* infection, presumably in the formation of immune complexes that influence antigen presentation, and that the natural IgM antibodies are important for early IL-1a and IL-6 responses from innate cells against this opportunistic fungal pathogen *in vivo*.

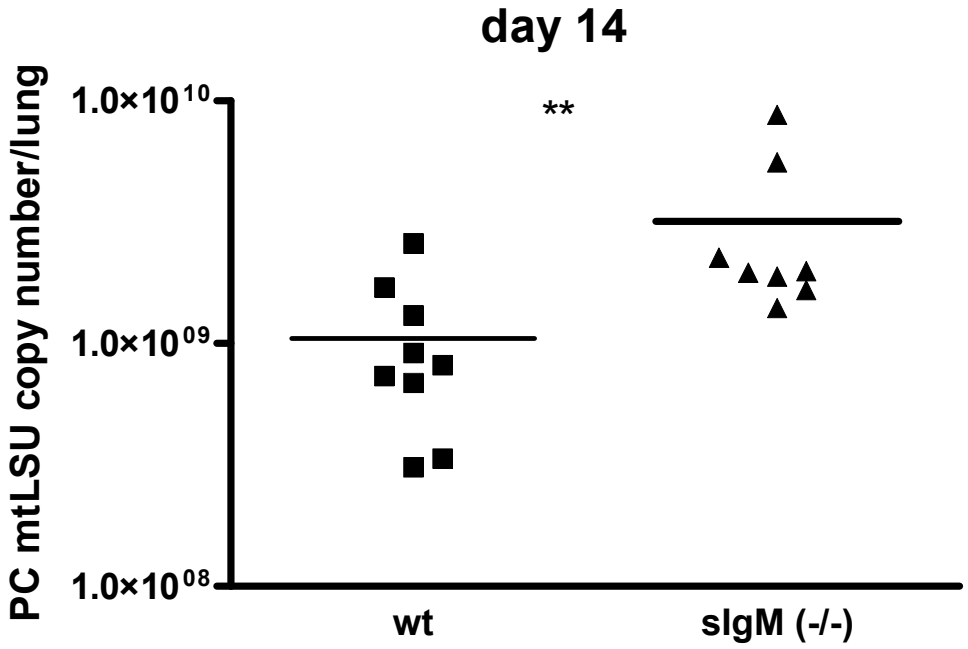


Figure 15: **Impaired clearance of *Pneumocystis* from the lungs of C57/BL6J sIgM (-/-) mice.** wt or sIgM (-/-) mice were challenged with *Pneumocystis* intratracheally and assessed for pathogen burden 14 days after infection by real-time PCR assessment of PC mtLSU copy numbers. Individual mice are represented by the symbols, 4-5 mice per group were evaluated per timepoint, and data are pooled from two independent experiments. ** indicates P<.01

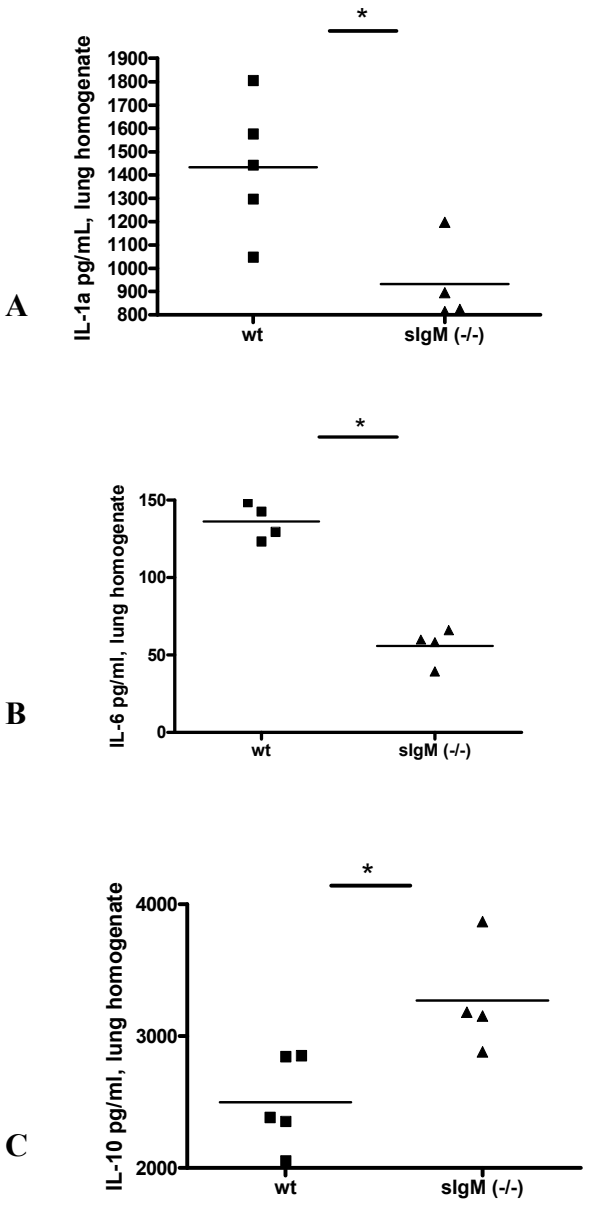


Figure 16: **Importance of the IgM isotype for the production of pulmonary proinflammatory cytokines after *Pneumocystis challenge*.** Studying mice as presented in Figure 15, lung homogenates were assessed for quantities of (A)IL-1α, (B) IL-6 and (C) IL-10 by luminex assay. Individual mice are represented by symbols. * indicates P<.05.

3.3.8 sIgM (-/-) mice have impaired trafficking of CD11c+ cells carrying zymosan to lymph nodes, but normal recognition in the lungs by CD11c+ cells

We have demonstrated that serum containing natural IgM antibodies bind fungi and inhibit the growth of *Pneumocystis* infection in the lungs of susceptible mice, and that mice lacking secreted IgM, the primary isotype for natural antibodies, have impaired clearance of *Pneumocystis* in the lungs as observed at intermediate timepoints after infection. Additionally, natural antibodies regulate inflammatory responses against *Pneumocystis*, inhibiting early pulmonary chemokine induction, while later enhancing the production of IL-1α and IL-6 in the lungs. As natural IgM appears to alter the manner in which *Pneumocystis* antigen presentation is occurring, we sought to assess whether mice deficient in secreted IgM have impaired presentation of fungal cell wall antigen to CD11c+ APCs *in vivo*. Wt or sIgM (-/-) mice were intratracheally challenged with FITC-zymosan and 16 hours later assessed for quantities of CD11c+ cells that were FITC positive as a function of zymosan endocytosis, in the lungs and in draining lymph nodes. In the lungs, we observed that almost all CD11c+ cells were FITC positive after FITC-zymosan challenge, and equivalent quantities of FITC+ CD11c+ cells were observed in wild type compared to sIgM (-/-) mice (Figure 17), suggesting that secreted IgM is not important for CD11c+ cell recognition of zymosan in the lungs. Additionally observed, mice lacking sIgM had greater quantities of FITC-zymosan in CD11c- cell populations in the lungs. However, when we compared quantities of CD11c+ cells in the mediastinal draining lymph node, we observed that the presence of FITC+ CD11c+ cells was somewhat diminished in sIgM (-/-) mice, suggesting that the migration of CD11c+ antigen presenting cells to the lymph nodes is impaired in the absence of secreted IgM (Figure 18). These data suggest that IgM coated zymosan guides

the recognition of antigen through pathways that do not as readily mature dendritic cells for migration to lymph nodes, and that natural IgMs are involved in the very earliest aspects of fungal carbohydrate antigen recognition in the host, influencing CD11c+ cell trafficking to lymph nodes.

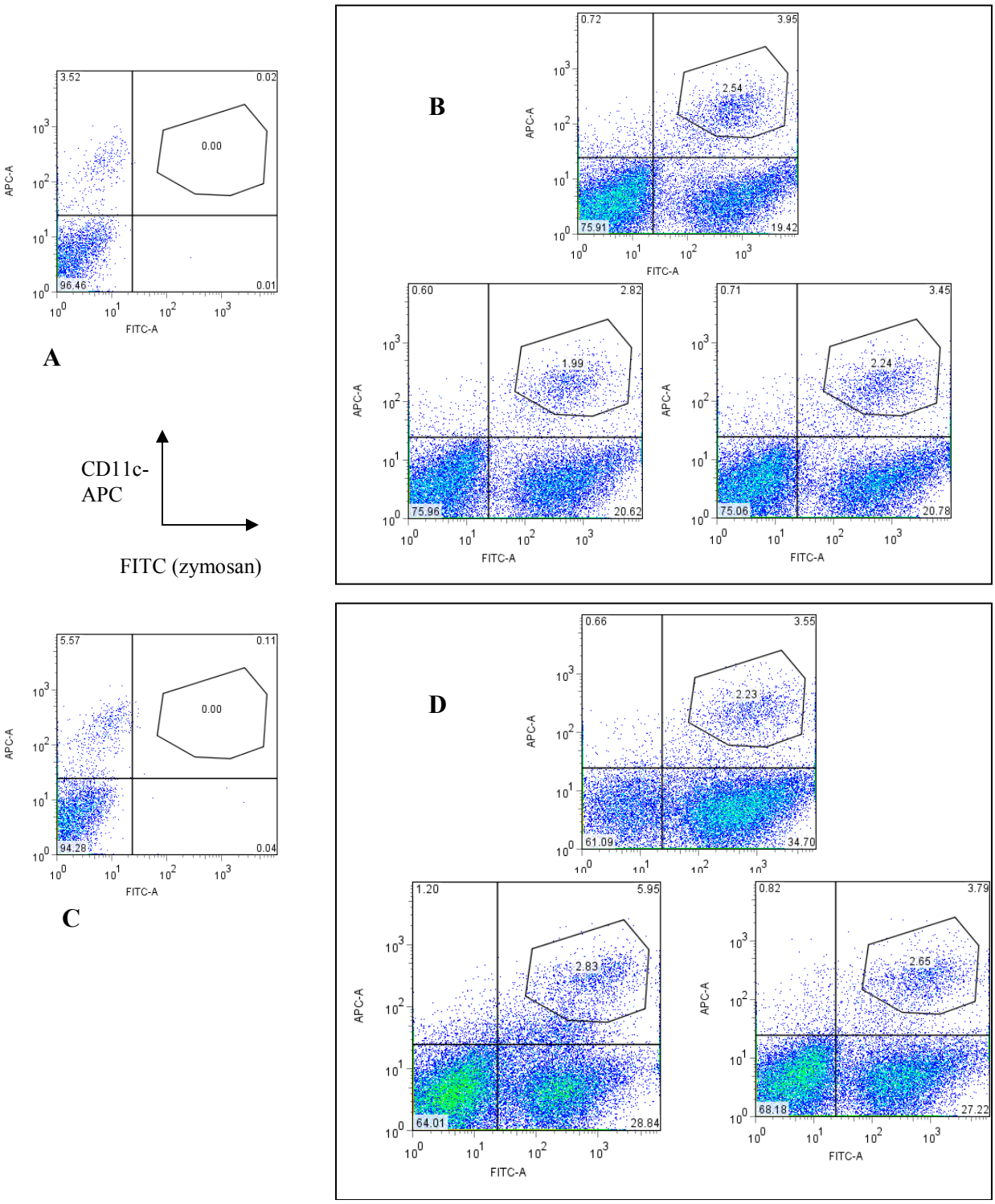


Figure 17: **FITC-zymosan internalization by CD11c+ cells in the lungs of wt vs sIgM (-/-) mice.** Wt or sIgM (-/-) mice were challenged with 500µg of FITC-zymosan or unlabelled zymosan intratracheally. 16 hours after challenge, mice were sacrificed, and lung cells were stained with CD11c, and analyzed for CD11c+/FITC+ cells by flow cytometry. Individual flow cytometry plots/mouse are presented. A) wt and C) sIgM (-/-) mice that received unlabelled zymosan. B) wt and D) sIgM (-/-) mice that received FITC-zymosan.

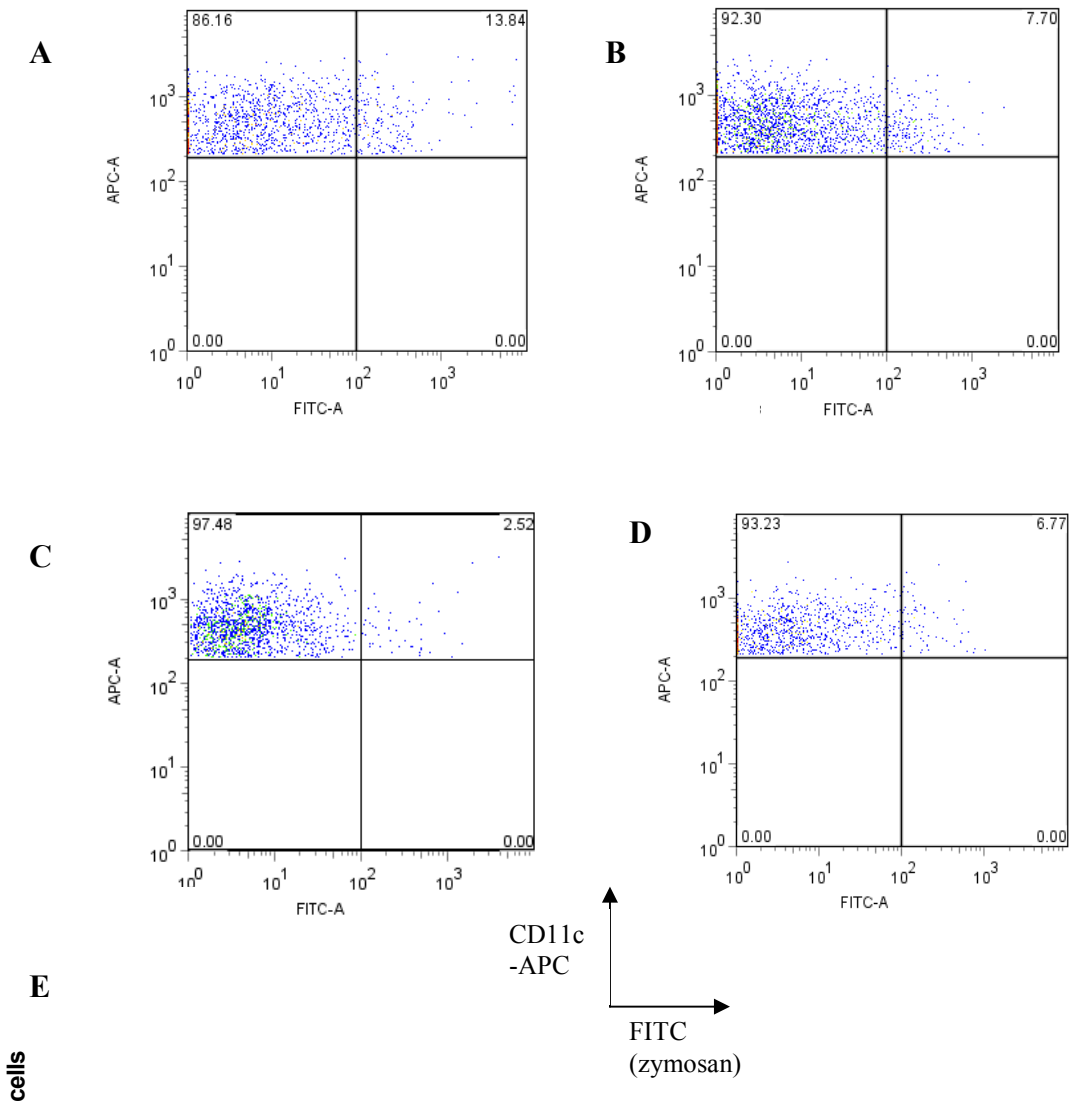


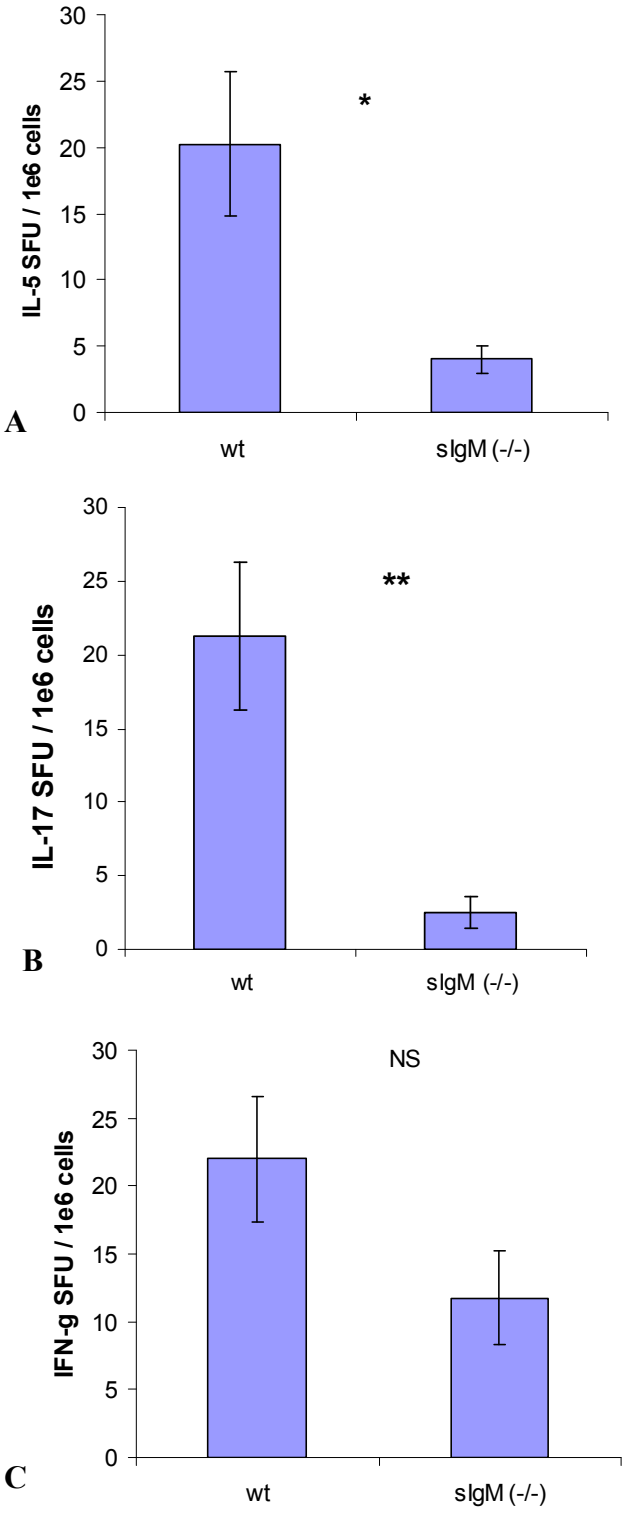
Figure 18: **Evaluation of trafficking of zymosan loaded CD11c+ cells to mediastinal lymph nodes in sIgM (-/-) vs. wt mice.** Wt and sIgM (-/-) mice were challenged with 500 µg of FITC-zymosan intratracheally. 16 hours later, individual mediastinal lymph nodes were collected, dispersed into single cell suspensions, stained with CD11c-APC, and analyzed by flow cytometry. After gating on CD11c cells, percent of cells which were FITC high was quantified and expressed as a fraction of CD11c cells. A) and B) Representative plots from individual wt mice as gated on CD11c+ cells C) and D) Representative plots from individual sIgM (-/-) mice as gated on CD11c+ cells. E) Summary of average frequency of CD11c+/FITC hi+ cells from n=5 or more individual lymph nodes/mouse per group, error bars represent the SEM. * indicates P<.05 by Mann Whitney test.

3.3.9 sIgM (-/-) mice have impaired IL-5 and IL-17 production in draining lymph nodes after Pneumocystis challenge

As mice unable to produce sIgM have diminished fungal cell wall carbohydrate antigen presentation by CD11c+ cells in draining lymph nodes, and delayed clearance of *Pneumocystis* from the lungs at 14 days, coupled with altered inflammatory responses in the lungs such as diminished IL-6 and enhanced IL-10, we hypothesized that perhaps the environment influencing Th priming in the lymph nodes might be affected by the absence of sIgM. As sIgM may have the function of affecting *Pneumocystis* antigen presentation by CD11c+ cells in lymph nodes as demonstrated with zymosan, and absence of sIgM results in the decrease in potent Th2 skewing cytokines such as IL-6 in the lungs, we hypothesized that perhaps Th2 and Th17 polarization in the lymph nodes might be affected by the absence of sIgM. Mice were challenged with *Pneumocystis* intratracheally, and 14 days after challenge, cells isolated from individual mediastinal lymph nodes were compared for their relative production of three different cytokines associated with CD4+ Th polarization, IFN-γ (Th1), IL-5 (Th2), and IL-17 (the recently described Th17 subset (272, 273). To determine frequencies without expanding the population ex vivo, lymph node cells were not re-stimulated with antigen. We observed that lymph node cells from sIgM mice challenged with *Pneumocystis* had significantly lower frequencies of IL-5 and IL-17 producing cells relative to wt mice (Figure 19 a, b). However, frequencies of IFN-γ producing lymph node cells from sIgM (-/-) and wt mice while trending lower in sIgM mice, were similar (Figure 19 c). Hence we conclude that at this timepoint, sIgM is involved in the

optimal production of IL-5 and IL-17 from lymph node cells as seen in wild type mice. Thus, we believe that sIgM, which likely partially involves natural antibodies targeting fungal cell wall carbohydrate antigens, are capable of shaping responses in lymph nodes guiding the adaptive immune response to *Pneumocystis*.

Figure 19: **Influence of sIgM on the production of cells producing cytokines associated with CD4 Th lineages in the lymph node 14 days after *Pneumocystis* challenge**. Wt or sIgM (-/-) mice were challenged with *Pneumocystis* intratracheally. 14 days thereafter, mice were sacrificed and the draining mediastinal lymph node was collected, individually dissociated into single cell suspensions, and studied for frequencies of single cells producing IL-5 (A), IL-17 (B), and IFN-γ (C) by ELISPOT assay. 4 mice were evaluated per group and average (+/-) SEM is presented. * indicates P<.05 and ** indicates P<.01 by Mann-Whitney test.



3.3.10 sIgM (-/-) mice have impaired serum IgG1 anti-Pneumocystis antibody responses, and enhanced anti-PC IgG2a antibody responses

The IgM isotype has been demonstrated to influence the kinetics of the evolving adaptive IgG antibody response to mucosal pulmonary pathogens such as influenza virus. Baumgarth et al observed that sIgM (-/-) mice have delayed serum kinetics of specific influenza IgG2a, and to a lesser extent delayed kinetics of specific IgG1 production , however the respective quantities observed in sIgM (-/-) mice reach equivalent levels to wt mice by 22 days after infection (198). As the role of IgM has not been assessed in the evolving antibody response against any fungal organism, we explored the kinetics and isotype production of antibodies from sIgM (-/-) mice. Unexpectedly, we observed that the production of anti-Pneumocystis IgG1 was significantly impaired in sIgM (-/-) mice, with the trend emerging as early as 10 days after challenge but notably continuing through 28 days after infection, with total quantities of IgG1 significantly lower (Figure 20a). Of note, IgG1 is the predominant anti-Pneumocystis isotype produced after infection (98) and is an antibody isotype associated with Th2 immunity. In comparision, serum anti-Pneumocystis IgG2a, which is an isotype associated with Th1 immunity, was significantly increased in the sIgM (-/-) mice, with the trend emerging as early as 10 days and observed through 28 days after infection (Figure 20b).

These data suggest that sIgM guides the adaptive immune response to *Pneumocystis* by influencing the isotype of anti-Pneumocystis IgG antibodies produced in the blood. As B cell isotype class-switching is influenced by many factors, including Th cells, we hypothesize that sIgM, by influencing aspects of antigen presentation, promotes Th2 responses after challenge

with fungi within lymph nodes, leading to the promotion of an adaptive anti-Pneumocystis immunoglobulin response promoting the production of IgG1 over IgG2a. As we observed heightened numbers of IL-5 producing cells in the mediastinal lymph nodes, at 14 days after *Pneumocystis* challenge, in wt mice relative to sIgM (-/-) mice (Figure 19), and we demonstrate that whole lymph nodes isolated from sIgM (-/-) mice upon *Pneumocystis* Ag restimulation produced diminished quantities of IL-5 and IL-9, (Figure 23a) we believe that a Th2 dominant response in the lymph nodes in wild type mice influences the B cell germinal center reaction leading to enhanced class-switch recombination towards the IgG1 isotype, while impaired class-switch recombination towards the IgG2a isotype. Additionally, IL-21 has been demonstrated to significantly influence the production of IgG1, and synergizes with Th2 cytokine IL-4 in mice (274) to promote this class-switch, and IL-21 production is associated with Th17 cells (275). As we observed 14 days after *Pneumocystis* challenge diminished quantities of IL-17 producing cells in the lymph nodes isolated from sIgM (-/-) mice (Figure 19), and impaired production of IL-17 from whole lymph nodes isolated from sIgM mice (-/-)restimulated with *Pneumocystis* antigen (Figure 23a), it is possible that impaired generation of Th17 cells via IL-21 production may also contribute to impaired anti-PC IgG1 production.

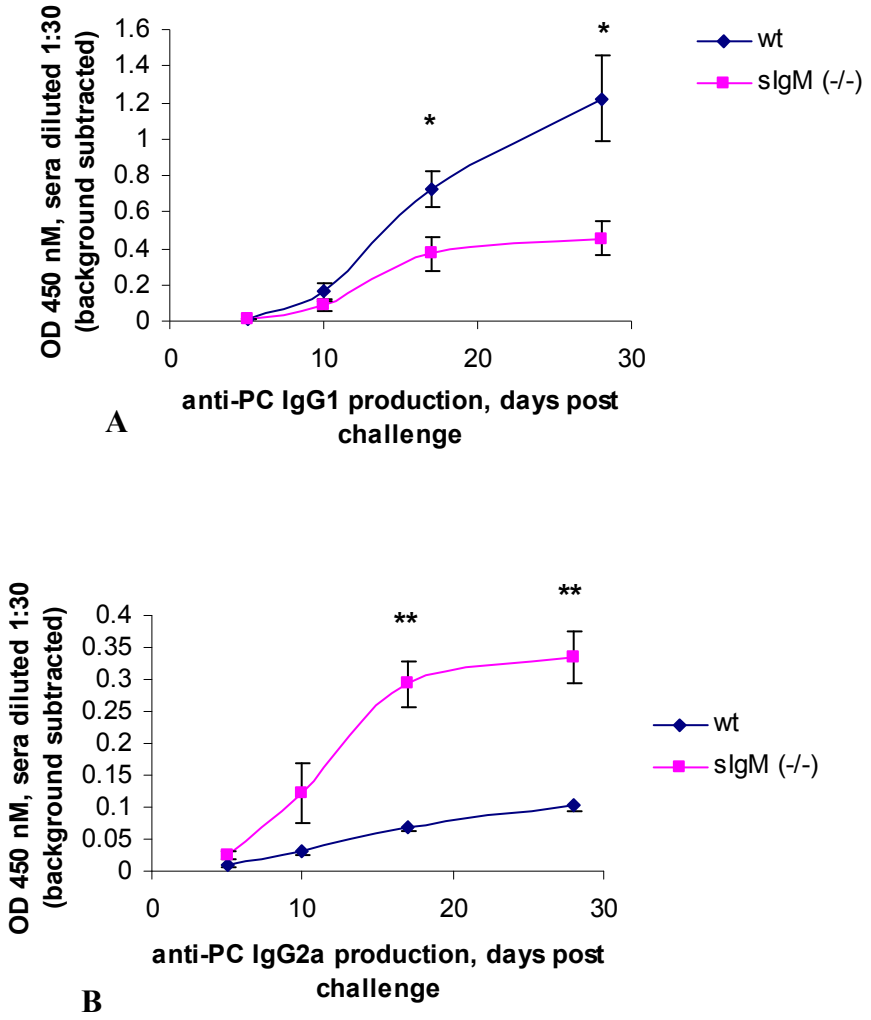
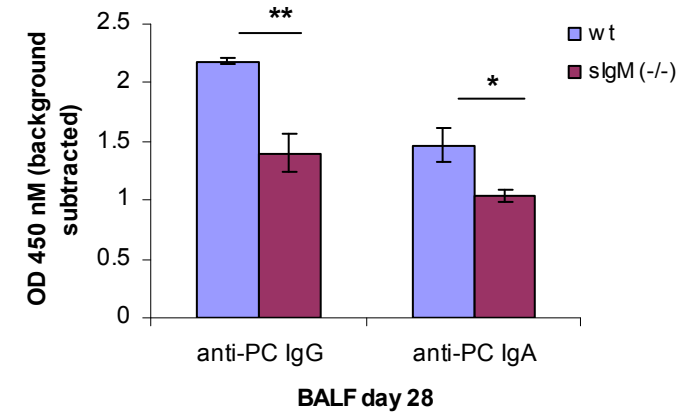


Figure 20: Influence of sIgM on adaptive serum antibody responses against *Pneumocystis*. Wt or sIgM (-/-) mice were challenged with *Pneumocystis* intratracheally. Mice were bled at the indicated timepoints, and serum was assessed for IgG1(A) or IgG2a (B) reactivity against *Pneumocystis* (PC) antigen by ELISA. Average responses from 4-5 mice per group per timepoint are presented. Error bars represent SEM. * indicates $p<.05$, ** indicates $p<.01$

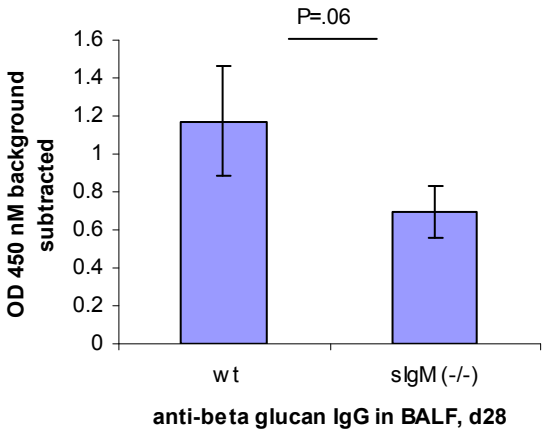
3.3.11 Mucosal antibody responses against Pneumocystis protein and carbohydrate antigens are impaired in sIgM (-/-) mice

The respiratory mucosa is in continual contact with environmental antigens, hence local immune responses at these sites are among the most important in host defense against pulmonary infections. The most abundant antibody isotype in the body is IgA, with the vast majority localized to mucosal surfaces, and produced by local B cells, though IgG and IgM are also significant components of mucosal secretions. We questioned whether the production of *Pneumocystis* specific immunoglobulins at the mucosa was affected by the absence of sIgM, given that sIgM influenced production of systemic immunoglobulins against *Pneumocystis*. Wt and sIgM (-/-) mice were challenged with *Pneumocystis* intratracheally, and 28 days after infection bronchoalveolar lavage fluid was collected and assessed for the presence of specific IgG and IgA responses against the fungal pathogen. Notably, we observed that quantities of anti-Pneumocystis specific IgG and IgA at the pulmonary mucosa were significantly decreased in mice lacking sIgM 28 days after infection (Figure 21a). As IgA induction in the serum against *Pneumocystis* Ag is essentially absent, these data suggest that sIgM is involved in optimizing local adaptive mucosal B cell responses against *Pneumocystis* protein antigens. Additionally, mucosal, induced IgG responses against the conserved fungal cell wall carbohydrate β-1,3 glucan also trended towards lower quantities in the absence of IgM, hence induced antibody responses targeting fungal carbohydrate antigens may also be influenced by sIgM (Figure 21b).

As the IgM isotype is not as abundant an isotype relative to IgA at mucosal surfaces, its importance in host defense, and in particular in guiding adaptive mucosal responses has not been deciphered. We demonstrate that mucosal IgG and IgA responses are enhanced by the presence of IgM, and we believe this to be a function of enhancing immune-complex generation, and increasing Th2 responses, which promote antibody-based immunity.



A



B

Figure 21: Influence of sIgM on induced mucosal antibody responses against *Pneumocystis* and β-glucan. Wt or sIgM (-/-) mice were challenged with *Pneumocystis* intratracheally and at 28 days were sacrificed and BALF analyzed. A) BALF was assessed for IgG and IgA reactivity against *Pneumocystis* (PC) antigen by ELISA. B) BALF was assessed for IgG reactivity against the β-1,3 linked glucan laminarin. Average responses from 4-5 mice per group are presented. Error bars represent SEM. * indicates p<.05, ** indicates p<.01, otherwise p value is presented

3.4 DISCUSSION

Immunity against fungal infections involves numerous host defense mechanisms, of varied importance, operating simultaneously or within temporal proximity and often with the capacity to influence one another. Some of these immune responses are required for effective host defense against fungi, others are contributory, while still others are detrimental. Here, we report on the significant contributory functions of natural antibodies and particularly the IgM isotype in host defense against the pulmonary mucosal opportunistic fungal pathogen *Pneumocystis carinii.* While adaptive immune defense mechanisms involving CD4+ T cells and B cells are central to host defense against *Pneumocystis* pneumonia, we have identified a significant function for secreted IgM (sIgM) in host defense against fungi, and demonstrate that natural, innate antibodies and sIgM are involved in immunologic responses at the earliest stages of infection, enhance clearance of infection, and shape the evolving adaptive immune response mounted by the host.

Almost all fungi possess a cell wall with conserved composition, consisting of a core of β-1,3 linked glucan interlinked with chitin (102). These polysaccharides, which notably cannot be synthesized by vertebrates, provide important structural function to fungal cell walls, and when studied in isolation are highly immunogenic in mammals (122, 123, 132). While the manner in which these carbohydrates initiate and enhance immune responses likely involves recognition by membrane associated pattern recognition receptors, such as CD11b (123, 276),

dectin-1 (145), toll-like receptors, and cellular enzymes such as chitinases (132, 277), the role of innate antibodies in the identification of these structures has not been evaluated. We demonstate that high quantities of IgM targeting β-glucan and chitosan/chitin are present in the serum of naïve wt mice, and that mice reared in germ-free conditions also contain these specificities. These data suggested to us that microbial stimulation is not required for the production of IgM targeting β-glucan and chitosan/chitin, and that these specificities are part of the innate, preexisting natural antibody repertoire.

Natural antibodies are molecules primarily of the IgM isotype, often with the capacity to bind more than one antigen, and usually encoded by a limited set of germ-line genes without N-region additions (169). Given their presence in the steady state, many groups have observed their importance in immediate immunologic responses such as delayed type hypersensitivity, (278) and in defense against infections (191, 196). The repertoire, while broad, is nonetheless highly restricted (279), as for example, mice produce natural antibodies targeting the carbohydrate antigen Galα1-3Gal (Gal) only after genetic deletion of an enzyme required for the production of this carbohydrate (185), and in our studies, we note that natural IgM is not reactive with mannan. As the natural antibody repertoire is likely to be the first representation of B cell immunity in evolution, we questioned whether more primitive organisms produce these specificities targeting conserved fungal cell wall carbohydrates. Catfish, among the earliest species containing immunoglobulins, and containing a tetrameric homolog of IgM, possessed serum IgM reactivity against β-glucan and chitosan/chitin, though notably reactivity with β-glucan was higher. While these catfish IgM may be innate or induced by antigenic stimulation, these data suggest that the capacity to make these specificities is conserved between species, likely due to important functions in host defense and/or homeostasis. Finally, we detected

natural IgM antibodies targeting β-glucan and chitosan/chitin in human umbilical cord blood, demonstrating that these specificities are conserved and also present in the absence of exogenous antigenic stimulation in humans. While these IgM specificities might be further enhanced by microbial exposure, we believe their production is partially represented in the innate IgM repertoire, which is predominantly produced by B-1 cells. We argue that these IgM represent soluble pattern recognition receptors for conserved fungal cell wall carbohydrates and may play a role in the observed immunogenicity of chitin and β-glucan.

As B-1 cells are the predominant B cell subset involved in the production of natural antibodies, we determined the localization of cells responsible for the secretion of IgM targeting β-glucan. We observed that production of these antibodies predominates in the spleen, and that cells isolated from the peritoneum had negligible frequencies of IgM secreting B cells specific for β-glucan. Our observations support studies demonstrating that B-1 cells, while primarily associated with the body cavitites such as the pleural and peritoneal space, migrate into secondary lymphoid organs after stimulation, where they differentiate into antibody secreting cells (184, 188) producing natural IgM, and it is perhaps this mechanism that leads to the substantial presence of β-glucan specific IgM in the serum. While it has been demonstrated that TLR ligand stimulation, particularly LPS or peptidoglycan stimulation, leads to B-1 cell CD11b downregulation and migration from the body cavities (189), as well as differentiation, it is unclear whether antigen specific signaling through the B cell receptor or through non-TLR pattern recognition receptors could potentially initiate this process. However, this mechanism, while likely having a steady state function, and hence involved in maintaining IgM within the narrow physiologic range observed in humans, demonstrates a pathway whereby natural IgM could be rapidly enhanced in settings of infection. Additionally, it underscores the presence of

B-1 derived IgM in secondary lymphoid organs, where adaptive immune responses are being initiated.

Natural IgM antibodies are opsonins in host defense against bacteria and viruses (191, 280), hence we sought to evaluate their capacity to bind fungal cell walls. We observed that serum IgM binds cell walls such as zymosan, suggesting that the carbohydrate components of fungal cell walls are specific targets of natural IgM. Additionally, natural IgM binds *Pneumocystis* organisms and growing *Aspergillus* conidia, suggesting that certain antigens targeted by natural antibodies may be more accessible at certain stages of fungal cell growth, for particular pathogens. As it has been observed that dectin-1 is accessible to *Aspergillus* β-glucan particularly as conidia mature, it suggests that the natural antibody repertoire may be targeting these same antigens. Of note, IgG present in specific pathogen free mouse serum has much lower affinity for fungal cell walls than IgM. Hence, we argue that specificities targeting fungal cell wall carbohydrates predominate in the IgM isotype in naïve, specific pathogen free mice.

We used naïve specific pathogen free serum, from either wt or SCID mice, to assess the effects of the natural antibody repertoire on host defense against *Pneumocystis* in susceptible SCID mice, a similar strategy to that employed by Ocshenbein et al in first defining functions of natural antibodies in restricting viral and bacterial dissemination (191). Wt serum significantly limited the growth of infection in SCID mice challenged with *Pneumocystis*, with the effect observed at intermediate timepoints after infection, 5 and 14 days thereafter. Additionally, naïve wt serum enhanced production in SCID mice infected with Pneumocystis of IL-1α and IL-6 in the lungs, cytokines important in host defense mechanisms against this infection. However, at earlier timepoints, in the immediate response to the inoculum, natural antibody containing serum limited the quantities of neutrophils as well as pulmonary chemokines associated with

neutrophil chemotaxis, while not detectably influencing rates of *Pneumocystis* clearance. Collectively, these data suggest that natural antibodies alter the routes of antigen presentation, perhaps partially inhibiting recognition of *Pneumocystis* directly through membrane bound pattern recognition receptors such as dectin-1, TLR2 or lactosylceramide, and perhaps neutralizing inflammatory components such as shed β-glucan resulting from *Pneumocystis* degradation, leading to the inhibiton of the production of signals for neutrophil chemotaxis into the lungs. Simultaneously, the rerouting of antigen through other recognition pathways leads to enhanced inflammation at later stages of infection, coupled with diminished pathogen burden. The hypothesized contribution of the IgM isotype in these responses was confirmed with studies in the sIgM (-/-) mouse, which also demonstrated impaired clearance of *Pneumocystis* at intermediate stages of infection and impaired production of IL-1α and IL-6 in the lungs. These models together demonstrate that natural antibodies, particularly IgM, are significant contributory effector molecules in host defense against *Pneumocystis*. Our data confirm reports from other groups demonstrating a value for natural antibodies and the IgM isotype in host defense against systemic and mucosal infections.

As the earliest aspects of *Pneumocystis* recognition were altered in the absence of natural antibodies, we hypothesized that the absence of sIgM may potentially have consequences in the production 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 draining lymph nodes after instillation of antigen into the lungs (281). By assessing how the trafficking of the fungal cell wall carbohydrate antigen particle zymosan occurs in the lungs in the absence of sIgM, we observed that pulmonary CD11c+ cells were equivalently capable of internalizing the particulate. However, analysis of CD11c+ cells in the

mediastinal draining lymph node, dendritic cells (DC's) that have migrated secondary to activation and maturation, demonstrated that the presence of these cells containing zymosan in inductive sites was impaired in the absence of sIgM. As dendritic cell migration is dependent on the process of cellular maturation and the expression of specific chemokine receptors mediating lymph node homing, which in turn is guided by the signaling pathways initiated by pathogen associated molecular patterns, we believe that fungal cell wall carbohydrate antigen coated in IgM are processed through pattern recognition receptors 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. It is possible that sIgM modulates zymosan recognition by DC through some pattern recognition receptors over others in the lung, and as sIgM is an excellent promoter of the classical complement pathway, it may be that sIgM enhances 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 more recently described Fcα/μ receptor identified in mice and humans on hematopoeitic and other cells (282, 283), could be preferentially involved in the recognition of IgM coated zymosan by DCs, shaping the cell maturation program upon activation. Recognition of sIgM coated Pneumocystis, for example, could lead to differential production of activation molecules on airway DCs such as OX40L(284), chitinases (285), or leukotriene receptors (286) which could significantly alter DC priming towards Th2 adaptive immune responses. Of note, we also observed diminished quantities of chemokines MCP-1 and MIP-1α, and chemokine regulating cytokine IL-1β from whole lymph nodes isolated from sIgM (-/-) mice 14 days after challenge with *Pneumocystis* and restimulated with Ag in vitro

(Supplemental Figure 23b), and this phenotype may contribute to aspects of DC homing over the course of infection.

The inflammatory environment of the airway is widely considered to be Th2 inclined, though host responses to pulmonary fungal infections involve the production of Th1, Th2 and Th17 type inflammatory responses (287-293). While 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 dominates in response to this infection (98, 294, 295). We demonstrate that sIgM plays an important role in the production of the Th environment in the draining pulmonary lymph nodes, as absence of this antibody isotype markedly impaired the production of cells producing IL-17 and IL-5, while not significantly influencing the generation of IFN-γ producing cells in the lymph node. As we observed heightened quantities of IL-6 in the lungs of wt relative to sIgM mice after *Pneumocystis* challenge, it may be that this potent cytokine promoting Th-2 responses influences this process. Our studies suggest that DC modulation of Th priming is influenced by the presence of sIgM, and an important component of sIgM at this early stage are natural antibodies targeting conserved fungal cell wall carbohydrates. While we cannot distinguish whether antigen dose in draining lymph nodes or aspects of antigen presentation guides this phenotype, we hypothesize that IgM antibodies play a significant role in the Th2 bias of the respiratory mucosa.

Given that sIgM deficient mice produce less IL-5 and IL-17 in draining lymph nodes, at a timepoint as the adaptive immune response is shaping, and have diminished trafficking of zymosan to inductive sites at early timepoints, we hypothesized that the production of adaptive B cell responses against *Pneumocystis* might be diminished, and the antibody isotype profile

skewed towards a Th1 type response, as Th derived cytokines play a critical role in B cell differentiation and isotype class-switch recombination (296, 297). We observed that the generation of Pneumocystis specific IgG1 antibodies was significantly diminished in the absence of sIgM, while IgG2a was enhanced. The dramatic alteration in isotype prevalance against *Pneumocystis* is similar to what is observed in IL-4 (-/-) mice challenged with *Pneumocystis* (98), with strong inhibition of IgG1 production and enhanced production of IgG2a. Our data support the concept that IgM present at early stages of infection promote Th2 effector functions while diminishing Th1 effector functions, as manifested in selective Th help promoting class-switch recombination towards IgG1. As these trends emerge as early as 10 days after infection, we believe that the natural antibody repertoire, combined with early induced sIgM contributes to this phenotype. 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 in memory responses against Pneumocystis. Hence, sIgM influences both specific isotype production as well as overall presence of antibodies against *Pneumocystis* in the mucosa, and we believe this is 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. 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 infection, suffer from polyclonal B cell activation and polyclonal gammopathy, with poor antibody responses to specific antigens (298). Dysregulation of natural and adaptive 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.

In summary, we report on the presence of conserved natural IgM targeting the abundant and conserved carbohydrate antigens present in fungal cell walls. We demonstrate that natural antibodies and sIgM are involved in the earliest stages of fungal recognition and host defense, and identify the critical importance of sIgM in promoting adaptive Th2 type immune responses locally and systemically in response to *Pneumocystis* fungal infection of the lungs.

3.5 SUPPLEMENTAL DATA

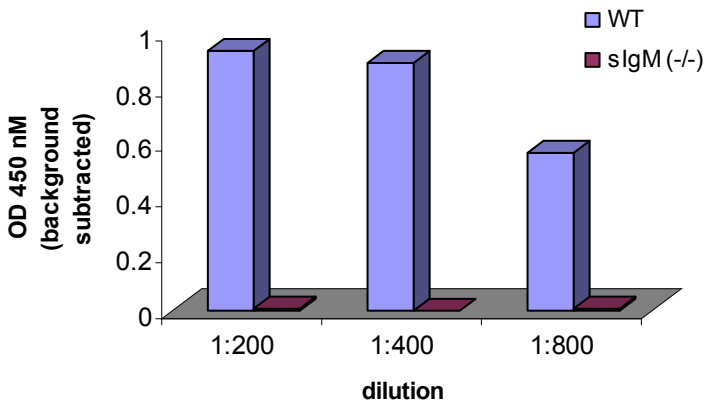


Figure 22: Supplemental Data. Confirmation of absence of secreted IgM in sIgM (-/-) mice on the C57BL/6J background. Serum from naïve wt and sIgM mice were compared for the production of total serum IgM. Average data from 2 mice/group/dilution are presented.

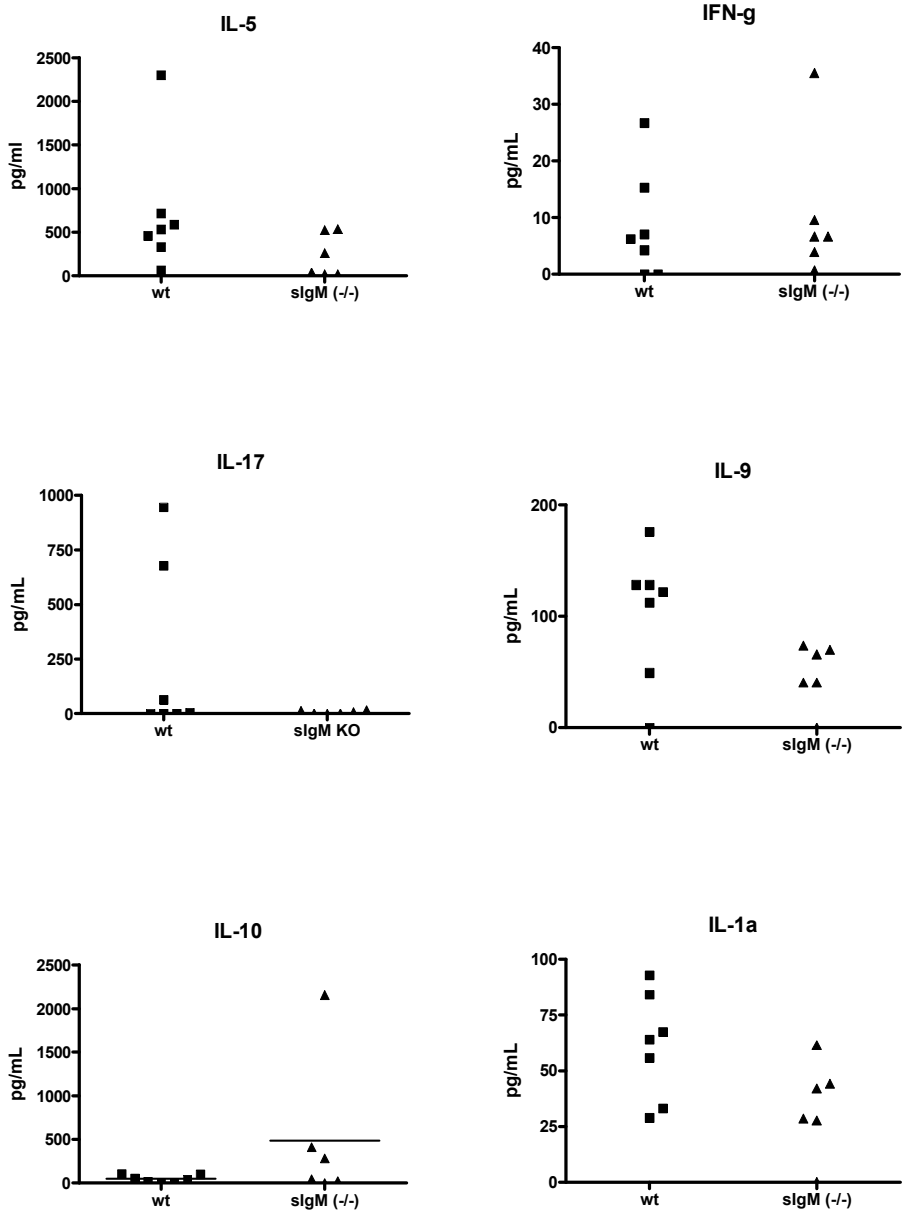


Figure 23a: Supplemental Data. Cytokine profiles of mediastinal lymph nodes from sIgM and wt mice challenged with Pneumocystis and restimulated with Pneumocystis Ag in vitro. Whole lymph nodes were isolated from wt of sIgM (-/-) mice 14 days after intratracheal *Pneumocystis* challenge. Single lymph nodes were teased apart, and restimulated with *Pneumocystis* antigen in vitro and cultured for 3 days. Cytokine responses from individual lymph nodes are represented by the symbols.

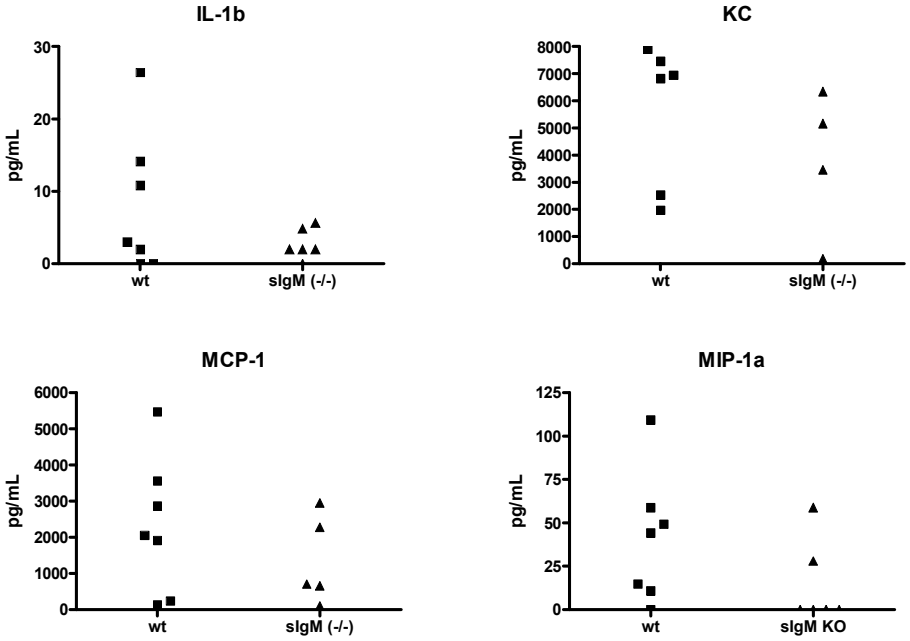


Figure 23b: Supplemental Data. Chemokine/Cytokine profiles of mediastinal lymph nodes from sIgM and wt mice challenged with Pneumocystis and restimulated with Pneumocystis Ag in vitro. Whole lymph nodes were isolated from wt or sIgM (-/-) mice 14 days after intratracheal *Pneumocystis* challenge. Single lymph nodes were teased apart, and restimulated with *Pneumocystis* antigen in vitro and cultured for 3 days. Cytokine responses from individual lymph nodes are represented by the symbols.

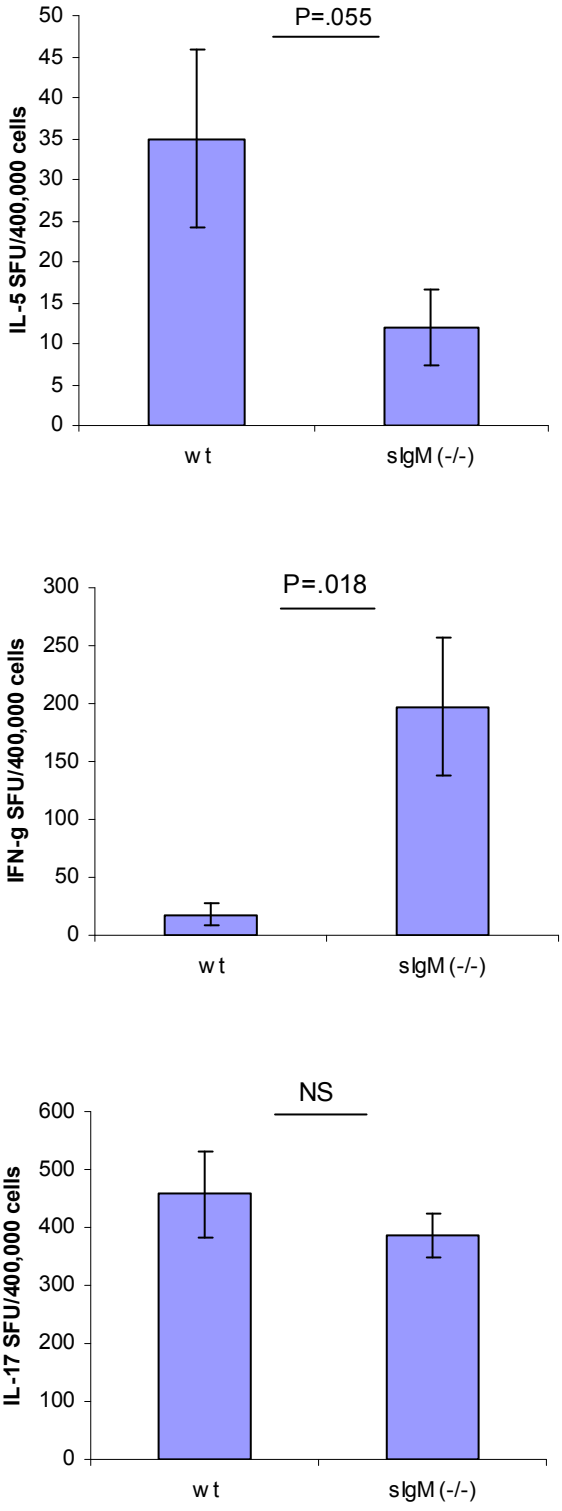


Figure 24: Supplemental Data. Quantitative ELISPOT analysis of single cells in the lung producing cytokines associated with Th1, Th2, and Th17 lineages 28 days after Pneumocystis challenge in wt vs sIgM (-/-) mice. Wt of sIgM (-/-) mice were challenged with Pneumocystis intratracheally. 28 days thereafter, lungs were digested, dispersed into single cells suspensions, enumerated, and analyzed for production of IL-5, IFN-γ, and IL-17 by ELISPOT assay. Average of responses from 5 individual mice/group, and error bars represent SEM. Mann-Whitney test P values presented above respective graphs.

4.0 ADAPTIVE ANTIBODY RESPONSES AGAINST *PNEUMOCYSTIS* CONSERVED FUNGAL CELL WALL CARBOHYDRATES AND REGULATION OF RESPONSES BY CD4+ T CELLS

4.1 INTRODUCTION

Host defense against the opportunisitic pulmonary fungal pathogen *Pneumocystis jirovecii* involves the interplay between innate and adaptive immune responses, which are ultimately initiated through the detection of specific *Pneumocystis* antigens. Currently, very few protein antigens from this complex pathogen have been identified as capable of initiating adaptive host defense responses with some protective benefit (92, 299-301), and many of these *Pneumocystis* proteins are generated from multi-copy gene families or demonstrate significant diversity between *Pneumocystis* species, impairing the assessment of potential vaccine candidates (302-304). The evaluation of potential antigens in adaptive immune responses, with greater conservation, and perhaps a less critical requirement for CD4+ T cells in the development of responses, may be provide an alternate approach for the development of therapies in settings of disease susceptibility.

The fungal cell wall of *Pneumocystis* consists of the conserved carbohydrates, mannan, β-glucan, and chitin, found in most all fungi (111, 116, 305). The carbohydrate components of the *Pneumocystis* cell wall have been studied as targets of various soluble and membrane-bound pattern recognition receptors (48, 49, 72, 138), but the potential role of these carbohydrates as targets of adaptive humoral responses in Pneumocystis infection has not been explored. Structurally, these fungal cell wall carbohydrates are comparable to typical thymus-independent type II (TI-2) antigens, given their large size, highly repetitive structures, and non-protein nature. In these studies, we assessed whether adaptive antibody responses are generated against these conserved carbohydrates antigens, and we sought to define the role of CD4+ T cells in the generation of these responses. Additionally, in the study of a mucosal disease such as *Pneumocystis*, where the a significant portion of the infection consists of trophozoites tightly adhering to the apical surface of epithelial cells, we sought to specifically understand mucosal antibody production against these carbohydrates, as it is unclear what role CD4+ T cells have in guiding aspects of potential TI-2 antibody responses in lungs. Further, as the contribution of CD4+ T cells may be restricted to various stages of B cell function, we assessed whether memory B cells require a CD4+ T cell sufficient environment for the production of antibodies targeting Pneumocystis fungal cell wall carbohydrates.

While there is abundant evidence that antibodies sufficient for host defense against *Pneumocystis* are generated in a CD4+ T cell sufficient environment (82, 97, 167), it is unclear whether antibodies generated in a CD4+ T cell deficient environment also have some protective efficacy, or modulate aspects of the host immune response. Here we demonstrate that chitosan/chitin, and β-glucan are targets of adaptive antibody responses against *Pneumocystis*,

and we dissect the requirements for CD4+ T cells in isotype class-switching, mucosal antibody production, and functional B cell memory against these antigens.

4.2 MATERIALS AND METHODS

4.2.1 Mice

Male BALB/c mice, 6-8 weeks in age, were purchased from Jackson Immunoresearch Laboratories, and Male BALB/c.*scid* mice 6-8 weeks of age were obtained from Taconic Farms. All mice were maintained in a specific pathogen-free environment in microisolator cages within the animal care facilities of Children's Hospital of Pittsburgh under protocols reviewed and approved by the Animal Research and Care Committee. Mice were provided with water and food ad libitum and received 12-h light/dark cycles

4.2.2 Chronic *In vivo* CD4+ T cell depletion

BALB/c mice were chronically depleted of CD4+ T cells using the rat mAb GK1.5 obtained from National Cell Culture Center. Mice received 200 μg GK1.5/weekly, intraperitoneally, for two weeks prior to *Pneumocystis* challenge, and weekly treatments of GK1.5 were continued through the course of the infection. This dose of mAb was confirmed to be sufficient for the depletion of CD4+ T cells to less than 0.5% of the original quantity of CD4+ T cells in the spleen, as measured with staining of CD4 with RM4-4 (BD Pharmingen). Additionally, this dose leads to sustained depression of CD4+ T cells in the spleen through at least 7 days (at which

point GK1.5 is again administered), and *Pneumocystis* susceptibility. Control mice received weekly treatments of Rat IgG at the same dose and administered in the same schedule (Sigma). Both antibodies were dissolved in sterile PBS. GK1.5 and control antibody were kept at -80°C until needed.

4.2.3 P. carinii f. sp. muris isolate

The *Pneumocystis* inoculum was prepared as previously described (42, 89). Briefly, B6.*scid* and BALB/c.*scid* mice with *Pneumocystis* pneumonia were injected with a lethal dose of ketamine/xylazine and the lungs were aseptically removed and frozen in 1 ml of PBS at –80°C. Lungs were mechanically dissociated in sterile PBS, filtered through sterile gauze, and pelleted at 500 x *g* for 10 min at 4°C. The pellet was resuspended in sterile PBS and a 1/5 dilution was stained by a modified Giemsa stain (Diff-Quik; Baxter). The number of *Pneumocystis* cysts was quantified microscopically and the inoculum concentration was adjusted to 2×10^6 cysts/ml. Gram stains were performed on the inoculum preparations to exclude contamination with bacteria.

4.2.4 Pneumocystis infection

Mice were anesthetized and intratracheally challenged with the cyst enumerated preparation of 2×10^5 *Pneumocystis* cysts in 100 µL PBS.

4.2.5 Serum and BALF collection

Fluid from the lower respiratory tract was obtained by bronchoalveolar lavage of mice anesthetized with i.p. ketamine/xylazine as described previously (89). 1 mL of BALF was collected from mice using sterile PBS. Lavage fluids were centrifuged at 350 x *g* and supernatant was stored at –80°C until use.

For serum collection in longitudinal studies with mice, small volumes of blood were collected from the periorbital sinus. At terminal sacrifice of mice, blood was obtained by caudal venipuncture under anesthesia. Serum was isolated from collected blood by use of serum separator tubes, as per protocol. Serum was stored at –80°C until use. Additionally, in various timecourse studies as noted, after *Pneumocystis* challenge mice were sacrificed and serum was pooled per timepoint.

4.2.6 Detection of antibody responses against Pneumocystis, beta-glucan, mannan, and chitosan/chitin

Pneumocystis (PC) antigen was prepared as previously described (57) and derived from the lungs of infected BALB/c.*scid* and C57BL/6.*scid* mice by differential centrifugation to derive organisms, which were then sonicated. PC antigen was normalized to protein concentration after sonication of organisms. PC antigen was dissolved in carbonate buffer, pH 9.5, and seeded to Nunc-Polysorp 96 well plates at a concentration of 1 μg/mL. Laminarin (Sigma), derived from the brown algae *Laminaria digitata*, is composed primarily of β-1,3 linked glucan, the predominant β-glucan linkage found in fungal cell walls (102). Medium molecular weight

chitosan (Sigma), derived from crab shells, is polymer of chitin that is 75-85% deacetylated. Chitosan/chitin was dissolved in 2% acetic acid/PBS (v/v) at a concentration of 0.5-1mg/mL and thereafter diluted into PBS to final quantity of 25 μg/mL. α-1,6 linked mannan (Sigma) derived from *Saccharomyces cerevesiae* was dissolved in PBS. Carbohydrate antigens were seeded at a concentration of 25 μg/mL to Nunc-Polysorp 96 well plates and kept overnight at 4 degrees. 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 was applied in serial dilutions. BALF was run neat. HRP-conjugated secondary antibodies against murine IgG, IgA, and IgM were obtained from Santa Cruz Biotechnology and Southern Biotech. Plates were developed with TMB, and the reaction was quenched with 2N sulfuric acid. Background for all ELISAs was typically less than .05 at an OD of 450 nM. Estimated endpoint titer was determined by assessing the lowest concentration at which signal was obtained that was 2 times higher than background. If this value fell between 2 concentrations, then the titer was approximated relative to the numerical distance between the reading of the upper and lower concentration. Optical density values were reported at 450 nM, absolute or with background subtracted, as indicated per graph.

4.2.7 Measurement of BAFF/BlyS and active TGF-β

BAFF levels in serum, lung homogenates, and BALF were measured by ELISA (R&D Systems). Active TGF-β levels in serum and lung homogenates were measured by ELISA using a TGF-β1 DuoSet (R&D Systems).

4.2.8 Adoptive transfer studies in SCID mice

Wild type BALB/c mice were challenged with Pneumocystis intratracheally and rested for 3.5 months (PC memory mice). Spleens were collected from PC memory mice or age-matched naïve mice, pushed through 70-µm pore size cell strainer, and RBC's lysed with sterile NH_4Cl/Tris solution for 5 min on ice. Thereafter, cells were simultaneously stained with fluorochrome conjugated antibodies against B220 and CD4, and typically a combination of APC-anti-B220 and PE-anti-CD4 was used. B220+/CD4- and CD4+ /B220- populations were each collected by FACS and typically the purity of B220+ cells was ~98% and ~92% for CD4+ T cells. Cells were suspended in sterile PBS. Depending on the group, 10^6 B cells +/- 500,000 CD4+ T cells were administered to SCID mice intravenously. Three days after cell transfer, SCID mice were challenged with *Pneumocystis* intratracheally. 50 days after challenge, mice were harvested for analysis of systemic and mucosal antibody responses.

4.2.9 Statistical Analysis

Data were analyzed using GraphPad statistical software. Comparisons between groups were made with the Student *t* test or Mann-Whitney *U* test. Significance was accepted at a value of $p < 0.05$.

4.3 RESULTS

4.3.1 Induction of IgG responses against beta-glucan and chitosan/chitin in the blood and enrichment of mucosal of IgA, IgG, and IgM isotypes targeting fungal cell wall carbohydrates after Pneumocystis challenge

To assess whether adaptive antibody responses are generated against the conserved fungal cell wall carbohydrates β-glucan, chitosan/chitin, and mannan, Balb/c wt mice were challenged with *Pneumocystis* intratracheally. At serial timepoints, 2-3 mice were sacrificed, and serum was pooled and assessed for Ag reactivity in various ELISAs. First, we observed that naïve mice possess low levels of IgM reactive with Pneumocystis Ag (PC) prior to challenge at a titer of 1:150, whereas anti-PC IgG is entirely absent (Figure 25 a). However, both the IgG and IgM response rapidly enhance after challenge, with the IgM preceding the IgG response, plateauing at 22 and 36 days respectively. When we probed this same serum for IgG reactivity against β-glucan and chitosan/chitin, we noted that the IgG responses against these antigens occurred as well, plateauing somewhat later, at 42 days after infection (Figure 25 b). There were no IgG responses against mannan (data not shown). Specific IgG induced against β-glucan and chitosan/chitin after PC challenge is of significantly lower quantities than polyclonal responses against IgG produced against PC protein antigen. However, we demonstrate that IgG responses targeting conserved fungal cell wall carbohydrates are induced as a consequence of *Pneumocystis* challenge.

To assess how these serum responses might translate into changes in mucosal Abs lining the lung epithelium, we compared the IgG, IgA, and IgM responses to β-glucan and

chitosan/chitin in the BALF of naïve mice and mice that had been challenged with *Pneumocystis* 28 days earlier. We observed that Pneumocytis challenge significantly enhanced quantities of these antibodies targeting fungal cell wall carbohydrates in the lung (Figure 25c,d). Of note, these dynamics differ from studies of BALF reactivity against PC sonicate, where no reactivity of any isotype is observed at baseline (data not shown), hence *Pneumocystis* exposure is not required for the production of the IgG, IgA, and IgM against β-glucan and chitosan/chitin at baseline. These data suggest that Pneumocystis exposure leads to the increased presence of mucosal antibodies targeting Pneumocystis fungal cell wall carbohydrates.

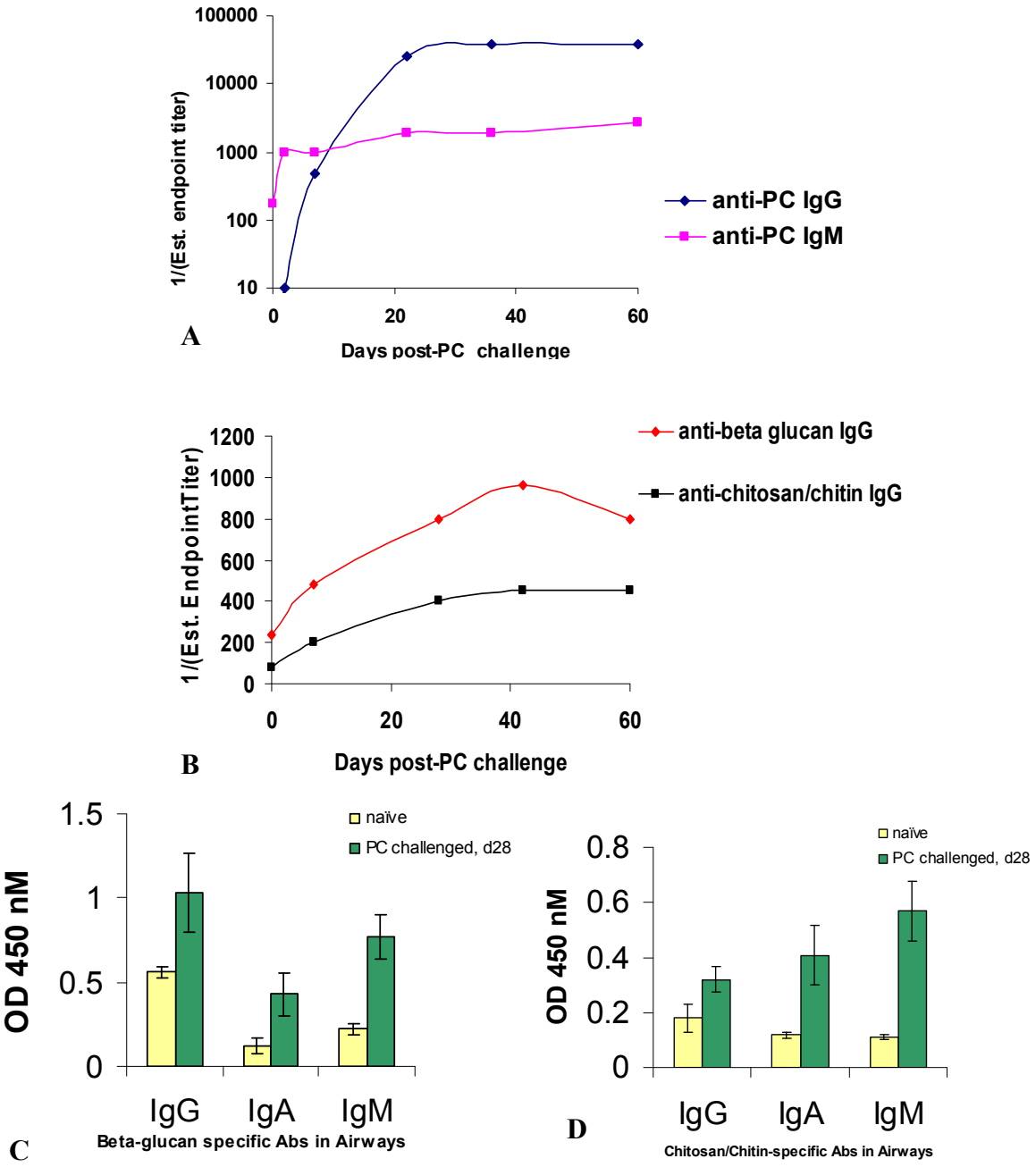


Figure 25: Systemic and mucosal immunoglobulins targeting beta-glucan and chitosan/chitin are enhanced after Pneumocystis challenge. BALB/c wt mice were challenged with Pneumocystis intratracheally. Pooled serum from n=2-3 sacrificed mice per timepoint was A) probed for IgG and IgM reactivity against Pneumocystis sonicate (PC) or B) IgG reactivity against beta-glucan or chitosan/chitin. C & D) Mice were sacrificed 28 days after challenge and BALF was compared to naïve age-matched mice for IgG, IgA, and IgM reactivity against chitosan chitin (C) or beta-glucan (D). 4-5 mice per group. Average values presented, error bars represent SEM.

4.3.2 CD4+ T cell depletion impairs production of serum isotypes targeting Pneumocystis protein Ag but does not impair serum responses against fungal cell wall carbohydrate antigen: Beta-glucan and chitosan/chitin are TI-2 Ags

We sought to assess the role of CD4+ T cells in the production of antibodies targeting fungal cell wall carbohydrates after *Pneumocystis* challenge. Utilizing a monoclonal Ab that specifically targets CD4, GK1.5, we chronically depleted wt mice of CD4+ T cells in secondary lymphoid organs, leading to a phenotype of significant *Pneumocystis* growth in the lungs after intratracheal challenge, as previously described (42). Mice were treated mice with 200 μg of GK1.5, or Rat IgG as a control, weekly. After two weeks of treatment, mice were challenged with *Pneumocystis* intratracheally. Weekly GK1.5 and Rat IgG treatments were continued through the duration of the experiment to maintain chronic CD4+ T cell depletion. Mice were sacrificed 28 days after challenge, and blood and BALF were analyzed for antibody reactivity against *Pneumocystis* Ag (PC) as well as β-glucan and chitosan/chitin. In the serum, we observed that chronic CD4+ T cell depletion significantly impairs the production of anti-PC IgG isotypes, of which the IgG1 isotype is most dramatically affected, while not influencing quantities of anti-PC IgM (Figure 26a). These data support studies by other groups on the effect of CD4+ T cell depletion on the production of Abs targeting Pneumocystis Ag (97, 98), and suggest that the majority of the polyclonal response against PC Ag is directed against CD4+ T cell dependent antigens, likely of protein nature. Yet, when we probed the serum for anti-beta glucan and anti-chitosan/chitin IgG responses, we noticed that CD4+ T cell depletion had no effect on its

production, and additionally did not influence quantities IgM (Figure 26b,c), which in Chapter Three, we argued were likely innate Abs. Hence we conclude that the induced IgG response occurs in the absence of specific requirement for CD4+ T cells, and given the structural nature of these carbohydrates, we conclude that these are thymus-indpendent type II antigens.

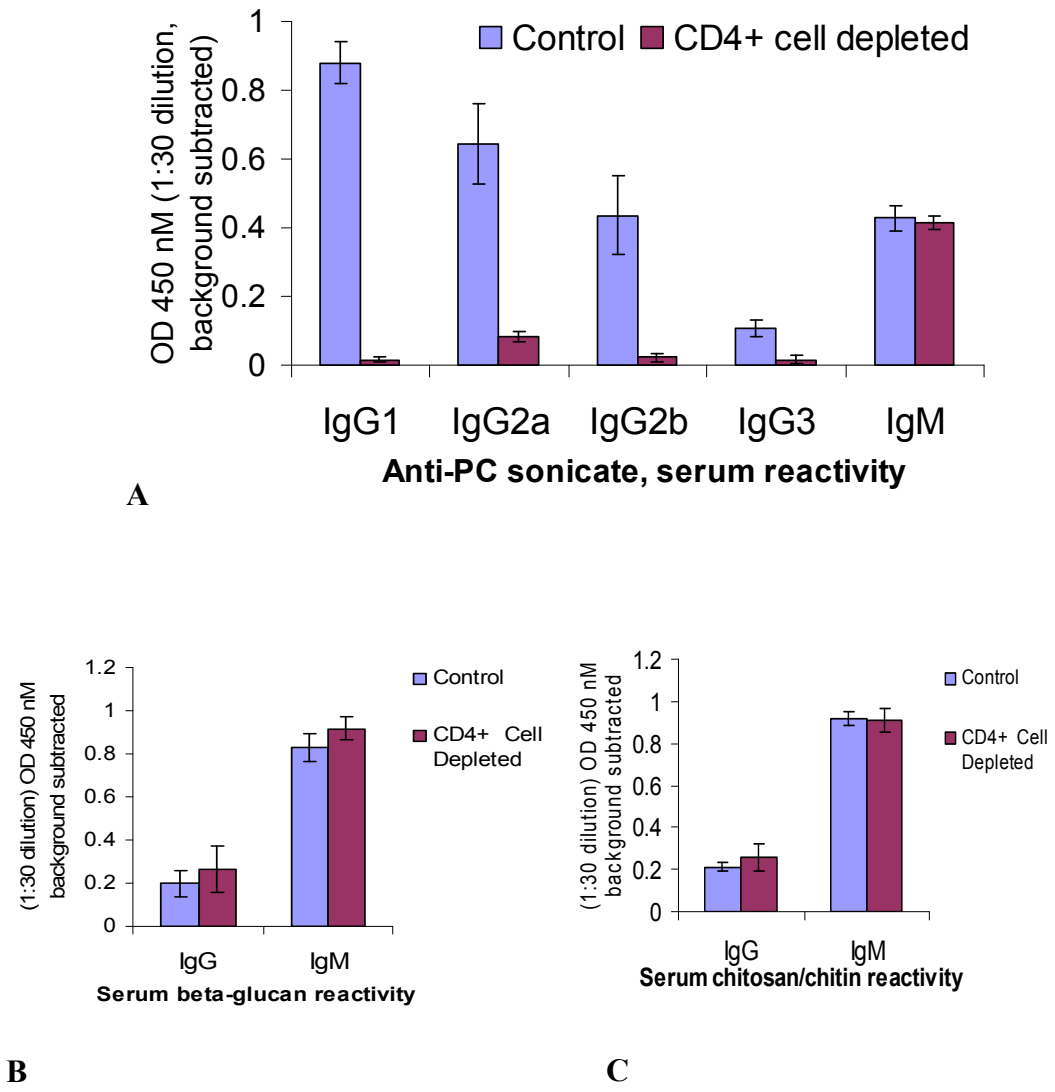


Figure 26: Effect of CD4+ T cells on the presence of antibodies targeting PC or fungal cell wall carbohydrates in the serum. Mice were challenged with *Pneumocystis* intratracheally. 28 days thereafter, mice were sacrificed and serum collected and probed for reactivity against A) PC sonicate, with specific analysis of isotype reactivity, or B) Beta-glucan, for IgG or IgM reactivity, or C) Chitsan/chitin, for IgG or IgM reactivity. 4-5 mice/group. Average responses are presented, with error bars representing the SEM.

4.3.3 Absence of CD4+ T cells significantly influences mucosal Ab responses against fungal cell wall carbohydrate antigen

We next sought to assess how antibody responses targeting fungal cell wall carbohydrates might be generated at the mucosa, in the absence of CD4+ T cells. We analysed BALF collected from mice described in Figure 26. First in our analyses, we assessed reactivity against PC Ag, and observed that significant quantities of induced IgG and IgA against PC Ag are present in the mucosa, and that these responses are highly impaired in the setting of CD4+ T cell deficiency (Figure 27a), while the anti-PC IgM response is unaffected.

At the mucosa, CD4+ T cell depletion notably affected the presence of various isotypes targeting the fungal cell wall conserved carbohydrates, but in a different manner than which anti-PC antibodies were affected. While CD4+ T cell depletion did not reduce quantites of IgG targeting β-glucan and chitosan/chitin at the mucosa, quantities of IgA were significantly impaired (Figure 27 b, 27c). These data suggest that the production of specific IgA targeting conserved fungal cell wall carbohydrates at the mucosa crtically involves CD4+ T cells, suggesting perhaps that Th cells are critically involved in IgA class-switching at the mucosa in the production of these antibodies. Alternatively, CD4+ T cells might influence survival or homing signals to IgA antibody secreting cells of the lung.

Additionally, we observed specific enhancement of quantities of IgM targeting fungal cell wall carbohydrates in the mucosa, and of note, we did not observe enhanced production of these carbohydrates in the serum, suggesting that the phenotype is manifested by local IgM producing cells in the mucosa (Figure 27 b, 27c). These data suggest that in the setting of impaired host defense against *Pneumocystis* and consequently enhanced PC Ag in the lungs, as

occurs in the absence of CD4+ T cells which possess required effector function in host defense (96), antibody secreting cells producing IgM targeting fungal cell wall carbohydrates preferentially home to the lung, where they influence mucosal IgM responses against *Pneumocystis.* This finding is striking in light of the observation that anti-PC IgM is not affected by CD4+ T cell depletion in the serum or in the lung mucosa. It suggests to us that perhaps different B cells are responsible for the production of IgM targeting fungal cell wall carbohydrates vs IgM targeting PC antigen, accounting for their differences in regulation. We hypothesize that the enhanced IgM production against fungal cell wall carbohydrates at the mucosa may represent a primitive host defense pathway, which is activated in the setting of impaired pulmonary defense against *Pneumocystis* and increased pulmonary burden in the lungs

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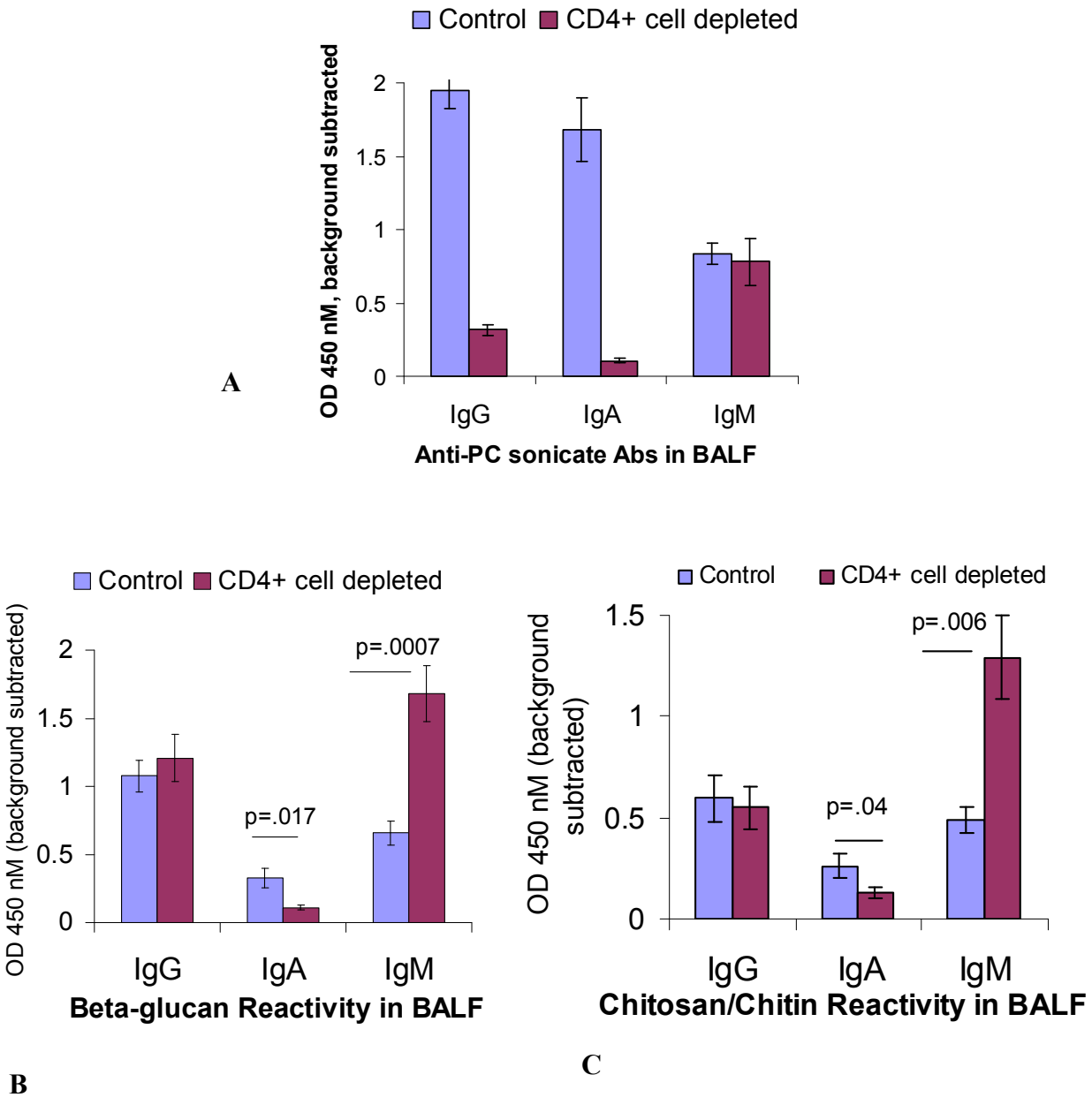

Figure 27: Effect of CD4+ T cells on the presence of antibodies targeting PC or fungal cell wall carbohydrates in the BALF. BALF was collected from mice as described in Figure 26, 28 days after infection. BALF was probed for reactivity, by isotype, against A) PC sonicate, B) Beta-glucan, and C) Chitosan/chitin. All data are pooled from two independent experiments, n=8-10 mice/group. Mean value with SEM are presented. p values were calculated with Students t test.

4.3.4 Assessment of the regulation of potential IgA class-switch factors in mice challenged with Pneumocystis in the setting of CD4+ T cell deficiency

We sought to understand factors that might be responsible for the perturbation of IgA responses in the lung against fungal cell wall carbohydrates. As class-switching towards IgG occurred normally, we questioned whether specific IgA isotype-class switch signals might be impaired in the setting of CD4+ T cell deficiency. Mucosal IgA class switch factors include TGF-β, which is considered a CD4+ T cell related isotype class-switch factor, and the cytokines BAFF/Blys and APRIL, which have recently been demonstrated to be released by the mucosal epithelium in a CD4+ T cell-independent, TLR-dependent manner (306, 307). We hypothesized that factors promoting CD4+ T cell-dependent and CD4+ T cell-independent class-switching towards IgA would both be diminished, leading to the diminished quantities of IgA targeting beta-glucan and chitosan/chitin that we observed. We found that while TGF-β levels were decreased in the serum and lung homogenate, quantities of BAFF/BlyS were very high, significantly enhanced in the setting of CD4+ T cell depletion (Figure 28). Hence, impaired release of BAFF/BlyS likely does not account for the impaired IgA response against these carbohydrate Ags, but perhaps decreased TGF-β accounts may play a role in these differences. Additionally, other IgA class-switch recombination factors such as IL-5 (308) or iNOS (309) may be relevant.

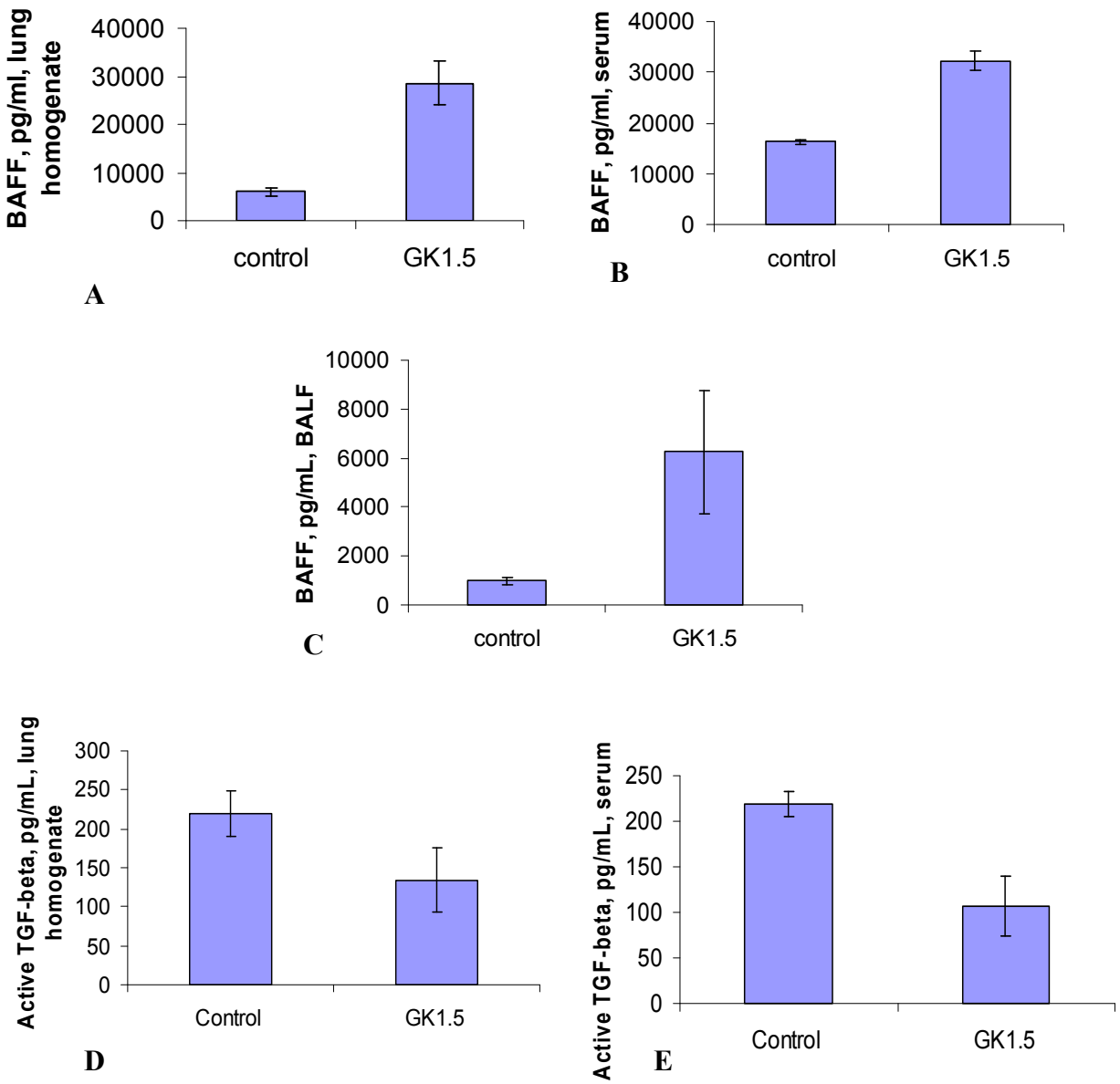

Figure 28: Presence of IgA class-switch recombination factors in tissues of mice challenged with *Pneumocystis* in the setting chronic CD4+ T cell depletion. BAFF/BlyS measured from A) lung homogenate, B) serum, and C) BALF. Active TGF-β measured from D) lung homogenate, and E) serum. Quantities of active TGF-β were negligible in BALF. 4-5 mice were studied/group. Mean with SEM are presented

4.3.5 Memory B cells targeting fungal cell wall carbohydrates are produced as a consequence of Pneumocystis challenge, and do not require CD4+ T cells for reactivation

Our studies thus far have indicated that while the conserved fungal cell wall carbohydrates β-glucan and chitosan are TI-2 antigens, the production of antibodies at mucosal sites is regulated by CD4+ T cells. Hence, we sought to understand the importance of CD4+ T cells in potential memory B cell responses against these carbohydrates as a consequence of *Pneumocystis* challenge. wt mice develop *Pneumocystis* infection in the lungs and ultimately clear it while generating protective adaptive immune responses within 4-5 weeks (96, 310), hence we challenged BALB/c mice with *Pneumocystis* intratracheally, and allowed rodents to rest 110 days thereafter in order to provide sufficient time for resolution of the infection, and we hereafter refer to these rodents "PC memory mice". As memory B cells have been described to be localized primarily in secondary lymphoid organs in mice (214, 218), we took splenocytes from the *Pneumocystis* memory mice, or from age-matched naïve mice "PC naïve mice", and selected either B220+ cells or CD4+ cells by FACS. Mice received combinations of CD4+ T cells and B cells from either group, or B cells alone. Three days later, BALB/c.scid mice were challenged with *Pneumocystis* intratracheally. 50 days after challenge, tissues were collected and analyzed for the production of Abs targeting fungal cell wall carbohydrates. In a first analysis, we compared the ability of SCID mice receiving B cells from PC memory mice or PC naïve mice to produce antibody responses targeting fungal cell wall carbohydrates in the serum and blood. We found that mice receiving B cells from PC memory mice have higher quantities of IgG antibodies targeting β-glucan and chitosan/chitin than mice receiving B cells from PC naïve mice

in the serum and at mucosal sites. Interestingly, chitosan/chitin responses dominate in SCID mice relative to the β-glucan response, which dominate in the wt mouse, suggesting that even the factors guiding B cell function between these two specificities may differ, as well as the manifestation of responses as evaluated over different timecourses, with this study evaluating presence at day 50 after challenge, in SCID mice. These data suggested to us that *Pneumocystis* challenge in wt mice enriches the spleen for B cells capable of producing antibody responses against fungal cell wall carbohydrates. We argue that this constitutes a functional immunologic memory, as IgG responses against fungal cell wall carbohydrates are induced, and *Pneumocystis* challenge led to the alteration of the rodent's B cell population such that it possessed a greater ability to produce antibody against these carbohydrate specificities when transferred into SCID hosts and challenged with *Pneumocystis*. As we also demonstrate that B cells from Pneumocystis naïve mice are capable of producing a *de novo* IgG responses to fungal cell wall carbohydrates, in the absence of CD4+ T cells, it underscores the capacity of these B cells to produce IgG in a CD4+ T cell independent manner in a primary response. Hence, the primary and memory anti-beta glucan and chitosan/chitin IgG responses do not require CD4+ T cells, and *Pneumocystis* challenge enriches the spleen for cells capable of producing IgG against these carbohydrates.

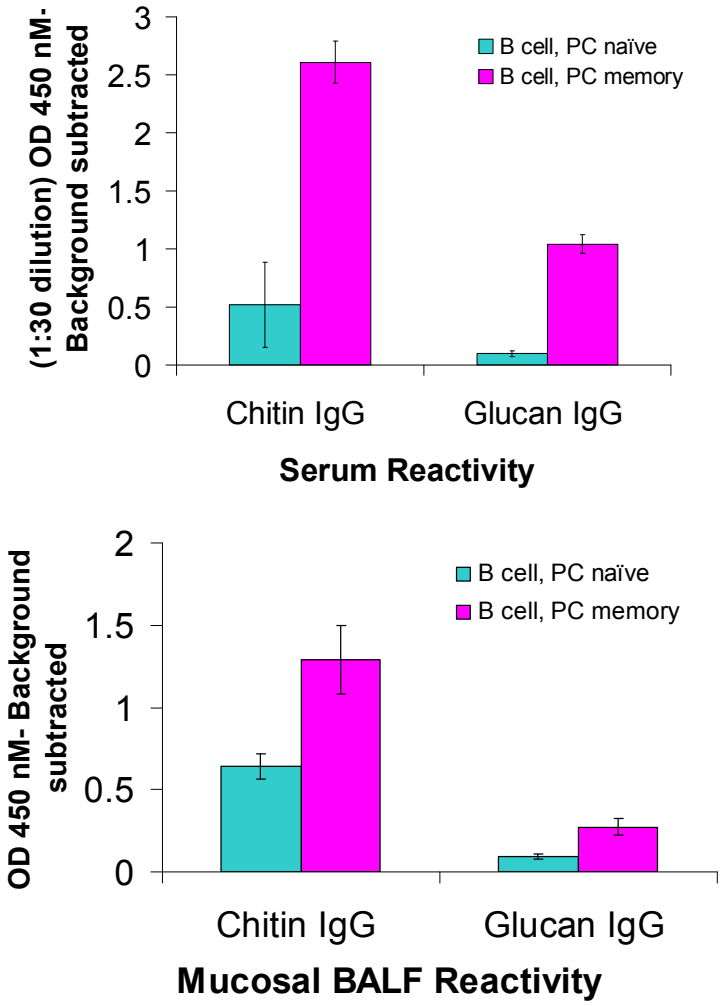

Figure 29: Functional memory IgG responses as a consequence of PC challenge against conserved carbohydrates, and operativity in the absence of CD4+ T cells. Wt mice were challenged with Pneumocystis intratracheally and rested for 110 days. 1 million B220+ cells sorted from pooled splenocytes from either PC memory mice (B cell, PC memory) or PC naïve, unchallenged mice (B cell, PC naïve) were transferred into individual SCID mice and 3 days later mice were challenged with Pneumocystis intratracheally. Mice were sacrificed 50 days thereafter, and BALF and serum analyzed by ELISA to detect IgG responses against fungal cell wall carbohydrates. Average +/- SEM for n=2 (B cell, PC memory) and n=3 (B cell, PC naïve) individual mice per reactivity assessed per graph.

4.3.6 Optimal IgA production against fungal cell wall carbohydrates requires CD4+ T cells in an adoptive transfer model

Given the observation that the absence of CD4+ T cells impairs the production of specific IgA against fungal cell wall carbohydrates, we sought to confirm this requirement by assessing whether IgA production against fungal cell wall carbohydrates occurs in the BALF of SCID mice transferred with B cells but in the absence of CD4+ T cells. Cells from PC memory and PC naïve splenocytes are those collected in section 4.3.5. Comparisions were made between mice receiving 1 million B cells from either PC memory or PC naïve mice; mice receiving 1 million B cells from PC memory mice supplemented with 500,000 CD4+ T cells from PC naïve mice; and mice receiving 1 miilion PC naïve B cells supplemented with 500,000 CD4+ T cells from PC memory mice. We found that neither group where B cells were transferred alone was capable of producing significant quantities of mucosal IgA targeting fungal cell wall carbohydrates, while both groups receiving B cells and CD4+ T cells were capable of producing an IgA response against these antigens (Figure 30). We conclude that the IgA responses observed were the result of *de novo* B cell priming and homing to the lung mucosa, requiring CD4+ T cells as helper cells, as there was no significant difference between the mice receiving naïve B cells or PC B cells in mucosal IgA responses if simultaneously provided CD4+ T cells.

As patients with HIV have diminished quantities and functions of CD4+ T cells , and our studies demonstrate that CD4+ T cell deficiency leads to impaired production of mucosal IgA

targeting fungal cell wall carbohydrates, we hypothesize that this response may be an important pathway that is perturbed in the setting of CD4+ T cell deficiency. IgA may have unique effector functions. Additionally, the quantities of IgA that occur at baseline targeting these carbohydrates could be perturbed in the setting of HIV, leading to impaired defense in the setting of acute exposure, allowing for escape of Pneumocystis organisms from humoral host defenses.

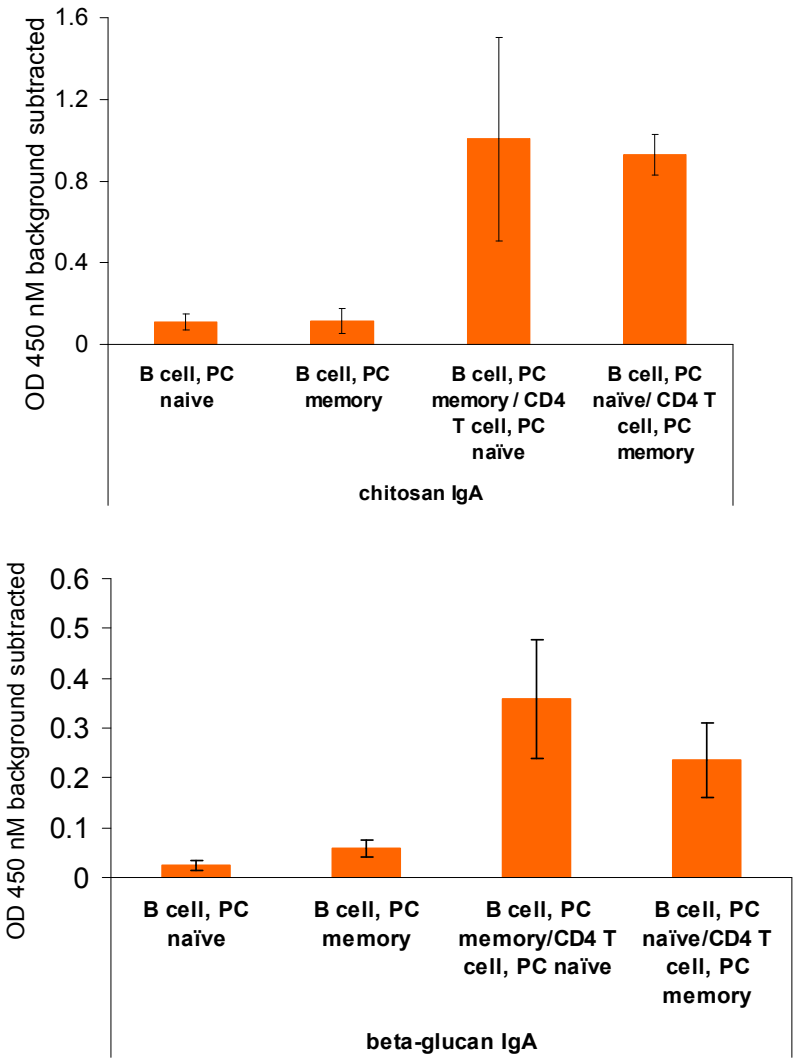

Figure 30: Mucosal IgA production in the BALF of SCID mice transferred with B cells, and requirement for CD4+ T cells for optimal IgA production against targeting fungal cell wall carbohydrate antigen. Wt mice were challenged with Pneumocystis intratracheally and rested for 110 days. SCID mice were transferred with 1 million B220+ cells sorted from pooled splenocytes from either PC memory mice (B cell, PC memory) or PC naïve, unchallenged mice (B cell, PC naïve). Additionally cohorts containing an additional 500,000 CD4+ T cells from either source PC memory or PC naïve were generated: (B cell, PC memory/CD4 T cell, PC naïve) and (B cell, PC naïve/ CD4 T cell, PC memory). Cells were transferred into individual SCID mice and 3 days later mice were challenged with Pneumocystis intratracheally. Mice were sacrificed 50 days thereafter, and BALF analyzed by ELISA to detect IgA responses against fungal cell wall carbohydrates. Average +/- SEM for n=2 mice (B cell, PC memory) and n=3 mice for all other groups, per reactivity, per graph.

4.4 DISCUSSION

The generation of immunoglobulins targeting carbohydrate antigen are among the most important protective immune responses generated in humans. Some of the most essential vaccines in clinical use induce protective antibodies against carbohydrate antigens in the cell walls of bacteria such as *Streptococcus pneumoniae*, *Hemophilus influenzae*, or *Neisseria meningitidis*, preventing against human diseases ranging from pneumonia to meningitis (201). Despite the importance of these responses in host defense against infection, the mechanisms regulating the induction of antibodies against carbohydrate antigens in the setting of an actual infection are poorly characterized. With recent studies in murine models demonstrating that a β-glucan-diphtheria toxoid conjugate vaccine enhanced host defense against systemic and/or mucosal *Aspergillus* and *Candida* infection (154), and that a fusion protein with the ability to recognize β-glucan as well as bind FcγR's could enhance host defense against *Pneumocystis* (270), we sought to understand whether antibody responses targeting the conserved fungal cell wall carbohydrates of Pneumocystis are induced in the setting of actual pathogen infection. Further, as Pneumocystis pneumonia susceptibility correlates with major defects in adaptive immune responses, and most clearly with diseases that specifically perturb CD4 + T cell function, we questioned how such antibody responses might be affected by absence of these cells. Finally, to design any vaccine in the setting of such immunodeficiency, we must understand the factors influencing memory responses, and how CD4+ T cells factor into the maintenance phase of responses. Hence, these studies sought to make initial characterizations of the antibody responses generated against the conserved fungal cell wall carbohydrates, and their

regulation, after *Pneumocystis* challenge, with implications relevant for host defense against *Pneumocystis* and potentially other fungal pathogens.

We observed that intratracheal *Pneumocystis* challenge led to the slow induction of specific IgG targeting β-glucan and chitosan/chitin in the serum, and additionally, 28 days after infection, quantities of IgG, IgA, and IgM against these antigens were enhanced at the mucosal surface of the lung. These data demonstrate, first, that specific anti-carbohydrate antibodies targeting *Pneumocystis* carbohydrates are synthesized by the host after challenge with intact pathogen. Additionally, as IgA responses were absent in the serum against these carbohydrates, it demonstrates that the induction of local B cell immune responses occurs against β-glucan and chitosan/chitin, with enrichment of specific IgG and IgM as well at the mucosa, either through systemic or local processes. As these carbohydrates are conserved between most all fungal species, we hypothesize that production of these antibodies may occur after exposure to other pathogenic fungi, and that in mucosal infections, these responses may particularly accumulate at mucosal sites.

Second, we demonstrate that β-glucan and chitosan/chitin are TI-2 independent antigens. CD4+ T cells are not required for the production of antibodies targeting these antigens, and there is no perturbation of quantities of IgG or IgM against these antigens in the blood, while IgG isotype responses are significantly impaired againt PC sonicate, likely representing protein antigens that generate strong, high-affinity antibody responses. Hence, antibody responses targeting conserved fungal cell wall carbohydrates may occur in settings of CD4+ T cell insufficiency.

We observed heightened mucosal IgM targeting fungal cell wall carbohydrates in the setting of CD4+ T cell deficiency and *Pneumocystis* challenge. Taken with the observation that

there was no enhancement of IgM responses against PC sonicate at the mucosa, and no difference between control mice and CD4+ T cell depleted mice in quantities of β-glucan and chitosan/chitin IgM in the serum, we argue that perhaps the enhanced IgM mucosal response specific to carbohydrates in the setting of persisting antigen is a primitive host defense mechanism. To reconcile the observation of enhanced quantities of carbohydrate-specific IgM secreting cells in the mucosa, but not in the serum, we argue that this response requires specific trafficking of IgM antibody-secreting cells to the mucosa.

Our studies suggest that CD4+ T cells are required for the production of fungal cell wall carbohydrate directed IgA, while not IgG, at the mucosal surfaces of the lung. We observed significantly diminished quantitites of IgA in the BALF of β-glucan and chitosan/chitin specific antibodies after Pnuemocystis challenge in the models of chronic CD4+ T cell depletion and after adoptive transfer of B cells without CD4+ T cells into SCID mice. IgA responses are local responses, presumably generated as a consequence of regional priming, and we observed no appreciable IgA in the serum against these carbohydrates, thus, we did not consider CD4+ T cell derived migration of IgA antibody secreting cells to play a major role in this process. Hence, we hypothesized that CD4+ T cells may make signals that are required for local IgA class-switch recombination. We evaluated whether class-switching factors might impair the production of IgA, and evaluated quantities of TGF-β, which is involved in the CD4+ T cell dependent IgA class-switching and BAFF/BlyS/Tnfsf13b, a B cell survival factor which is involved in CD4+ T cell independent IgA class-switch recombination at the mucosa (309). We demonstrate that in mice depleted of CD4+ T cells and challenged with Pneumocystis, that active TGF-β is decreased, suggesting that CD4+ T cell depletion itself may reduce production of TGF-β. On the other hand, while we anticipated that factors guiding non-CD4+ T cell dependent IgA class

switch recombination would be reduced, these BAFF quantities are significantly enhanced in CD4+ T cell depleted mice challenged with *Pneumocystis*. Hence, we believe that CD4+ T cell dependent IgA class-switch recombination is perturbed in this model, and that IgA class-switch recombination factors that are considered CD4+ T cell independent, are unable to compensate for the deficiency of CD4+ T cell derived factors. While the majority of mucosal IgA is produced in a CD4+ T cell independent manner, our data suggests that some antigen induced carbohydrate-directed mucosal IgA is produced in a CD4+ T cell dependent manner.

Finally, we provide evidence that functional B cell memory responses are generated after *Pneumocystis* challenge against fungal cell wall carbohydrates. Splenic B cells from mice previously challenged with *Pneumocystis*, when transferred into SCID mice and challenged with the fungal organism, produce significantly more specific IgG against β-glucan and chitosan/chitin in the serum and at the lung mucosa, compared to SCID mice receiving B cells from mice that had not been challenged with *Pneumocystis*. Importantly, SCID mice receiving B cells from mice not previously challenged with *Pneumocystis,* produced significant quantities of IgG against β-glucan and chitosan/chitin in SCID mice after *Pneumocystis* challenge, underscoring our observation that the production of these antibodies does not require CD4+ T cells. These data suggest that CD4+ T cells are not required for the induction of IgG targeting fungal cell wall carbohydrates, and additionally, that memory B cells producing antibodies that target β-glucan and chitosan/chitin do not require a CD4+ T cells for activation and functions after their generation.

These data describe novel roles for fungal cell wall carbohydrates as targets of adaptive antibody responses and underscore the complexity of T cell involvement on the production of antibody targeting carbohydrates antigens at mucosal sites.

5.0 SUMMARY

The core conclusions from these investigations are summarized below:

1) A molecule with the capacity to recognize β-1,3 linked glucan, and bind to FcγRII and FcγRIII receptors on antigen presenting cells, was able to enhance recognition of fungal cell wall carbohydrates by macrophages, enhance macrophage effector function against *Pneumocystis*, and impair the growth of *Pneumocystis* infection in the lungs of SCID mice. We hypothesized that the host may recapitulate this effective strategy to limit growth of fungal organisms by the production of antibodies targeting fungal cell wall carbohydrates.
2) We observed that natural IgM antibodies target the conserved fungal cell wall carbohydrates β-glucan and chitosan/chitin. Probing for these IgM specificities in germ-free mice, and in human cord blood, we determined that microbial stimulation is not required for the production of these specificities. Underscoring their conservation, these IgM specificities were observed in catfish, which contain a primitive immune system and a tetrameric homolog of IgM. In mice, natural antibody containing serum limits rapid recruitment of neutrophils as observed in the acute challenge, and at intermediate timepoints after infection, limits the growth of *Pneumocystis* organisms in the lungs and leads to enhanced IL-1α and IL-6 in the lungs. Mice unable to secrete IgM, sIgM (-/-), and hence lacking the major isotype normally consisting the bulk of natural antibodies,

have impaired clearance of *Pneumocystis* at intermediate timepoints and impaired IL-1α, IL-6 in the lungs. As IgM appears to influence the earliest host responses against *Pneumocystis,* we assessed aspects of fungal cell wall carbohydrate antigen trafficking in sIgM (-/-) mice. We observed that sIgM (-/-) mice have normal uptake of zymosan in the lungs by CD11c+ cells, but impaired trafficking of CD11c+ cells containing the particle to the mediastinal lymph nodes. Fourteen days after infection, sIgM (-/-) mice have decreased quantities of IL-5 and IL-17 producing cells in the lymph node, while comparable quantities of IFN-γ producing cells. The apparent involvement of IgM in producing a dominant Th2 response was additionally manifested in the adaptive humoral response, as the production of serum anti-*Pneumocystis* IgG1 was significantly impaired while IgG2a much enhanced. Additionally quantities of anti-*Pneumocystis* IgG and IgA at the mucosa were impaired. Hence, we conclude that natural and early induced IgM play a significant role in host defense against *Pneumocystis* and in the types of adaptive immune responses mounted by the host.

3) Pneumocystis challenge results in induced/adaptive antibody responses that target conserved fungal cell wall carbohydrates β-glucan and chitosan/chitin. Particularly, serum levels of IgG against these carbohydrates increase, and quantities of specific IgG, IgA, and IgM increase in the lung after Pneumocystis challenge. CD4+ T cells are required for mucosal IgA production, but are not required for mucosal IgG production against fungal cell wall carbohydrates. Additionally IgM responses against these carbohydrates are significantly enhanced at the mucosa in the absence of CD4+ T cells, but not in the blood, suggesting that these cells are recruited into the lungs as a potential host defense mechanism to deal with increased Ag load in the setting of CD4+ T cell

deficiency. Lastly, we observe functional memory B cell responses against these carbohydrates, as a long term consequence of PC challenge, and provide evidence that these responses do not require CD4+ T cells for functions in the memory phase.

APPENDIX: PUBLICATIONS

Zheng, M., A. J. Ramsay, M. B. Robichaux, K. A. Norris, C. Kliment, C. Crowe, R. R. Rapaka, C. Steele, F. McAllister, J. E. Shellito, L. Marrero, P. Schwarzenberger, Q. Zhong, and J. K. Kolls. 2005. CD4+ T cell-independent DNA vaccination against opportunistic infections. *J Clin Invest* 115:3536-3544.

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