Pneumocystis

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Since its initial misidentification as a trypanosome some 100 years ago, *Pneumocystis* has remained recalcitrant to study. Although we have learned much, we still do not have definitive answers to such basic questions as, where is the reservoir of infection, how does *Pneumocystis* reproduce, what is the mechanism of infection, and are there true species of *Pneumocystis*? The goal of this review is to provide the reader the most up to date information available about the biology of *Pneumocystis* and the disease it produces.

Because it was first recognized in an animal also infected with *Trypanosoma cruzi*, *Pneumocystis* was initially mistaken for a morphologic form in the life cycle of *T. cruzi*. Antonio Carini, an Italian working in Brazil, saw the same organism-like cysts in the lungs of rats experimentally infected with *Trypanosoma lewisi*. The Delanoës and their colleagues at the Pasteur Institute recognized that these alveolar cysts were present in the lungs of local Parisian sewer rats, and established that the organisms were unique and distinct from trypanosomes. They proposed the name *Pneumocystis carinii* for the new species.

Over the next quarter century, there were occasional histological descriptions of *Pneumocystis* in the lungs of humans, but it was not until 1942 that Van der Meer and Brug reported the initial epidemics of interstitial plasma cell pneumonia among premature debilitated babies in nurseries and foundling homes in central Europe (Van der Meer and Brug 1942). In 1952

in Czechoslovakia, Vanek and Jirovec provided the most convincing demonstration of the etiologic relationship of *Pneumocystis* to this disease in an autopsy study of 16 cases (Vanek and Jirovec 1952).

Based on morphology, *Pneumocystis* was originally thought to be a protozoa. However, in 1988, DNA analyses revealed that the genomic structure of *Pneumocystis* was more closely related to fungi than to protozoa (Edman et al. 1988). Most recently a change in nomenclature for the organism infecting humans from *P. carinii* to *Pneumocystis jirovecii* has been proposed (Stringer et al. 2002). This name was chosen in honor of the parasitologist Otto Jirovec, who is credited by some, likely in error, with the original description of this organism in humans. The biologic rationale for this change is the unique antigenic and genetic properties, and restricted infectivity profile of the *Pneumocystis* organisms associated with each mammalian species.

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SYSTEMATIC POSITION OF *Pneumocystis* SPECIES WITHIN THE KINGDOM FUNGI

The genus *Pneumocystis* represents related fungal species that are members of the phylum/division Ascomycota, the subphylum Taphrinomycotina, class Pneumocystidomycetes, order Pneumocystidales, and family Pneumocystidaceae, all within the kingdom Fungi. Genetic investigations indicate that individual *Pneumocystis* species coevolved and coexisted with their respective mammalian hosts over many thousands of years (reviewed in Aliouat-Denis et al. 2008). Indeed, the genus *Pneumocystis* encompasses a variety of ubiquitous fungi that colonize and infect a wide variety of mammalian host species. The species *P. jirovecii* specifically infects humans, whereas the well-studied species of *P. carinii* and *Pneumocystis murina* are the genus members associated with rats and mice, respectively. Relevant *Pneumocystis* species have been identified in rabbits, ferrets, sheep, monkeys, and even in waterborne mammalian hosts such as cetaceans. The various *Pneumocystis* species are also quite rigid with respect to their unique specificity for any given mammalian host. For example, the human-specific species *P. jirovecii* is only capable of infecting humans and is not capable of infecting other animals, and vice versa. Species specificity of *Pneumocystis* members across various host species was studied by Wakefield and colleagues using polymerase chain reaction (PCR) with primer pairs specific for the mitochondrial large subunit rRNA genes of all known species of *Pneumocystis*. When primers specific for nonhuman *Pneumocystis* species were used on human clinical isolates, no amplification was detected. Conversely, primers specific for human *Pneumocystis* failed to amplify any product from nonhuman isolates (reviewed in Stringer 2002). Interestingly, human *P. jirovecii* was found to have closest homology with *Pneumocystis* organisms derived from primates. Furthermore, cross-infection of a given *Pneumocystis* species into its non-native mammalian host has never been conclusively proven.

Additional investigations defining the molecular genetics of various *Pneumocystis* species

have further indicated that rats can be infected with *P. carinii*, *Pneumocystis wakefieldiae*, or with both species concurrently (Icenhour et al. 2006). The general term *Pneumocystis* simply refers to the genus name, and unless otherwise specified, denotes qualities that are shared by all of the specific *Pneumocystis* species studied.

EPIDEMIOLOGY

There is no known environmental reservoir for *Pneumocystis*. DNA sequences identical to those of *Pneumocystis* have been detected by PCR in samples of ambient air, but intact organisms have not been visualized in environmental samples nor has disease been transmitted by such samples. Animal-to-animal transmission of *Pneumocystis* by the airborne route has been shown, and human-to-human transmission is presumed based on animal studies (Hendley and Weller 1971; Hughes 1982; Gigliotti et al. 2003). Consistent with this concept are the many clusters of cases of *Pneumocystis* pneumonia (PCP) that have been reported in immunocompromised patients (Sassi et al. 2012). As noted, animal experiments have shown that *Pneumocystis* from one mammalian host is not able to infect a different mammalian host, thereby making animal-to-human transmission unlikely (Gigliotti et al. 1993; Durand-Joly et al. 2002). *Pneumocystis* infection has global distribution among humans and most individuals show serologic evidence of infection by 2 years of age (Vargas et al. 2001). The incidence of PCP is related to the extent of immunosuppression, especially impairment in cell-mediated immunity, as evidenced by the frequent occurrence of PCP in patients with AIDS.

***Pneumocystis* SPECIES (LIFE CYCLE, MORPHOLOGICAL CHARACTERISTICS, SPECIES-SPECIFIC DIAGNOSTIC FEATURES DISTINGUISHING THE RELATED SPECIES)**

The study of the *Pneumocystis* life cycle has remained a significant challenge over many years owing to the fact that an environmental reservoir or niche has still not been defined, and that

a long-term in vitro culture system has also remained elusive. Transient proliferation of *Pneumocystis* has been achieved on lung epithelial and other feeder cells; however, such axenic cultures cannot be maintained beyond a few weeks (Cushion et al. 1985; Limper et al. 1997). Furthermore, despite scattered reports of short-term in vitro culture in systems that do not require feeder cells (Merali et al. 1999), continuous *Pneumocystis* cultures have not been consistently observed in major research laboratories. This may be explained by the observation that *Pneumocystis* appears to lack genes involved in de novo synthesis of amino acids (Hauser et al. 2010). Therefore, *Pneumocystis* investigators remain hamstrung without a valid and reproducible culture system, or with reliable methods to genetically manipulate this organism. Despite these obstacles, considerable advances have been made over the past decade using molecular approaches often in heterologous systems (Hauser et al. 2006; LoPressi et al. 2007). Immune-suppressed animal models of *Pneumocystis* pneumonia, particularly using rats and mice, continue to serve as a reliable source of organisms for study. In fact, all currently available agents for the treatment of *P. jirovecii* pneumonia in humans were initially derived from the study of *P. carinii* in rats and *P. murina* in mice (Thomas and Limper 2007).

Light microscopic and ultrastructural analyses of animal- and human-derived materials have provided some initial insight into the life cycle of *Pneumocystis*. Such studies reveal that there are at least two different life cycle forms of *Pneumocystis* organisms, namely, the trophic form and the cyst (Fig. 1). The trophic form generally measured $\sim 2 \mu\text{m}$ in greatest diameter is relatively heterogonous in shape, and is surrounded only by a plasma membrane, lacking a rigid cell wall. The trophic forms contain a single haploid nucleus (1C), and are lacking in a rigid cell wall structure (Wyder et al. 1994). In contrast, the cyst is significantly larger than the trophic form, measuring $\sim 8\text{--}10 \mu\text{m}$ in greatest diameter, is more uniform in shape, and contains up to eight intracystic bodies. The nuclear content in cysts varies between diploid (2C), tetraploid (5C), or octaploid (8C) DNA con-

Figure 1. Electron micrographic visualization of typical *Pneumocystis* life cycle forms. (A) Reveals a trophic form of *P. carinii* adherent to a type I alveolar epithelial cell, mediated by interdigitation of the organism's plasma membrane with that of the host cell. (B) Shows a thick-walled cyst form of *P. carinii*. Scale bars, $\sim 1 \mu\text{m}$. (From Limper et al. 1997; reprinted, with permission, from the author.)

tent. Rupture of the mature cyst containing eight separate nuclear intracystic bodies, a process termed excystment, releases the intracystic bodies to become new trophic forms of the organism. The rigid *Pneumocystis* cyst wall is formed of β -glucan, a complex branching polysaccharide containing other components such as mannoproteins including glycoprotein A/major surface glycoprotein, chitins, and other proteins expressed on the surface of the organism. The main function of the cyst wall is to confer rigidity and support for the organism, and putatively, to protect the organism from harsh environmental conditions outside the host. Additional studies indicate that interactions of the cyst wall with lung cells can also

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activate innate immune responses in the host (Carmona et al. 2012; Evans et al. 2012).

Unlike many other fungi, the route of *Pneumocystis* infection is not well defined. Current concepts suggest that infectious *Pneumocystis* forms are transmitted as aerosolized particles from host to host. Although some evidence supports the presence of *Pneumocystis* forms and DNA outside the host, an environmental reservoir or environmental phase of the life cycle has not been defined. Some investigators have proposed a model of initial infection early during infancy or childhood, with later reactivation during periods of immune suppression. Human serologic evidence of anti-*Pneumocystis* antibodies or positive PCR in healthy children supports early exposure to this organism (reviewed in Carmona and Limper 2011). Additional data further support the notion of colonization of patients with underlying lung disease such as chronic obstructive pulmonary disease (COPD) and also transiently in otherwise healthy individuals (Morris et al. 2004). Although some evidence suggests in utero transmission of *Pneumocystis* organisms, careful studies in rodents indicate that infants are likely exposed to the infectious agents very early in life (Icenhour et al. 2002).

Once inhaled, *Pneumocystis* has substantial tropism for alveolar epithelial cells of the lung and rarely disseminates to other organs, although occasional cases of extrapulmonary dissemination have been reported specifically in patients with profound immunosuppression such as during advanced HIV infection (Dietrich et al. 1992; Guttler et al. 1993; Bartlett and Hulette 1997; Ruggli et al. 1997; Hagmann et al. 2001; Panos et al. 2007). *Pneumocystis* trophic forms bind tightly to type I alveolar epithelial cells, in part utilizing extracellular matrix proteins (Walzer 1986). Interactions of *Pneumocystis* with extracellular matrix proteins, particularly fibronectin and vitronectin, facilitate binding of *Pneumocystis* to lung epithelial cells (Limper et al. 1993). A *Pneumocystis* surface integrin-like extracellular matrix adhesion receptor, termed PcInt1, has been described. This protein is expressed only on trophic forms and mediates attachment of the organism to fibro-

nectin (Kottom et al. 2008). Adhesion of *Pneumocystis* to lung epithelial cells triggers several specific signaling pathways in the fungus, involving activation of conserved mitogen-activated protein (MAP) kinases, namely, PcSte20 and PcCbk1 that promote organism mating, proliferation, and cell wall remodeling (Kottom et al. 2003; Kottom and Limper 2013). It is proposed that a sexual conjugation mating of trophic forms occurs after binding of the organisms to lung cells, which is then followed by meiosis and mitosis. These processes result in generation of the mature cyst, containing the eight intracystic forms (Burgess et al. 2008). *Pneumocystis* life cycle forms that may represent conjugated trophic forms, zygotc forms, and other forms in transition to acquiring a mature cell wall have been generally referred to as “precyst” forms.

Pneumocystis cysts and trophic forms have identical morphology irrespective of whether they are derived from human (*P. jirovecii*), rat (*P. carinii*), mouse (*P. murina*), or other mammalian hosts. In human clinical samples, *Pneumocystis* trophic forms or cysts can be identified in induced sputum, bronchoalveolar lavage fluid, or lung tissue using a variety of tinctorial histochemical stains. Trophic forms can be detected with Papanicolaou, Gram-Weigert, or Wright Giemsa. Cysts can be stained with Gomori methenamine silver (GMS), cresyl echt violet, toluidine blue O, or calcofluor white (CW) fungal stains. The use of any of these tinctorial stains requires expertise on the part of the investigator to differentiate *Pneumocystis* from artifacts, from other endemic mycoses (such as *Histoplasma capsulatum*), and from nonspecific staining. Immunofluorescent antibody (IFA) stains, which use monoclonal antibodies directed against human *Pneumocystis* epitopes, can enhance direct detection of this organism in clinical specimens, because they have high specificity and because they detect both the cyst and trophic forms of *Pneumocystis*.

DISEASE

Defects in CD4⁺ T-cell-mediated immunity render hosts susceptible to *Pneumocystis* infection. However, innate immune mechanisms also

contribute to host defense by limiting fungal growth in immunocompromised hosts. For example, accelerated *Pneumocystis* growth has been observed in CD4⁺ T-cell-deficient mice that also lack components of innate immunity (Inoue et al. 2011; Kelly et al. 2013; Bello-Irizarry et al. 2014). Although hypoxia is the hallmark of PCP, the clinical features vary with immune status in individual patients. The disease onset may be insidious with a clinical course of 3 or more wk, or fulminant and rapidly progressive over a few days. Fever generally is present and often precedes the onset of nonproductive cough, tachypnea, and severe dyspnea. Classic physical findings at the time of initial evaluation include tachypnea, nasal flaring, and intercostal, subcostal, or supracostal retractions. Cyanosis may be present or may develop rapidly. Auscultation of the chest frequently is characterized by a conspicuous absence of adventitious sounds. Scattered rales, rhonchi, or wheezes most often are detected later in the clinical course as resolution occurs. Radiographic abnormalities vary. Bilateral diffuse parenchymal infiltrates occur most commonly, but no pattern is sufficiently specific either to exclude or to confirm a diagnosis of PCP. Although initially a reticulogranular interstitial process, *Pneumocystis* pneumonitis progresses to a predominantly alveolar process with coalescence and air bronchogram formation. Late in the course of the disease, lung fields may opacify completely. Residual interstitial fibrosis occurs in a small percentage of patients. The timing of onset of clinical disease in non-AIDS, high-risk patients is unpredictable, but disease often occurs after discontinuance or reduction in the dose of corticosteroid therapy or after engraftment in bone marrow transplant recipients. These observations support the hypothesis that the clinical features early in the course of PCP are dependent to a large extent on the patient's ability to mount an inflammatory response. A more robust pulmonary inflammatory response, rather than organism burden, portends a worse prognosis. For example, AIDS patients who develop PCP typically have higher organism burdens, but lower mortality rates than non-AIDS patients (Limper et al. 1989). This is fur-

ther evidence that the patient's inflammatory response plays an important role in the pathogenesis of PCP.

Diagnosis of PCP requires the demonstration of *Pneumocystis* in the lungs of a patient with compatible pulmonary signs and symptoms. An open lung biopsy is the most reliable method, although bronchoalveolar lavage is generally more practical and nearly as sensitive. Induced sputum samples are gaining popularity, but are helpful only if positive. The yield of various diagnostic specimens is approximately as follows: induced sputum 20%–40%, tracheal aspirate 50%–60%, bronchoalveolar lavage 75%–95%, transbronchial biopsy 75%–95%, and open lung biopsy 90%–100%. Four commonly used stains to identify *Pneumocystis* are GMS and toluidine blue that stain only cyst forms, polychrome stains such as Giemsa that stain both trophozoites and sporozoites, and fluorescein-labeled monoclonal antibody that stains for both trophozoites and cysts (Procop et al. 2004). PCR analysis of respiratory specimens offers promise as a rapid diagnostic method, but a standardized system for clinical use has not been established.

The drug of choice for prophylaxis against or treatment of PCP is trimethoprim-sulfamethoxazole (tmp-smx). The duration of treatment is generally 3 wk for patients with AIDS and 2 wk for other patients. Adverse reactions occur frequently, more so in adults than children. These include rash, fever, and neutropenia. These side effects are less common in non-AIDS patients. For patients who cannot tolerate or fail to respond to tmp-smx after 5–7 d, pentamidine isethionate may be used. Atovaquone has been used primarily in adults with mild to moderate disease. Administration of corticosteroids in addition to anti-*Pneumocystis* drugs increases the chances for survival in moderate and severe cases of PCP. Detailed guidelines for the management of PCP can be found at www.aidsinfo.nih.gov.

PATHOGENESIS

In the immunocompromised host, PCP is uniformly fatal if untreated. However, in contrast to

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conventional thinking, recent evidence indicates that immunocompetent hosts are also transiently infected with *Pneumocystis*. Infection of the normal host results in a self-limited mild or subclinical lower respiratory tract infection, and the organism is not overtly pathogenic in hosts with intact immune systems (Vargas et al. 2001; Gigliotti et al. 2003; Gigliotti and Wright 2012). Sequencing of the *P. jirovecii* genome determined that the organism lacks obvious virulence genes or toxins, and does not possess many metabolic enzymes typically found in free-living fungi (Cisse et al. 2012). In contrast to other human fungal pathogens, *Pneumocystis* grows slowly and noninvasively in its permissive host. These findings suggest that *Pneumocystis* have adapted to survive and propagate within mammalian lungs without causing obvious cellular damage, which is clearly beneficial for organisms that rely on the host lung environment for survival.

Early ultrastructural studies described the tight attachment of *Pneumocystis* to type I pneumocytes in vitro as well as in vivo (Murphy et al. 1977; Lanken et al. 1980; Yoneda and Walzer 1980; Long et al. 1986; Benfield et al. 1997). Despite this close host–pathogen interaction and the observation of projections emanating from the organism to the host cell, these early studies indicated that *Pneumocystis* causes little damage to the host and incites remarkably little inflammation. Only during advanced infection were pathogenic changes such as degeneration of type I pneumocytes, hyperplasia of type II pneumocytes, and disruption of the alveolar–capillary barrier noted. In the chronically immunosuppressed host, the pathophysiology of PCP appears related to physical disruption of gas exchange through direct attachment of *Pneumocystis* to alveolar epithelial cells (AECs), and the accumulation of organisms, cell debris, and a characteristic foamy exudate in the alveolar lumen. As the infection progresses, the interaction of *Pneumocystis* with lung epithelial cells may impede lung repair by inhibiting epithelial proliferation (Limper et al. 1998).

Pneumocystis lacks many characteristics that contribute to the virulence of other human fungal pathogens, but nonetheless PCP remains a

life-threatening opportunistic respiratory disease. An intact immune system resists *Pneumocystis* infection, and fulminant PCP is only observed in immunocompromised individuals. However, the host's immune system is also a major contributor to the pathogenesis of PCP. Clinical evidence has offered insight into the nature of PCP-related lung injury. For example, the severity of PCP correlates positively with the degree of pulmonary inflammation, but not lung fungal burden (Limper et al. 1989; Benfield et al. 1995; Bang et al. 2001). Comparison of AIDS and non-AIDS PCP patients revealed that profoundly immunosuppressed AIDS patients present with higher lung burdens, but display a subtler onset of disease with better pulmonary function and improved prognosis relative to non-AIDS patients (Limper et al. 1989). The onset of clinical PCP has also been noted when corticosteroids are tapered, or when bone marrow engraftment is evident (Sepkowitz 1992, 1993), suggesting that restoration of the immune system drives pathogenesis. The significance of immunopathogenesis has gained more attention with the recognition of PCP-related immune reconstitution inflammatory syndrome (IRIS) in HIV-positive patients (Barry et al. 2002; Koval et al. 2002; Jagannathan et al. 2009). The rapid recovery of CD4⁺ T cells following initiation of combined antiretroviral therapy causes an intense pulmonary immune response and rapid pulmonary decompensation. This pathological immune response is driven by a preexisting pulmonary infection, and *Pneumocystis* has been identified as one of the causative agents. These observations support the immunopathogenic nature of PCP in that infection with *Pneumocystis* is necessary to cause pneumonia, but the *Pneumocystis*-driven immune response is responsible for the associated pathophysiology. The failure of current therapies to adequately combat immunopathogenesis during PCP may partly explain treatment failures in patients. Even in the absence of viable organisms, the presence of *Pneumocystis* antigen may continue to drive immunopathogenesis.

Animal models of PCP have proven remarkably accurate mimics of human disease,

and have provided a platform for the identification of immune effector mechanisms that contribute to immunopathogenesis. Severe combined immunodeficient (SCID) mice are extremely susceptible to *Pneumocystis* infection because they are unable to mount an adaptive immune response. Roths and Sidman found that transferring functional lymphocytes to *Pneumocystis*-infected SCID mice causes a lethal hyperinflammatory response in the lung (Roths and Sidman 1992). Subsequent studies show that immune reconstitution of infected SCID mice induces a rapid pulmonary inflammatory response with deleterious effects on pulmonary function and oxygenation (Wright et al. 1997, 1999). Although immune reconstitution restores an effective CD4⁺ T-cell-dependent immune response against *Pneumocystis*, it also has profound effects on physiology, including severe weight loss, tachypnea, and hypoxia. Non-reconstituted SCID mice with similar *Pneumocystis* burdens exhibited few signs of PCP-related respiratory disease, again supporting the concept that *Pneumocystis* itself is not potentially toxic or damaging to the lung. The immune reconstituted SCID mouse model of PCP is similar to the IRIS presentation of PCP in human patients, and provides a model for elucidating mechanisms of PCP-related IRIS. Recent discoveries using this model have determined that CD4⁺ T cells are critical for mediating immunopathogenesis, whereas CD8⁺ T cells serve a regulatory function by limiting the CD4⁺ T-cell response (Bhagwat et al. 2006; Swain et al. 2006). Pharmacological manipulation of the organism in vivo found that the cyst form of *Pneumocystis*, which contains proinflammatory cell wall components, drives immunopathogenesis during IRIS (Linke et al. 2013). Immunomodulatory strategies that alter T-cell and macrophage effector phenotype during IRIS hold promise for enhancing host defense while reducing disease severity and promoting resolution (Wang et al. 2010, 2011).

The classic presentation of AIDS-related PCP has also been effectively modeled in animals. Although much work has been performed in rodents, SIV-infected nonhuman primates have also been used to model PCP (Board et

al. 2003). Systemic administration of anti-CD4 monoclonal antibody maintains mice in a chronic CD4⁺ T-cell-depleted state. CD4-depleted mice develop a clinical syndrome very similar to AIDS-related PCP in humans (Harmesen and Stankiewicz 1990; Shellito et al. 1990; Wright et al. 1999). In the absence of CD4⁺ lymphocytes, CD8⁺ T cells are recruited to the lung in response to *Pneumocystis* infection (Wright et al. 1999, 2004; Meissner et al. 2005). These lymphocytes are unable to control *Pneumocystis* infection, but directly contribute to PCP-related lung injury. Infected mice depleted of both CD4⁺ and CD8⁺ lymphocytes are significantly healthier than mice depleted of only CD4⁺ lymphocytes (Wright et al. 1999). The mechanism of CD8⁺ T-cell-mediated lung injury during PCP is antigen specific and requires TNF receptor signaling (Wright et al. 2004), MHC class I expression on resident lung cells (Meissner et al. 2005), and the presence of *Pneumocystis* antigen in the lung (Meissner et al. 2005). It has also been suggested that *Pneumocystis* itself enhances the immunosuppressive environment in CD4-depleted mice by inducing alveolar macrophage apoptosis (Lasbury et al. 2006, 2007). Despite the fact that neutrophil numbers in the lungs of humans and animals with PCP strongly correlate with the severity of disease, these cells do not appear to directly contribute to either host defense or immunopathogenesis (Swain et al. 2004).

Surfactant dysfunction has been noted in humans and animals suffering from PCP (Sheehan et al. 1986; Escamilla et al. 1992; Hoffman et al. 1992; Aliouat et al. 1998; Atochina et al. 2000; Schmidt et al. 2006), and surfactant therapy has shown therapeutic benefit in both patients and experimental animals (Eijking et al. 1992; Creery et al. 1997). Dramatic alterations in surfactant phospholipid and protein composition, as well as impairment of physiological surfactant function are associated with PCP. *Pneumocystis* may disrupt the pulmonary surfactant system through several mechanisms. *Pneumocystis* may act directly on AECs to modulate surfactant phospholipid and protein synthesis; *Pneumocystis* organisms may directly interact with surfactant components in the al-

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veolus to disrupt function; or *Pneumocystis* may affect surfactant through disruption of the alveolar–capillary barrier resulting in leakage of inhibitory plasma proteins into the alveolar lumen (Wang et al. 2005). Furthermore, a potential mechanism of PCP-related immunopathogenesis is impairment of normal surfactant function. Pulmonary inflammation elicited during *Pneumocystis* infection was found to directly disrupt surfactant function, contributing to PCP-related respiratory impairment (Wright et al. 2001). Thus, surfactant dysfunction is a physiological consequence of *Pneumocystis* infection, which likely contributes to impaired gas exchange and hypoxia that is a hallmark of PCP.

Current research suggests that in addition to causing active PCP, *Pneumocystis* infection may also be an underappreciated determinant of chronic lung disease. Recent clinical evidence suggests that *Pneumocystis* infection or colonization exacerbates other forms of chronic lung disease. COPD patients who are also colonized with *Pneumocystis* show an accelerated disease progression with worse pulmonary function compared with noncolonized controls (Morris and Norris 2012). Recent work in a nonhuman primate model of SIV infection determined that *Pneumocystis* colonization drives the development of COPD (Shipley et al. 2010). *Pneumocystis* colonization has also been reported in cystic fibrosis patients (Calderon et al. 2010), although the significance of this finding is unclear. Animal studies have shown that transient *Pneumocystis* infection can promote allergic sensitization of the lung (Swain et al. 2011), induce chronic pulmonary hypertension (Swain et al. 2007), and also exacerbate pulmonary fibrosis (Bruckner et al. 2006). *Pneumocystis* may also affect HIV-related disease by exerting unique effects on bone marrow precursor cells (Taylor et al. 2011). Many of these potential complicating effects persist long after the infection has been cleared.

Although the specific mechanisms of PCP-related immunopathogenesis are not fully defined, the contribution of inflammation and the immune response to this disease process is clear, and standard treatment of moderate to

severe PCP typically includes corticosteroids as adjunctive therapy to dampen inflammation. Although infection is necessary to cause PCP, certain aspects of the immune response cause or exacerbate disease symptoms. Differences in the degree of immunosuppression among PCP patients likely affect their ability to produce an immunopathogenic response to infection, and may account for variability in the severity of PCP between different patient groups.

UNIQUE CHARACTERISTICS DISTINGUISHING *Pneumocystis* FROM OTHER RELATED MOLDS OR YEASTS

As ascomycetous fungi, *Pneumocystis* species bear certain morphological similarities to other related organisms. Although an extremely close fungal relative to *Pneumocystis* has not yet been defined, sequencing of rRNA from *P. carinii*, as well as more extensive sequencing studies undertaken during the *P. carinii* genome project, indicates the closest phylogenetic relative to be *Schizosaccharomyces pombe*. In addition, some genetic similarities have also been noted to *Neurospora* species. Functional genetic studies further show close parallels in certain *Pneumocystis* functional metabolic systems (e.g., cell wall generation and integrity) to those systems utilized by *Saccharomyces cerevisiae*. Similar to other ascomycetes, *Pneumocystis* have a life cycle form consisting of an ascus or spore case, which in the case of *Pneumocystis* has classically been referred to as the cyst. However, unlike other phylogenetically related organisms, *Pneumocystis* also has the separate life cycle stage, the trophic form, that lacks a rigid cell wall. The trophic form is known to extend cell membrane projections, which interdigitate with host lung cells and mediate firm attachment of the *Pneumocystis* trophic forms to the alveolar epithelium (Limper and Martin 1990). The *Pneumocystis* trophic forms, however, do not proceed to show either pseudohyphal or true hyphal growth morphologies. Hence, *Pneumocystis* cannot be viewed as a dimorphic fungus in the classical sense. Although the typical *Pneumocystis* cyst forms that are observed on silver staining are structurally reminiscent of yeast forms

present in other fungal infections, during active infection, the overwhelming majority (>90%) of *Pneumocystis* organisms present in the lung are actually of the trophic form morphology.

In further contrast to other ascomycetous fungi, and despite extensive and ongoing efforts, *Pneumocystis* remains nonculturable in defined media. Although this may in part be nutritional, efforts to supplement defined media with lung extracts and with varying oxygen tensions and pH have been disappointingly unsuccessful (Thomas and Limper 2007). One clue to the elusive life cycle of *Pneumocystis* may be the organism's ability to sense contact with lung epithelial cell surfaces. This contact sensing is termed thigmotropism, and is a phenotypic quality not widely observed in other ascomycetous fungi. Contact sensing by *Pneumocystis* initiates signaling pathways in the organism essential for cell wall remodeling and fungal proliferation (Kottom et al. 2003, 2011a; Kottom and Limper 2013). Also distinguishing *Pneumocystis* from other ascomycetes is a lack of response to traditional antifungal agents, including polyenes such as amphotericin and extended spectrum azoles. The lack of responsiveness of *Pneumocystis* to amphotericin and other polyenes is likely related to the lack of ergosterol in the organism's cell wall. In addition, sequencing data indicate that *Pneumocystis* natively shows resistance to extended spectrum azole such as itraconazole widely used against other fungi (Morales et al. 2003). Therapies against *Pneumocystis* largely rely on treatment with trimethoprim-sulfamethoxazole or with pentamidine (Thomas and Limper 2004).

ANALYTICAL MANIPULATION (MOLECULAR GENETICS INCLUDING PARADIGMS THAT DEFINE VIRULENCE FACTORS OR DRUG TARGETS)

Even though *Pneumocystis* is nonculturable, and is not genetically tractable, significant advances in understanding the molecular genetics of the organism have occurred over the past decade by directly sequencing *Pneumocystis* genes, and by studying *Pneumocystis* protein functions using heterologous gene expression, largely in

S. pombe and *S. cerevisiae* (Burgess et al. 2008; Kottom and Limper 2013). The rat-derived *P. carinii* genome project (pgp.cchmc.org) has been widely utilized by the *Pneumocystis* research community. More recently, sequencing of both the *P. murina* and the *P. jirovecii* genomes has been reported (Cisse et al. 2012; Ma et al. 2013). The *P. carinii* genome contains ~8 million base pairs of DNA divided into 15 linear chromosomes ranging from 300 to 700 kb (Stringer and Cushion 1998). The genes have a high A:T content of 65%, and the majority of genes are interrupted by introns ranging from 38 to 424 bp (Thomas et al. 1999). Using these sequence resources and heterologous expression strategies, key molecules in the organism's mitotic cell cycle, cell wall assembly, signal transduction cascades, and metabolic pathways have been identified.

A significant fraction of the *Pneumocystis* genome encodes for surface membrane proteins and glycoconjugates. The most plentiful surface antigens, termed glycoprotein A (gpA) or major surface glycoprotein (MSG), have molecular masses of 95–120 kDa and are heavily mannosylated. These glycoproteins are proposed to participate in attachment of *Pneumocystis* to host lung cells, as well as in evading host defenses (Gigliotti et al. 1986, 1988; Linke et al. 1989; Lundgren et al. 1993; O'Riordan et al. 1995; Vuk-Pavlovic et al. 2001). The gpA/MSG isoform expressed is immunogenic and antigenically distinct in the different species of *Pneumocystis* that infect different mammalian hosts (Kovacs et al. 1989; Gigliotti 1992; Kovacs et al. 1993; Stringer and Keely 2001). Of the >80 gpA/MSG found in the *P. carinii* genome, only a single isoform of gpA/MSG is expressed at any given time. This may enable the organism to evade host defense and establish infection. Also expressed by the organism is the subtilisin-like serine proteases Prt1 (also termed Kex1) in both human and mouse-derived *Pneumocystis* (Lugli et al. 1997, 1999; Lee et al. 2000; Kutty and Kovacs 2003). These proteases are thought to function in processing of the *Pneumocystis* surface antigens.

The major structural component of the *Pneumocystis* cyst wall is β -glucan. This form

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of glucan consists of polymers of D-glucose arranged with a β -1,3-linked carbohydrate core and side chains of β -1,6-linked glucosyl residues (Douglas 2001). The cyst wall also contains other complex carbohydrate polymers, chitins, and accessory proteins. *P. carinii* β -glucan provides stability and structural support in the infected lung, but also elicits significant inflammatory responses in the infected host, which enhance immunological damage to the alveolar epithelium (Vassallo et al. 2000). Isolated *P. carinii* β -glucans elicit inflammatory responses by alveolar macrophages and respiratory epithelial cells, promoting respiratory damage in patients with severe PCP (Limper et al. 1989; Vassallo et al. 1999a,b; Lebron et al. 2003; Carmona et al. 2006). In addition, *Pneumocystis* β -glucans also activate dendritic cells and T-cell responses (Carmona et al. 2006) that participate in the clearance of *Pneumocystis* organisms. *Pneumocystis* β -glucans further prime dendritic cells in a manner that polarizes T cells toward Th17 differentiation, further augmenting host antifungal resistance (Zelante et al. 2007). The synthesis of *Pneumocystis* β -glucan is mediated by *Pcgscl*, a β -1-3-glucan synthetase gene and *Pckre6*, which confers β -1-6-glucan synthetic activity. Inhibition of the *P. jirovecii* cell wall with agents that suppress these pathways is a potential novel treatment target for *Pneumocystis* pneumonia, although this has yet to be studied in humans (Schmatz et al. 1990; Powles et al. 1998).

Additional studies have focused on the mechanisms that regulate *Pneumocystis* cell-cycle control and proliferation, as these might provide new understanding for treating these infections. *Pneumocystis* shows both mitotic and meiotic cell replication machinery (Kottom et al. 2000; Burgess et al. 2008). Analogous to other ascomycetes, particularly *S. pombe*, *Pneumocystis* cell-cycle control is precisely regulated by cell division cycle (cdc) cyclin-dependent kinases and their related cyclins and regulatory inhibitors. These molecules include *P. carinii* cdc2 cyclin-dependent kinase, the cdc13 B-type cyclin, and the regulatory cdc25 mitotic phosphatase (Thomas et al. 1998; Kottom et al. 2000; Gustafson et al. 2001). These mole-

cules are essential in mitotic proliferation. In addition, regulated gene expression and chromosomal replication further requires regulation of *Pneumocystis* histone acetylation, which is mediated both in *P. carinii* and *P. jirovecii* by Rtt109 histone acetyltransferases and related proteins (Kottom et al. 2011b). These molecules appear to be a novel new treatment target with activity in many major fungi (Pupaibool et al. 2013). Additional evidence supports the existence of meiotic life cycle machinery in *Pneumocystis* species required for sexual reproduction. This system in *P. carinii* utilizes PcMei2 and PcRan1, molecules highly analogous to the meiotic regulatory systems present in *S. pombe* (Burgess et al. 2008, 2009). Taken together, these studies indicate that *Pneumocystis* species have a life cycle with both mitotic and meiotic phases analogous to other ascomycetous fungi, but with unique adaptations to interact with host cells and proliferate within the unique niche of the mammalian lung.

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