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Current understanding of *Pneumocystis* immunology

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Abstract

Pneumocystis jirovecii is the opportunistic fungal organism that causes *Pneumocystis* pneumonia (PCP) in humans. Similar to other opportunistic pathogens, *Pneumocystis* causes disease in individuals who are immunocompromised, particularly those infected with HIV. PCP remains the most common opportunistic infection in patients with AIDS. Incidence has decreased greatly with the advent of HAART. However, an increase in the non-HIV immunocompromised population, noncompliance with current treatments, emergence of drug-resistant strains and rise in HIV⁺ cases in developing countries makes *Pneumocystis* a pathogen of continued interest and a public health threat. A great deal of research interest has addressed therapeutic interventions to boost waning immunity in the host to prevent or treat PCP. This article focuses on research conducted during the previous 5 years regarding the host immune response to *Pneumocystis*, including innate, cell-mediated and humoral immunity, and associated immunotherapies tested against PCP.

Keywords

adaptive immunity; chemokine; cytokine; fungal; HAART; HIV⁺; inflammatory response; innate immunity; *Pneumocystis* pneumonia

Pneumocystis has been identified in every mammalian species studied thus far. The diversity within the genus became clear when DNA sequence analysis established that *Pneumocystis* is species specific, with each host species harboring a distinct genetic variation of the organism [1,2]. The *Pneumocystis* organism whose primary host is man was renamed *Pneumocystis jirovecii*, in honor of Otto Jirovec, who is credited with describing the microbe in humans [3], with the rat-specific fungus termed *Pneumocystis carinii*, and the mouse *Pneumocystis murina* [4]. It is important to note, however, that the acronym for *Pneumocystis* pneumonia (PCP) is still in common use.

The inability to consistently culture the organism *in vitro* or isolate it from the environment has made defining the complete lifecycle of *Pneumocystis* species difficult. *Pneumocystis* may have an *ex vivo* spore phase [5,6], and there is recent evidence that like other fungi, *Pneumocystis* is able to form biofilms containing a branch-like morphology in cell-free

medium [7]. However, what is generally accepted regarding the *Pneumocystis* lifecycle comes primarily from microscopic observations of animal models of infection. Within the mammalian host, *Pneumocystis* has a tropism for the alveoli of the lung where it exists in two stages: the small (1–4 μm) amoeba-like trophozoite form and the large (5–8 μm) cyst form (Figure 1) [8]. The haploid trophozoite can divide asexually by binary fission [9], or two trophozoites may conjugate (sexual phase), giving rise to a diploid cell that then goes through a process of meiosis, eventually giving rise to a cyst containing eight sporozoites [8,10]. Evidence supporting the existence of a sexual cycle came in 1984 when it was found that *Pneumocystis* forms a synaptonemal complex leading to meiotic nuclear division [11]. In addition, sequencing of the *Pneumocystis* genome has led to the discovery of genes coding for proteins involved in mating and pheromone responsiveness in other fungi [12,13].

The mode of *Pneumocystis* infection is not fully understood and, for many years, it was speculated that this opportunistic pathogen may be an obligate parasitic organism [3]. It was believed that humans were infected very early in life and, in an immunocompetent individual, the infection would be controlled and the organism would go into a quiescent state. PCP was, therefore, believed to be a result of reactivation of latent infection comparable to *Toxoplasma gondii* [14]. However, continued emerging evidence suggests that PCP is not a result of a reactivation of a dormant infection, but rather a reinfection [15] from an as of yet unknown human or environmental source [16].

Interestingly, colonization, or the presence of *Pneumocystis* without signs or symptoms of PCP, has been heavily documented in humans. Using a sensitive molecular technique to detect the organisms, Vargas *et al.* found a significant fraction (36%) of children with mild respiratory disease were carriers of *P. jirovecii* [17]. Colonization has also been found at autopsy of infants who died of sudden infant death syndrome [18]. In healthy, immunocompetent individuals, the colonized state tends to be transient [19–22]. However, in the HIV-infected population, colonization is much more persistent, with rates varying from 10 to 70%, depending on the state of their immunodeficiency and lung health [23–27]. Colonization is also common in the non-HIV-infected immunosuppressed population, particularly in individuals with chronic obstructive lung disease [26,28–32].

The incidence and outcome of PCP has shifted several times over the course of the HIV epidemic. Clinically, PCP in HIV⁺ patients is characterized by progressive dyspnea, a nonproductive cough or cough productive of clear sputum, malaise and low-grade fever (Figure 2) [33]. Before the advent of routine prophylaxis, PCP was the most common cause of death among HIV⁺ patients [34]. *Pneumocystis* infection dropped off significantly in HIV⁺ patients post-1995 with the routine use of HAART [35,36]. HAART is credited with being the most crucial factor in the control of PCP in HIV⁺ individuals owing to the enhanced immune function that accompanies treatment [37]. Despite these advances, there continues to be a significant mortality associated with *Pneumocystis* infection [38,39]. Insight into the persistence of PCP in HIV⁺ patients comes from a recent large-scale retrospective study. Walzer *et al.* studied 494 patients with 547 episodes of PCP from a single HIV treatment center over a 21-year period. Surprisingly, the authors found that the mortality rate associated with PCP infection in patients not receiving HAART had not significantly changed since the beginning of the HIV epidemic (1985–2006) [40]. Some interesting demographic changes were found from the pre- to post-HAART era in patients presenting with PCP, including an increase in cases in heterosexual men and women, increase in age at time of infection, a decrease in PCP prophylaxis use and a decrease in awareness of HIV status [40,41]. Therefore, it appears that current cases of PCP now result from failure to diagnose HIV infection, rather than from the inevitable progression of AIDS seen before the availability of current treatments [41]. Efforts should be made for early

diagnosis and treatment of HIV both in traditional at-risk populations and populations that historically have not been associated with HIV infection.

The incidence of PCP in the non-HIV⁺ population has historically been a rare occurrence, with *Pneumocystis* causing cases of ‘plasma cell pneumonia’ in premature and malnourished children, as well as in cancer patients on chemotherapy. The increased use of immunosuppressive or chemotherapeutic agents either for long periods of time or in high doses has resulted in a rise in PCP cases [42,43]. The severity of PCP in non-HIV patients is greater than in those infected with HIV, with a more rapid and fulminant onset [44,45]. Patients present with symptoms of fever, dry cough, shortness of breath and can quickly progress to respiratory failure [33]. Most at risk are bone marrow and solid organ transplant patients, as well as cancer patients, particularly those with hematological malignancies [46–48]. Other conditions associated with a higher risk for PCP include collagen vascular disease, mainly Wegener’s granulomatosis, and inflammatory bowel disease [49–51]. Recently, PCP has been reported in patients with rheumatoid arthritis and Crohn’s disease who are receiving anti-TNF- α therapies [52–54].

Currently, treatments for *Pneumocystis* infection are all chemically based antimicrobials, with the most effective treatment being trimethoprim–sulfamethoxazole. However, there is a high incidence of intolerance to this medication owing to allergies against the sulfa component of the drug. In addition, nonadherence with prophylaxis and/or long-term prophylaxis use in *Pneumocystis*-colonized individuals have led to the selection of mutations giving rise to drug-resistant strains. These include mutations in the targets for trimethoprim and pyrimeth-amine [55], atovaquone [56] and sulfa drugs [57–59], leading to treatment failure and increased mortality [45]. With drug resistance threatening the most effective treatments, novel therapies are in great demand. The inability to culture the organism *in vitro* has been a major obstacle to studying therapies for this opportunistic pathogen. Most recently, Cushion *et al.* have demonstrated the ability to passage the organism in cell-free media and shown that the passaged organisms were able to cause infection in rodents [7], suggesting a novel *in vitro* system to study *Pneumocystis*. Currently, however, the infected animal model remains the main source of both organism and laboratory studies and researchers have made great strides in understanding the host–pathogen relationship with *Pneumocystis*, particularly in the area of immunology, despite the experimental limitations. One of the benefits of animal models is the ability to investigate novel treatment strategies and there have been several tested against *Pneumocystis* infection, which are discussed throughout the text and summarized in Tables 1 & 2.

Innate immunity

Alveolar macrophages

Pneumocystis has a tropism for the alveolar spaces of the lung and the alveolar macrophage (AM) is the first line of host defense to control this microorganism and prevent infection. The importance of AMs lies in their ability to directly kill both trophozoites and cysts, and there are many studies that show an inverse correlation between macrophage numbers and severity of PCP [60–62]. Being professional phagocytic cells, AM recognize pathogen-associated molecular patterns on the surface of microorganisms through pattern-recognition receptors (PRRs). Attachment results in activation of the macrophage and/or phagocytosis and subsequent phagolysosomal fusion leading to degradation of foreign organisms. In addition, activated AMs further activate CD4⁺ and CD8⁺ T cells, leading to adaptive immune responses.

Attachment/ingestion—Several receptors on the AM and the corresponding surface molecules on *Pneumocystis* that are associated with attachment and ingestion/phagocytosis have been identified. A major surface antigen of *Pneumocystis* is gpA, also named major surface glycoprotein (MSG) [63]. This surface protein is heavily glycosylated with mannose residues and although there are over 80 genes that code for gpA, only one isoform is expressed at any one time [64]. Therefore, similar to other fungal organisms with mannosylated glycoproteins contained in the cell wall, *Pneumocystis* binds to the mannose receptor (MR) on the AM [65]. Indeed, the significance of the MR–gpA interaction was clarified when Koziel *et al.* reported that relative to healthy controls, AM phagocytosis of *Pneumocystis* organisms in HIV⁺ individuals was reduced by up to 74%, which correlated with an 80% decrease in MR surface expression and endocytosis by AMs [66]. Cells from patients with clinical AIDS (CD4 T cells <200 cells/mm³) demonstrated the lowest *Pneumocystis* phagocytosis and the lowest MR endocytosis [66]. It was later found that *Pneumocystis* infection induced shedding of a soluble form of the MR (sMR) into the extracellular alveolar fluid of HIV-infected patients [67]. *In situ* experiments by Fraser *et al.* showed that *Pneumocystis* organisms that were coated with exogenous sMR were less likely to be phagocytosed than uncoated organisms in experimental mice with PCP [67]. A mechanism of phagocytosis of unopsonized *Pneumocystis* was described by Zhang *et al.* who showed that the MR is associated with focal F-actin polymerization, RhoGTPase activation and phosphorylation of the downstream effector molecule PAK-1, and these mechanisms are impaired in AMs from HIV⁺ individuals [68]. In addition to endocytic and phagocytic effector functions, binding of *Pneumocystis* to MR leads to activation of NF- κ B, resulting in production of the proinflammatory chemokines IL-8 and matrix metalloproteinase-9, and this response is abrogated in HIV⁺ individuals [69]. Further studies revealed that in AMs from healthy individuals, *Pneumocystis*–MR binding was not associated with induction or release of key cytokines in the host innate response to *Pneumocystis*, namely IL-1 β , IL-6 or TNF- α , with the authors speculating that the MR may function to regulate potentially damaging innate inflammatory responses to infectious challenge in the lungs [70]. However, the role of the MR in host defense against *Pneumocystis* infection was brought into question when animal studies using MR-deficient mice revealed that there was no significant difference in susceptibility to *Pneumocystis* infection or clearance of organisms compared with immunocompetent mice [71]. Furthermore, there was no significant difference in organism burden between MR^{−/−} and wild-type mice when CD4 T cells were depleted [71]. As is frequently seen with nonspecific innate immune responses, redundant mechanisms are often at play, and the lack of this receptor may have been compensated for in the transgenic mice.

β -glucan receptors are an additional class of nonspecific PRR on professional phagocytic cells recognizing the β -glucan moieties in the cell wall of fungal organisms. Indeed, β -glucan is a significant component of the cell wall of *Pneumocystis* [11,72,73] and has been shown to induce the production of TNF- α and the murine homolog of IL-8, macrophage inflammatory protein (MIP)-2, from AMs [74] through NF- κ B translocation [75]. It was further shown that the serum glycoproteins vitronectin and fibronectin bind to *Pneumocystis* cell wall β -glucan, augmenting the AM response and resulting in significant production of IL-6 [76]. Although β -glucans are known to bind to receptors, such as complement receptor 3 [77], Brown and Gordon were the first to identify dectin-1 as the major β -glucan receptor on macrophages, with highest expression in lung, liver and thymus tissues [78]. Furthermore, the authors were able to show that dectin-1 mediated nonopsonic phagocytosis of the opportunistic fungal pathogen *Candida albicans* [78]. When investigated in *Pneumocystis*–AM interactions, Steele *et al.* found that dectin-1 mediates nonopsonic phagocytosis and subsequent killing of the fungus through the generation of reactive oxygen species [79]. In addition, the authors demonstrated that dectin-1 is required for *Pneumocystis*-induced MIP-2 release [79]. Recently, Saijo *et al.* found that dectin-1-

knockout mice were more susceptible than wild-type to *Pneumocystis* infection, even though their cytokine production was normal [80]. However, *Pneumocystis*-infected dectin-1-knockout macrophages did show defective production of reactive oxygen species [80]. In addition to nonopsonic AM receptor recognition and uptake of *Pneumocystis*, antibody-mediated uptake through Fc receptors (i.e., opsonized killing) has also been reported [81]. Combining the extracellular domain of dectin-1 with the Fc portion of murine IgG1 in a fusion protein, Rapaka *et al.* were able to demonstrate enhanced uptake of *Pneumocystis* by AM and diminished severity of disease in a PCP model in severe combined immunodeficiency (SCID) mice [82].

Another class of PRR on AM that are known to play important roles in the inflammatory response to, but not phagocytosis of, pathogenic fungi are the Toll-like receptors (TLRs). Lebron *et al.* found that in response to *Pneumocystis* β -glucan, macrophages from TLR-4-knockout mice behaved similarly to those of wild-type mice. By contrast, macrophages retrieved from mice deficient in MyD88, (the downstream regulatory protein of TLR pathways) were abrogated in their response to *Pneumocystis* cell-wall β -glucan, indicating that TLRs other than TLR-4 are involved in the AM inflammatory response to the fungus [75]. However, in a murine model of PCP, TLR-4-knockout mice demonstrated a more fulminant infection relative to controls, with increased weight loss, increased production of proinflammatory cytokines, along with a decrease in downregulatory cytokines, suggesting that recognition of *Pneumocystis* by TLR-4 may help to regulate the host inflammatory responses through cytokine production by AMs [83]. Recently, TLR-2 has been shown to be the major TLR for *Pneumocystis* [84–87]. Normal mouse AMs were shown to express TNF- α and MIP-2 via NF- κ B activation through TLR-2, while transcription of TLR-2, but not TLR-4, was shown to be increased in AM in response to *Pneumocystis* [85]. In addition, the AM TNF- α and MIP-2 responses to the pathogen were blocked with anti-TLR-2 antibody [85]. Interestingly, Tachado *et al.* found that human macrophages exposed to *Pneumocystis* require the coexpression of TLR-2 and MR in order to express IL-8 [84]. The importance of TLR-2 is further highlighted by animal studies using TLR-2 knockout mice, which demonstrate decreased inflammatory responses during PCP relative to wild-type [85,87]. This decreased inflammatory response in TLR-2-knockout mice is concomitant with increased severity of PCP and organism burden. It has been shown that lack of TLR-2 does not affect phagocytosis of *Pneumocystis* [86], indicating that TLR-2-mediated inflammatory responses contribute to the clearance of the organism [87].

Similar to TLRs, scavenger receptor A (SRA) belongs to a class of pattern recognition receptors found on macrophages that control the inflammatory response to *Pneumocystis*. In an *in vivo* study, Hollifield *et al.* showed that SRA-deficient mice produced significantly more TNF- α , IL-12 and IL-18 in response to *Pneumocystis* infection than wild-type mice [88]. AMs from these mice displayed no difference in phagocytosis of the pathogen, indicating that SRA is not involved in uptake, but is important for controlling the inflammatory response to the fungal pathogen.

Another aspect of AM host defense against *Pneumocystis* is the role of surfactant proteins (SPs). Pulmonary surfactant is a complex of lipids and proteins secreted by alveolar type II cells that reduce surface tension at the air–liquid interface, prevent alveolar collapse at low lung volumes and have a role in innate lung immunity [89,90]. Functionally, the four major surfactant proteins are divided into two groups: the hydrophobic proteins, SP-B and SP-C, have a primary role in controlling surface tension, while the hydrophilic proteins, SP-A and SP-D, are important components of innate lung immunity. Studies have demonstrated that *Pneumocystis* infection results in downregulation of SP-B and SP-C, resulting in significant increases in surface tension, presumably contributing to hypoxemic respiratory failure observed in patients with PCP [91,92]. By contrast, SP-A and SP-D have been shown to

play roles in clearing the pathogenic fungi *Aspergillus fumigatus* and *Histoplasma capsulatum* from the lungs [93]. Both SP-A and SP-D bind mannose moieties of *Pneumocystis* [94,95], and infection has been shown to result in marked increases in production of SP-A and SP-D [96]. However, there have been conflicting reports on whether SP-A promotes [97] or inhibits [98] AM uptake of *Pneumocystis* organisms. Studies using SP-A-knockout mice support the protection theory in that infected SP-A-deficient mice exhibited an enhanced susceptibility to infection and attenuated production of proinflammatory cytokines and reactive oxygen and nitrogen species [99]. Further studies demonstrate that SP-A-knockout mice exposed to *Pneumocystis* were susceptible to colonization with the organism and, upon immunosuppression, were not able to clear *Pneumocystis* from their lungs [100]. Similarly, studies testing SP-D have shown that this surfactant protein binds to *Pneumocystis*, resulting in increased AM attachment, but not phagocytosis [94]. However, in a murine model of PCP, overexpression of SP-D resulted in markedly higher organism burden relative to control mice combined with a significantly higher levels of TNF- α and MIP-2, indicating that SP-D facilitates the development of *Pneumocystis* infection and related lung inflammation in an immunosuppressed mouse model [101].

Mechanism of killing—Once phagocytosed, *Pneumocystis* is readily killed by healthy AMs. A great deal of research interest has gone into understanding the mechanism of killing and potential defects associated with HIV⁺ infection. Neutralizing studies have shown that of the components of the macrophage oxidative burst, hydrogen peroxide is primarily responsible for the killing of *Pneumocystis* within the phagocyte [79]. Clinically, the importance of the macrophage oxidative burst as an effector function against *Pneumocystis* was revealed in a study by Koziel *et al.*, who showed that AM from HIV⁺ individuals with CD4 T-cell counts below 200 cells/mm³ have significantly reduced hydrogen peroxide production, and this effect is more pronounced in patients with active PCP [102]. Similarly, release of nitric oxide and reactive nitrogen intermediates (RNIs) is also an important component of the microbicidal effector function of macrophages and, indeed, it was found that the nitrosative burst is toxic to *Pneumocystis* organisms. However, the presence of the fungus alone does not induce its production [103]. It was later found that priming of the AMs by IFN- γ was necessary to produce RNIs [104]. In the same study, the authors also found that TNF- α plays a central role in mediating this microbicidal effect of IFN- γ -stimulated macrophages, as neutralization of TNF- α abrogated the killing [104].

Since AMs require lymphocyte-derived products, such as IFN- γ , to be maximally effective in host defense, immunosuppression, either due to HIV disease or immunosuppressive therapy, has severe consequences during infection. In addition, immunosuppression confers a direct effect on macrophage numbers. The importance of AMs in host defense against *Pneumocystis* is highlighted by a study that chemically depleted over 85% of AMs in normal rats, followed by *Pneumocystis* challenge. It was found that AM-depleted mice had a significantly higher fungal load in the lungs compared with nondepleted mice 24 h postchallenge [105]. Furthermore, a study by Lasbury *et al.* demonstrated a direct correlation between *Pneumocystis* organism burden and AM counts during infection and recovery [106]. It was found that dexamethasone-treated rats that were infected with *Pneumocystis* had a 58% decrease in the number of AMs compared with noninfected dexamethasone-treated animals, and that increased organism burden resulted in further decrease of AMs; conversely, infected healthy rats had a profound increase in AM in response to challenge. When the infected immunosuppressed rats were treated with anti-*Pneumocystis* drugs, there was slow recovery of AM numbers, while recovery from PCP by cessation of immunosuppression brought a rapid rebound in the number of AMs [106]. These results demonstrate not only that the immune state of the host affects AM number, but also that *Pneumocystis* organisms have a direct effect on AMs. Indeed, recently it has been shown

that the loss of AM during PCP infection is partly due to apoptosis, and that this is mediated by the proapoptotic enzyme caspase-9 [107]. In addition, polyamines are involved in cell-cycle regulation, and increased intracellular levels results in increased reactive oxygen species, leading to apoptosis [108]. Lasbury *et al.* found that high levels of polyamines were present within AMs and in the alveoli of *Pneumocystis*-infected mice, and that bronchial lavage fluid from infected mice caused macrophages to undergo apoptosis [108]. The mechanism for increased intracellular polyamines was found to be *Pneumocystis*-mediated overexpression of antizyme inhibitor (AZI) within the phagocyte, resulting in increased uptake of polyamines by the macrophages [109]. Another known mechanism of AM apoptosis is the decrease of survival signaling through PI-3K [110]. Active PI-3K signaling is controlled, at least in part, by the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) [111,112]. Most recently, it was shown in rodents that *Pneumocystis* infection induces the downregulation of the calcium-sensing protein calmodulin in AMs, resulting in increased apoptosis through the decrease of PI-3K and the cytokine GM-CSF [113].

Dendritic cells

In addition to AM, dendritic cells (DCs) are also important effector immune cells in the lung. Situated in airway epithelium, alveolar septae and around pulmonary vessels, they are quick to respond to inhaled antigens [114]. Their importance in fungal infections such as *A. fumigatus* and *C. albicans* is widely reported [115], but there have been few studies on the role of DC in immunity to *Pneumocystis*. Being professional antigen-presenting cells, activated DCs produce cytokines and migrate to the draining lymph nodes where they activate T-cell responses to antigens. Neonates, both HIV⁺ and HIV⁻, are more susceptible to *Pneumocystis* infection and colonization [116,117]. Confirming this, challenge studies on neonatal mice have demonstrated significant delay in *Pneumocystis* clearance compared with adult mice. The delayed clearance in the neonates was correlated with inefficient DC maturation factors, resulting in decreased phagocytosis and migration of DC to the draining lymph nodes [118,119]. The ability of DC to activate T cells is an essential component of immunity to pathogens and genetically modified DCs have been used as a CD4⁺ T-cell-independent vaccine against a murine *Pneumocystis* infection model by Zheng *et al.* [120]. This study showed that bone marrow-derived DCs genetically modified to express CD40 ligand became activated and expressed high levels of MHC class II and IL-12p70. When these DCs were pulsed with *Pneumocystis* antigens and administered to CD4⁺ T-cell-depleted mice, they were able to induce anti-*Pneumocystis* IgG antibodies and protect mice from PCP [120]. Similarly, Kobayashi *et al.* demonstrated that *Pneumocystis* interacts *in vitro* with murine bone marrow-derived DCs in part via MR, and DC exposed to *Pneumocystis* organisms displayed a Th2 cytokine response, secreting IL-4 [121]. The *in vivo* response was humoral, with production of IL-4 and induction of *Pneumocystis*-specific IgG responses, namely IgG1, IgG2a and IgG2b, but not IgG3. In addition, when CD4 T cells were depleted, mice treated with DCs exposed to *Pneumocystis* demonstrated suppression of growth of the pathogen after challenge [121]. Conversely, the interaction of *Pneumocystis* cell-surface β -glucans and DCs was studied by Carmona *et al.* who showed that this resulted in a Th1-patterned cytokine response [122]. IL-1 β and TNF- α were found to be the major cytokines secreted, but not the classic Th1 polarizing cytokine, IL-12. In addition, they found that DCs primed with β -glucan were able to induce T-cell proliferation via the T-cell stimulatory molecule Fas. When the Fas-Fas ligand interaction was blocked, IL-1 β and TNF- α secretion by DCs was impaired, indicating that β -glucan activation of DC and DC-T-cell responses are partially mediated by Fas-Fas ligand interactions [122]. Taken together, these results indicate that DCs may be protective against PCP, and that the key to protection depends on the type of antigen exposure as well as the immune status of the host.

Neutrophils

Unlike other opportunistic fungal infections such as *C. albicans*, the incidence of *Pneumocystis* infection is rare in immunodeficiency states associated with neutropenia [123]. Neutrophils are associated with inflammation and, therefore, have been implicated in severity of disease rather than resolution of infection. Indeed, inflammation and decreased pulmonary function caused by *Pneumocystis* infections in the lungs of HIV⁺ individuals have been correlated with the presence of elevated neutrophil counts [124,125]. In a thorough study to determine if neutrophils were actually responsible for the pulmonary damage seen in PCP, Swain *et al.* examined the effects of *Pneumocystis* infection in four mouse models of neutrophil dysfunction [126]. Surprisingly, the authors found that indicators of pulmonary damage were the same in knockout mice and comparable wild-type mice, and that there was no difference in the overall fungal burdens between groups. As with the human studies, they did find that neutrophil counts were a valid correlative marker of lung damage and outcome during infection. However, neither neutrophils nor reactive oxygen or nitrogen species appeared to be the causative agent of tissue damage, and this innate immune cell does not appear to play a major role in clearance of *Pneumocystis* [126].

Lung epithelial cells

Lung epithelial cells are critical in the pathogenesis of *Pneumocystis* infection as they are the predominant cell type for *Pneumocystis* trophozoite and cyst adherence. Like all epithelial cells, those of the lung are able to secrete inflammatory cytokines and chemokines in response to stressors. The lung epithelial cell line A549 has been shown to produce IL-6 in response to *Pneumocystis* organisms [127]. When incubated with the fungal surface protein gpA, these cells were shown to secrete the chemokines IL-8 and monocyte chemoattractant protein (MCP)-1 [128]. MCP-1 is involved in lung inflammation, immunity and lung epithelial repair, and was found to be induced by a JNK-mediated mechanism [129]. Alveolar epithelial cells isolated from rats were also shown to produce the neutrophil chemoattractant protein MIP-2 in response to *Pneumocystis* β -glucan moieties, which is thought to be mediated by the epithelial membrane glycosphingolipid lactosylceramide [130]. Further studies showed that *Pneumocystis* activated NF- κ B in lung epithelial cells [131], leading to the production of MIP-2 [132]. A recent study demonstrated increased apoptosis of lung epithelial cells from *Pneumocystis*-infected animals exposed to hyperoxia [133]. The authors established that the increase in epithelial cell apoptosis was mediated by activation of the Fas–Fas lig-activation of the Fas–Fas lig- and pathway, which led to increased mortality in a murine model of PCP [133].

Adaptive immunity

CD4⁺ T lymphocytes

The host immune response during PCP involves complex interactions between CD4⁺ T cells, CD8⁺ T cells, AMs, DCs, neutrophils and soluble mediators that together facilitate clearance of the infection. Of these cell populations, CD4⁺ T cells are absolutely critical for resolution of *Pneumocystis*, playing an essential role in memory-cell functions that coordinate host inflammatory responses by recruitment and activation of effector cells, which are responsible for elimination of the organism. Animal models of immunodeficiency using SCID, recombinant activating gene (*Rag*) 1^{-/-}, *Rag*2^{-/-} or lymphocyte-depleted mice clearly demonstrate that loss of CD4⁺ T cells renders mammals susceptible to *Pneumocystis* lung infection. Indeed, pneumonia caused by this fungal organism is most often observed when the CD4⁺ T-cell count falls below 200 cells/mm³ [134,135]

Many experiments have sought to determine how CD4⁺ T-cells control *Pneumocystis* infection. Recent studies focusing on T-cell activation through costimulatory molecules have

demonstrated that loss of the costimulatory receptor CD28 was sufficient to render the mice susceptible to acute *Pneumocystis* infection, which was eventually cleared, but was associated with a consistent increase in IL-10 and IFN- γ transcripts and an influx of naive CD8⁺ T cells [136]. Further depletion of CD2 and CD28 resulted in spontaneous development of lethal PCP associated with accumulation of CD8⁺ T cells in the lungs, marked reductions of antibody titers and dysregulation of cytokine production, particularly IL-10 and IL-15 [137], thus affecting immune downregulation and development of memory T cells. The authors speculate that despite normal numbers of CD4⁺ T cells, defects in costimulatory molecule function results in transient dysregulation of the cellular and cytokine environment, and this may lead to initial susceptibility to this opportunistic pathogen in HIV⁺ individuals before lymphopenia occurs [137].

Immune reconstitution disease—Although CD4⁺ T cells are necessary for clearance of *Pneumocystis*, additional investigations have shown that T-cell responses might also cause significant pulmonary impairment during PCP. It was found that despite active infection, *Pneumocystis*-infected SCID mice had very little lung damage until the late stages of disease [138]. However, when these animals were reconstituted with splenocytes, an intense T-cell-mediated inflammatory response consisting of both CD4⁺ and CD8⁺ T cells was initiated, causing substantially impaired gas exchange. Similarly, Roths and Sidman showed that CD4⁺ T cells adoptively transferred to SCID mice prior to induction of PCP protected them from infection, whereas adoptive transfer of CD4⁺ T cells after organism burden had already been established resulted in a fatal hyperinflammatory reaction [139]. This phenomenon, termed immune reconstitution disease (IRD), is prevalent in AIDS patients who demonstrate a rapid recovery of CD4⁺ T lymphocytes after HAART, as well as in non-HIV⁺ patients that experience immune recovery owing to termination of steroid use or cancer treatment [140]. At the onset of PCP-related IRD, patients no longer have heavy *Pneumocystis* infections, indicating that the severity of disease is directly related to the degree of immune recovery [141,142]. A murine model of IRD demonstrated that the pathology includes persistent parenchymal lung inflammation, increased macrophage and lymphocyte recruitment in the bronchoalveolar lavage fluid, decreased hydrophobic surfactant SP-B, impaired surfactant function and significant increases in the *S*-nitrosylated form of SP-D [143]. *S*-nitrosylated SP-D serves as a proinflammatory mediator, which results in enhanced macrophage migration and chemokine production, further exacerbating pulmonary inflammation [143]. In an attempt to decipher the role of CD4⁺ and CD8⁺ lymphocyte subsets in the pathophysiology of PCP-related IRD, Bhagwat *et al.* used an IRD model in SCID mice [144]. The authors found that CD4⁺ T cells were more abundant in the acute stage of IRD and this coincided with impaired pulmonary physiology and organism clearance. Conversely, CD8⁺ T cells were more abundant during the resolution phase following organism clearance. When splenocyte-reconstituted mice were further depleted of CD8⁺ T cells, they were able to clear the infection, but developed more severe disease, with increased IFN- γ production and a prolonged CD4⁺ T-cell response, than mice that were reconstituted with both T-cell subsets. By contrast, in the absence of CD4⁺ T cells, CD8⁺ T cells did not clear the organism and produced a nonprotective, pathological immune response, suggesting that the interplay between CD4⁺ and CD8⁺ T cells affects the ultimate outcome of PCP-related IRD [144]. Swain *et al.* used the murine model of PCP in B-cell-deficient mice to further examine the role of CD4⁺ and CD8⁺ T cells in the development of PCP pathology. Interestingly, he found that significant pulmonary injury occurred when only CD4⁺ T cells were present, and this pathology coincided with enhanced recruitment and activation of eosinophils and strong type 2 cytokine polarization in the alveolar environment [145]. Moreover, CD8⁺ T cells acted to moderate this CD4⁺ T-cell-mediated pathology possibly through increasing the ratio of CD25⁺ CD4⁺ regulatory T cells over CD25⁻ CD4⁺ effector T cells [145].

Regulatory T cells—Indeed, regulation of immunity to *Pneumocystis* is key to survival from PCP, and recent studies have further examined the role of CD4⁺ CD25⁺ regulatory T cells during such infections. This class of CD4⁺ T cells suppresses proliferation, cytokine production and cytotoxic activity of both CD4⁺ and CD8⁺ effector cells. Experiments have shown that adoptive transfer of CD4⁺ CD25⁺ regulatory T cells is tolerated by *Pneumocystis*-infected SCID mice and protects mice from IRD associated with transfer of CD4⁺ CD25[−] effector T cells [146,147]. Furthermore, CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells were found to be recruited to the lung during the course of *Pneumocystis* infection in immunocompetent mice, and loss of this cell population owing to anti-CD25⁺ antibody resulted in enhanced lung injury associated with increases in both Th2 and inflammatory cytokine production [147]. These experiments reveal that regulatory T cells control pulmonary inflammation and lung injury associated with *Pneumocystis* infection, both in the setting of IRD, as well as new acquisition of infection.

CD8⁺ T lymphocytes

As mentioned above, CD8⁺ T cells work in cohort with CD4⁺ T cells in the normal, effective immune response to *Pneumocystis* infection. However, there has been much debate on whether their involvement is protective or detrimental, particularly in the setting of CD4⁺ T-cell deficiency. Gigliotti *et al.* demonstrated that over a 6-week period in a murine model of PCP, there was no difference in organism burden in CD4⁺ T-cell-depleted versus CD4⁺, CD8⁺ T-cell-depleted animals, indicating that CD8⁺ T cells are not involved in fungal clearance [148]. Moreover, reconstitution with CD8⁺ T cells sensitized with *Pneumocystis* antigen failed to control organism burden but instead accelerated the onset of lung injury [148]. Similarly, in studies on the relationship of chronic obstructive pulmonary disease with HIV infection and *Pneumocystis* colonization, Norris *et al.* found that simian immunodeficiency virus-infected macaques develop a prolonged colonization state characterized by persistent influx of CD8⁺ T cells and neutrophils, as well as increases in IL-8, IFN- γ and TNF- α relative to simian immunodeficiency virus-infected monkeys [31]. This increased inflammatory state resulted in progressive pulmonary decline similar to that seen in emphysema. The mechanism of CD8⁺ T-cell damage in CD4⁺ T-cell-depleted animals infected with *Pneumocystis* was found to be dependent on antigen presentation by MHC class I expressing nonbone marrow-derived cells in the lung, and not by the CD8⁺ T-cell-derived cytotoxic mediators perforin, Fas or IFN- γ [149]. Additional experiments showed that the TNF receptor (TNFR) on lung parenchymal cells was needed for maximal CD8⁺ T-cell-dependent pulmonary inflammation and lung injury during PCP in CD4⁺ T-cell-depleted mice [150,151]. In these experiments, CD4⁺ T-cell-depleted infected animals were shown to have significantly greater pulmonary TNF- α levels, associated with increased lung concentrations of monocyte- and neutrophil-recruiting chemokines, than CD4⁺ and CD8⁺ T-cell-depleted mice. The increase in proinflammatory chemokines was abrogated when mice were depleted of TNFR, and the animals displayed improved surfactant activity, pulmonary function and diminished inflammation during infection [150]. Taken together, these studies suggest that although CD8⁺ T-cell cytotoxic effects are not the cause of lung injury, their presence in the lungs can be detrimental during *Pneumocystis* infection.

However, other studies have shown that CD8⁺ T cells can be protective against *Pneumocystis* infection, though this is dependent on their T cytotoxic (Tc)1-like phenotype, which is defined by high levels of endogenous IFN- γ production [51]. IFN- γ delivery via aerosol or by overexpression using adenoviral-mediated gene transfer using Ad-IFN [152] has been shown to result in clearance of *Pneumocystis* in the absence of CD4⁺ T cells, and this was associated with an increase in recruited IFN- γ -producing CD8⁺ T cells [152]. Exploring this further, McAllister *et al.* showed that CD8⁺ T cells recruited by Ad-IFN in

Pneumocystis-infected CD4⁺ T-cell-depleted mice enhanced macrophage-mediated killing of the pathogen *in vitro*. Interestingly, adoptive transfer of Tc1 CD8⁺ T cells into SCID mice protected them from infection, while transfer of Tc2-polarized CD8⁺ T cells resulted in lung injury [153]. Further experiments testing CD8⁺ T-cell subsets found that Tc1-polarized cells secreted higher levels of IFN- γ and GM-CSF and lower levels of the Th2 and regulatory cytokines IL-4, IL-5, IL-10 and IL-13 than Tc2 CD8⁺ T cells when stimulated with *Pneumocystis* antigen. The enhanced effector activity in the macrophage-mediated killing assay was found to be dependent on GM-CSF and not cell–cell contact. This indicates that antigen-specific secretion of GM-CSF is critical for effector activity of *Pneumocystis*-specific Tc1 CD8⁺ T cells *in vitro* [154]. In another study, cytotoxic CD8⁺ T cells were shown to have preferential expression of the lymphocyte-specific chemokine receptor, CXCR3, and it was found that overexpression of the chemokine IFN protein (IP)-10 in lungs of CD4⁺ T-cell-depleted *Pneumocystis*-infected mice resulted in accelerated clearance of the organism associated with increased recruitment of Tc1 CD8⁺ T cells [155]. Taken together, these results demonstrate that CD8⁺ T cells can either be protective or detrimental depending on their cytokine-associated phenotype and, thus, may in part explain the dichotomous role of CD8⁺ T cells and their clinical heterogeneity in the severity of PCP.

B cells & antibodies

Since PCP is so closely associated with HIV infection, the majority of research has been focused on CD4⁺ T cells. However, there is evidence that B cells and *Pneumocystis*-specific antibody responses also play a major role in immunity to this fungal pathogen. Clinically, PCP infections complicating B-cell-targeted chemotherapy for treatment of non-Hodgkin's lymphoma have been reported, demonstrating the importance of these lymphocytes as effector cells against *Pneumocystis* infections [156]. In addition, antibodies against *Pneumocystis* are readily detected in humans from a very young age. Although early reports suggested that HIV infection results in decreased anti-*Pneumocystis* antibody responses, more recent studies have determined that HIV⁺ PCP patients have higher [157] or equal [158] amounts of *Pneumocystis*-specific IgG than non-HIV⁺ PCP patients. Most recently, studies have found that geographical location [159], previous episodes of PCP and age [160] of HIV⁺ patients also play a role in serum responsiveness to *Pneumocystis* surface antigens.

Antibodies & vaccines—Animal studies have determined that the predominant serum antibodies are of the IgG class, but IgM is also present [161,162]. Early protection studies showed that IgM recognizing the surface glycoprotein gpA of *Pneumocystis* was partially protective against lung infection during steroid immunosuppression [163]. Additional studies using anti-*Pneumocystis* IgM showed protection when administered to infected, immunodeficient animals via an intranasal route [164]. Roths *et al.* found that anti-*Pneumocystis* hyper-immune serum was highly effective at reducing the number of organisms in all stages of PCP infection in SCID mice and increased the longevity of the animals [165]. Moreover, the hyper-immune serum was able to prevent IRD associated with transfer of *Pneumocystis*-sensitized lymphocytes [165]. In addition, Wells *et al.* were able to demonstrate passive prophylaxis using anti-*Pneumocystis* IgG1 against kexin, a serine protease [166]. Later studies by the authors found that the protective effects of antikexin IgG1 were mediated through its Fc portion as well as serum complement, indicating that Fc-mediated phagocytosis and complement activation rather than serum agglutination were necessary for the anti-*Pneumocystis* effects seen with the antibody [167]. Active immunization has also been demonstrated against *Pneumocystis* infection. Early studies by Harmsen *et al.* showed effective clearance through repeated exposure of mice to *Pneumocystis* organisms [168]. Mice that produced anti-*Pneumocystis* IgG antibodies were able to protect the animals from infection even when their CD4⁺ T cells were depleted. Protective active immunization was also demonstrated by Pascale *et al.* who, using multiple

intranasal immunizations with a *Pneumocystis* antigen-cholera toxin fragment B construct, were able to show significantly higher lymphoproliferative responses and increased titers of IgM, IgG and IgA, as well as complete clearance in CD4⁺ T-cell-depleted, *Pneumocystis*-infected mice [169]. Using a similar experimental design, Garvy *et al.* immunized mice genetically deficient in IFN- γ or IL-4 and then immunosuppressed them by CD4⁺ T-cell depletion. Interestingly, both groups of mice were able to resolve *Pneumocystis* infection without the presence of CD4⁺ T cells even though their IgG subclasses were generated by opposing Th1 (IL-4^{-/-}) and Th2 (IFN- γ ^{-/-}) responses [170]. Most recently, Wells *et al.* were able to demonstrate active immunization using a recombinant protein constructed of a *Pneumocystis* antigen, A12, and the reducing antioxidant, thioredoxin, resulting in a significant decrease in fungal burden during CD4⁺ T-cell depletion [171].

B-cell activation of T cells—Experiments blocking the interaction between B and T cells via the CD40–CD40 ligand pathway resulted in decreased organism clearance, correlated with both a decrease in antigen-specific IgG levels, as well as reduced T-cell activation [172]. The question arises as to whether B-cell-mediated protection against *Pneumocystis* is primarily antibody mediated, or is produced through B-cell activation of CD4⁺ T cells. In an attempt to answer this question, Lund *et al.* utilized the murine model of PCP using several genetically modified strains [173]. The authors showed that CD40-deficient mice, which are highly susceptible to *Pneumocystis* infection, produced normal levels of anti-*Pneumocystis* IgM, but were unable to clear infection. Second, CD40-deficient chimeric mice, which lack CD40 on B cells only and are unable to generate antigen-specific IgG, were more susceptible to infection than wild-type, but were eventually able to clear *Pneumocystis*. When B-cell-deficient or CD40-knockout chimeric mice were infected, their deficiencies resulted in reduced numbers of activated T cells in the lungs and draining lymph nodes during early PCP [173]. These results suggest that activation of T cells through CD40 is responsible for B-cell protection. Later studies by the authors found that B cells are also necessary for the generation of CD4⁺ memory T cells in response to *Pneumocystis* infection [174]. Further support for the importance of B-cell activation of T cells came in a multifaceted vaccination study by Zheng *et al.* who demonstrated a CD4⁺ T-cell-independent protection using CD40L combined with antigen [175]. A prototypic plasmid DNA vaccine was constructed encoding CD40L combined with the potent antigen ovalbumin, and was shown to elicit significant antigen-specific serum titers and Tc1 CD8⁺ T-cell responses in CD4⁺ T-cell-deficient animals. Using this approach, the authors replaced ovalbumin with the *Pneumocystis* antigen, kexin, and vaccinated CD4⁺ T-cell-depleted animals prior to *Pneumocystis* infection. The authors found that this vaccination strategy resulted in significantly higher anti-*Pneumocystis* IgG titers, as well as opsonic killing of the fungus compared with vaccination with kexin alone. Moreover, vaccinated mice demonstrated a three-log greater protection against PCP, and adoptive transfer of their B cells or IgG to SCID mice conferred protection against *Pneumocystis* challenge [175]. Taken together, these data demonstrate that *Pneumocystis*-specific IgG plays an important role, but clearance of *Pneumocystis* in mice is dependent on B-cell activation and expansion of effector and memory T cells. In addition, serum antibodies are becoming an important marker of *Pneumocystis* infection, and passive and/or active antibody immunization studies have clearly demonstrated at least some form of protection. Further determination of protective antigens and mode/location of delivery would be a great benefit in developing vaccine strategies against this opportunistic pathogen.

Cytokines & chemokines

Interferons

IFN- γ is one of the most studied cytokines with respect to opportunistic fungal infections, including *Pneumocystis*. The cytokine is produced primarily by CD4⁺ T cells and, thus, with the direct correlation between PCP infection and decrease in CD4⁺ T cells, there is an indirect link between IFN- γ and PCP. Indeed, experimental studies have shown that IFN- γ is made in the lung following *Pneumocystis* challenge [176]; however, IFN- γ was shown to not be directly toxic to *Pneumocystis* [177]. Blocking of IFN- γ in chronically infected SCID mice had no effect on the clearance of the organism, but the animals did demonstrate a prolonged and exacerbated *Pneumocystis*-driven interstitial pneumonia characterized by eosinophilia and formation of multinucleated giant cells [178]. Similarly, in a murine model of PCP following bone marrow transplantation, neutralization of IFN- γ resulted in exacerbated pulmonary inflammation [179]. However, as mentioned earlier, administration of adenoviral vector encoding IFN- γ was shown to protect CD4⁺ T-cell-depleted mice from *Pneumocystis* infection [152]. This effect has also been shown using recombinant IFN- γ treatment. Prophylactic treatment with recombinant IFN- γ to steroid-treated, *Pneumocystis*-infected rats has also been shown to reduce organism burden and prolong survival [180] and, similarly, aerosolized administration of the cytokine to CD4⁺ T-cell-depleted animals reduced the intensity of PCP [181]. Therefore, it appears that IFN- γ may not be entirely required for organism clearance, but is required for controlling the life-threatening *Pneumocystis*-driven inflammation in the lungs.

Recently, evidence has emerged that type I IFNs (IFN- α/β) play an important role in control and susceptibility to opportunistic infections [182,183]. Type I IFNs signal through one common receptor (IFN- α receptor; IFNAR) to induce DC maturation, resulting in secretion of cytokines that promote Th1 cell differentiation, and induce proliferation and expansion of memory CD8⁺ T cells [184,185]. Recent studies by Meissner *et al.* examined the role of IFNAR signaling during *Pneumocystis* infection and found that in the absence of an intact acquired immune system, type I IFN signaling is critical in maintaining bone marrow hemopoiesis during infection [186]. Additional studies demonstrated that IFNAR signaling is also correlated to hyperinflammatory responses during PCP. The authors found that in the absence of CD4⁺ T cells, IFNAR-knockout animals had a significant reduction in lung damage and proinflammatory cytokines, and improved lung function, which was associated with a decrease in CD8⁺ T-cell recruitment. However, in the presence of CD4⁺ T cells, IFNAR-knockout animals have an exacerbation of the Th2 response to *Pneumocystis*, characterized by persistent eosinophilia, mucus-cell metaplasia and IgE production, similar to asthma. Therefore, it appears that both CD4⁺ T-cell and type I IFN-mediated mechanisms can determine pulmonary complications from PCP [187].

TNF- α

TNF- α is a potent proinflammatory cytokine secreted primarily by AMs [188] and has several essential activities during *Pneumocystis* infection, but can also be detrimental owing to recruitment of neutrophils, which mediate lung injury, as well as further promotion of proinflammatory cytokines. In an animal model of PCP, Kolls *et al.* demonstrated that inhibiting TNF- α delayed clearance in normal mice and resulted in a more chronic infection when CD4⁺ T cells were depleted [189]. Similarly, neutralization studies in splenocyte-reconstituted SCID mice infected with *Pneumocystis* showed a complete inhibition of organism clearance [190]. Furthermore, TNF enhances the recruitment of neutrophils, lymphocytes and monocytes, which are required for optimal organism clearance [45]. TNF and its receptor signaling also induce the generation of other cytokines and chemokines from

epithelial cells and IFN- γ from lymphocytes, which further promote inflammatory cell activation and cellular recruitment during PCP [150].

IL-1, IL-6 & GM-CSF

IL-1 is an important cytokine that mediates inflammation and other host physiological responses to a variety of infections [191], and individuals infected with HIV demonstrate a significant reduction of IL-1 secretion from macrophages [192]. The importance of endogenous IL-1 in resistance to *Pneumocystis* infections was first demonstrated in a SCID model of PCP by Chen *et al.*, who showed that mice reconstituted with splenocytes have a significant increase in IL-1 levels [193]. Treatment of these mice with antibody blocking the IL-1 receptor completely inhibited the ability to clear the pathogen from the lungs, which correlated with reduced recruitment of lymphocytes, macrophages and neutrophils [193].

Like TNF- α and IL-1, IL-6 is a proinflammatory cytokine that participates in the host response to a variety of infections and has been shown to be made in high levels in response to *Pneumocystis* [194]. Unlike IL-1, neutralization of IL-6 does not affect *Pneumocystis* clearance, but results in augmented neutrophil and lymphocyte recruitment [194]. As mentioned earlier, IL-6 has also been shown to be critical for the production of the serum glycoproteins vitronectin and fibronectin [76] and, conversely, production of IL-6 was shown to be modulated by SP-A [195].

The hematopoietic growth factor GM-CSF, produced by macrophages epithelial and T cells, has potent stimulatory effects on monocytes and macrophages. The major effect is to enhance phagocytic and metabolic functions, including increased synthesis of molecules toxic to microbes, and to release other proinflammatory cytokines [196]. Mandujano *et al.* showed that administration of recombinant GM-CSF to chronically infected CD4⁺ T-cell-depleted mice enhanced *Pneumocystis* clearance [197]. In CD4⁺ T-cell-depleted, GM-CSF-deficient mice, PCP infection was exacerbated and AMs from these mice had impaired phagocytosis and TNF- α production in response to the fungus [198]. Furthermore, when GM-CSF was overexpressed in the lung, mice had decreased inflammation and augmented clearance [198]. Exogenous treatment with GM-CSF along with IL-4 was found to enhance clearance of *Pneumocystis* in neonatal mice, which are normally susceptible to PCP infection owing to lack of cellular maturation factors. Clearance was associated with marked expansion of activated DCs and activated CD4⁺ T lymphocytes [119]. As mentioned earlier, antigen-mediated GM-CSF secretion by *Pneumocystis*-specific Tc1 CD8⁺ T cells was shown to be critical for effector activity, as neutralization of the cytokine completely abrogated macrophage-mediated killing induced by the lymphocytes [154].

IL-12, IL-10 & the IL-23–IL-17 cytokine axis

IL-12 is produced mainly by macrophages and DCs and functions to promote T-cell differentiation to a Th1, IFN- γ -producing phenotype. Early clinical studies showed that AMs from AIDS patients with active PCP had reduced levels of IL-12 [199]. By contrast, however, a recent study demonstrated that IL-12 production is part of the normal host response to *Pneumocystis* infection [200]. In addition, local administration of IL-12 either with recombinant IL-12 or vector administration into the respiratory mucosa of normal or CD4⁺ T-cell-depleted, *Pneumocystis*-infected mice resulted in accelerated clearance [200]. This augmented clearance was associated with increased inflammatory cell recruitment and release of TNF- α , IL-12 and IFN- γ . These results indicate that IL-12 therapy can enhance host resistance to infection in both normal and impaired hosts.

Unlike IL-12, IL-10 is an anti-inflammatory cytokine whose function is to maintain a crucial balance between pathology and protection [201]. Like IL-12, IL-10 is produced primarily by

antigen-presenting cells and has also been shown to have defective expression in HIV⁺PCP⁺ individuals [199]. Ruan *et al.* have shown that administration of an adenoviral vector expressing IL-10 resulted in decreased *Pneumocystis*-driven pulmonary inflammation and improved survival, but did not improve fungal burden [202]. Further studies on IL-10 by Qureshi *et al.* showed that IL-10-deficient mice had increased clearance of *Pneumocystis* relative to control mice [203]. However, upon depletion of CD4⁺ T cells, IL-10-deficient mice were unable to clear the organisms from the lungs and although these mice had similar inflammatory responses to wild-type mice, lung injury was significantly higher in IL-10-deficient animals, indicating that IL-10 plays a critical role in controlling lung damage independent of modulating the inflammatory response [203].

IL-17 production by Th17 cells and their effects are well described in autoimmunity, but there is growing evidence that the Th17 lineage with its IL-23–IL-17 cytokine axis plays a significant role in mediating host mucosal immunity to a number of pulmonary pathogens, including pathogenic fungi [204–206]. IL-23 is a member of the IL-6 family of cytokines and has stimulating activity for IL-17-producing memory CD4⁺ T cells (Th17 cells) [207]. Like IL-12, IL-23 is produced by activated macrophages and DCs, and functions to expand committed Th17 effector cells to maintain and extend their function [208]. In an attempt to decipher the role of the IL-23–IL-17 axis in the host response to *Pneumocystis*, Rudner *et al.* found that AMs stimulated *in vitro* with *Pneumocystis* produced IL-23, and IL-23 transcript was expressed in the lungs of mice infected with the pathogen [209]. Furthermore, the authors found that IL-23-knockout mice infected with *Pneumocystis* had delayed clearance of the organism relative to wild-type infected mice, and this was associated with a decrease in CD4⁺ T cells, IL-17 and the lymphocyte chemokines IP-10, monokine induced by IFN- γ (MIG), MIP-1 α , MIP-1 β and RANTES. Similarly, depletion of IL-23 or IL-17 in infected wild-type mice resulted in increased fungal burden. Taken together, these results indicate that the IL-23–IL-17 axis participates in the host response to *Pneumocystis*; however, the detailed mechanism is still unknown.

RANTES, MCP-1, lymphotactin, MIP-1 α , MIP-1 β & MIP-2

Chemokines function to recruit effector cells to the site of injury, and multiple reports have found an increase in chemokine expression during *Pneumocystis* infection. In a SCID model of PCP, Wright *et al.* found that expression levels of the lymphocyte chemoattractants RANTES and lymphotactin, the monocyte and macrophage chemokines MCP-1, MIP-1 α and MIP-1 β , as well as the neutrophil chemokine MIP-2, were all upregulated upon lymphocyte reconstitution of the SCID mice [210]. Lack of detectable pulmonary inflammation in the nonreconstituted SCID mice correlated with a lack of pulmonary expression of the chemokines. Additionally, the time course of chemokine mRNA abundance coincided with the inflammatory response and organism clearance. Later studies by the author found that CD8⁺ T-cell-associated pathology required TNFR signaling [150]. Wright *et al.* showed that in CD4⁺ T-cell-depleted, *Pneumocystis*-infected mice, there were significantly higher TNF- α levels, which coincided with elevated levels of RANTES, MCP-1, MIP-2 and cytokine-induced neutrophil chemoattractant, which resulted in CD8⁺ T-cell-associated pathology. Using the same experimental model in TNFR-deficient animals resulted in diminished pulmonary inflammation, and improved surfactant activity and pulmonary function in the infected mice [150]. Combined, these results demonstrate that chemokines are essential for the resolution of infection, but can also result in a hyperinflammatory state.

IP-10, MIG & IFN-inducible T-cell α -chemoattractant

Overexpression of IFN- γ has been shown to ameliorate *Pneumocystis* infection in mice depleted of CD4⁺ T cells [152], and this clearance was associated with higher levels of the

CXCR3 glutamate, leucine and arginine (ELR)⁻ligands IP-10 and MIG [153]. Moreover, IFN- γ overexpression in the lung resulted in the increased recruitment of CXCR3⁺/IFN- γ -secreting Tc1 CD8⁺ T cells, and these cells were critical effector cells against *Pneumocystis* [153]. These chemokines, along with IFN-inducible T-cell α -chemoattractant (I-TAC), primarily recruit Th1 cells and, as their names imply, are induced by IFN- γ [211,212]. IP-10 has been shown not only to recruit and mobilize a Th1 response, but also subvert Th2 responses [213]. Clinically, in patients with HIV infection and T-cell alveolitis, expression of IP-10 and MIG were correlated to direct migration of pulmonary CXCR3⁺ Tc1 CD8⁺ T cells and excess pulmonary inflammation [214]. McAllister *et al.* found that that *Pneumocystis*-infected mice deficient in CXCR3 signaling, but CD4⁺ T-cell intact, had an initial delay, but were able to clear infection. However, when CD4⁺ T cells were depleted, decreased levels of IP-10, MIG and I-TAC were observed, and the mice were permissive to infection. In the absence of CD4⁺ T cells, overexpression of IP-10 using an adenoviral vector accelerated clearance of *Pneumocystis* from the lungs early in infection, and this was associated with increased recruitment of Tc1 CD8⁺ T cells [155]. Indeed, additional studies from our laboratory have shown that intranasal administration of recombinant I-TAC resulted in increased clearance in *Pneumocystis*-infected, CD4⁺ T-cell-depleted mice [KELLY M, UNPUBLISHED DATA]. In addition to their chemotactic properties, IFN-inducible ELR⁻CXC chemokines have also been shown to have defensin-like activity against *Escherichia coli* and *Listeria monocytogenes* *in vitro* [215], and *Bacillus anthracis* spores and bacilli *in vivo* [216]. Since augmented clearance was observed as early as 3 days postinfection in adenoviral IP-10-treated, CD4⁺ T-cell-depleted mice infected with *Pneumocystis*, McAllister *et al.* hypothesized that IP-10 may have a direct microbicidal effect on *Pneumocystis*. Confirming this, IP-10 added to *Pneumocystis in vitro* resulted in a dose-dependent effect on fungal viability [217]. Similarly, I-TAC was also found to have a direct microbicidal effect on *Pneumocystis* that was dose dependent and, like other defensins, addition of sodium chloride neutralized the microbicidal activity of the chemokine [KELLY M, UNPUBLISHED DATA]. Taken together, these results indicate that the IFN-inducible ELR⁻CXC chemokines contribute to host defense to *Pneumocystis*, both through recruitment of effector cells and, possibly, through direct antimicrobial effects. However, in the absence of secondary infection, these chemokines may lead to detrimental inflammatory effects.

Conclusion

Immunity to *Pneumocystis* is a dynamic interplay between nearly every arm of the immune system and animal models of infection have increased our understanding of host responses to this elusive fungal pathogen. Innate immunity is absolutely critical in resolution of *Pneumocystis* infection. Within the pulmonary environment, innate immune cells and associated cytokine and chemokine mediators are responsible for recognizing and clearing the organism, and for activating long-term adaptive responses, both cellular and antibody in nature. These adaptive immune responses are also critical for augmenting the innate responses as well as maintaining long-term memory protection. Yet, there remain many unanswered questions regarding *Pneumocystis*' basic biology, virulence attributes and pathogenesis. The increase in the non-HIV immunocompromised population, noncompliance with current treatments, emergence of drug-resistant strains and rise in HIV⁺ cases in developing countries, point to the need for continued research of this opportunistic fungal pathogen.

Future perspective

Research to date on host defense against *Pneumocystis* has revealed a surprisingly redundant system of checks and balances that prevent infection in the normal host and allow infection in the immunosuppressed host. Now that we have a better understanding of the immune

dysfunction associated with PCP, research is urgently required to uncover new ways to reverse or enhance these immune pathways to better treat established infection. This need is all the greater given the increased use of immunosuppressive therapy for non-HIV diseases (e.g., rheumatoid arthritis) and the emergence of *Pneumocystis* strains that may be resistant to conventional therapy with trimethoprim–sulfamethoxazole. Rapid identification of both infection- and drug-resistant organisms are especially needed. *Pneumocystis* is once again emerging from behind the shadow of HIV infection, with *Pneumocystis* colonization described in children and in patients with a variety of lung diseases. We need to better understand what host factors or environmental influences lead to colonization and the relationship between colonization and infection. Consistent *in vitro* culture of the fungal organism would be of great benefit in this regard as it would allow microarray and proteomic analysis of *Pneumocystis* during interactions with host cells, leading to identification of virulence attributes and immune evasion mechanisms of the organism. Finally, there are emerging data supporting a vaccine against *Pneumocystis*. We need better vaccine targets for *Pneumocystis* that transcend species specificity to allow for testing, and we need to understand when and how such a vaccine may be employed. The great hope with a vaccine is that we may one day eliminate *Pneumocystis* infection altogether and relegate *P. jirovecii* to a historical curiosity.

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Figure 1. Gomori's methenamine-silver stain of *Pneumocystis* cysts in lung tissue
Cysts contain one to eight sporozoites within. Arrow shows cyst containing two sporozoites.

Figure 2. Chest radiograph of *Pneumocystis pneumonia*

A 23-year-old male with 3-week history of fever, cough and shortness of breath. Chest x-ray shows bilateral ground glass infiltrates. Bronchoscopy revealed *Pneumocystis jirovecii*. Patient was found to be HIV⁺ on hospital admission.

Table 1Immunomodulation of *Pneumocystis* infection: cytokine strategies.

Treatment	Animal model of immunosuppression	Fungal burden	Effector cell recruitment	Tissue inflammation	Mortality	IRD	Ref.
Gene transfer							
Ad-IFN- γ	CD4+ T-cell depleted	↓	↑	n.d.	n.d.	n.d.	[152]
Ad-IL-12	CD4+ T-cell depleted	↓	↑	n.d.	n.d.	n.d.	[200]
Ad-IL-10	CD4+ T-cell depleted	Ø	↓	↓	↓	↓	[202]
Ad-i-TNF	CD4+ T-cell depleted	↑		↑	n.d.	n.d.	[189]
Ad-IP-10	CD4+ T-cell depleted	↓	↑	↓	n.d.	n.d.	[155]
Cytokine administration/ neutralization							
Recombinant IFN- γ	Steroid treated/CD4+ T-cell depleted	↓	n.d.	↓	↑	n.d.	[180,181]
Recombinant IL-12	CD4+ T-cell depleted	↓	↑	n.d.	n.d.	n.d.	[200]
Recombinant GM-CSF	CD4+ T-cell depleted	↓	n.d.	Ø	n.d.	n.d.	[197]
Recombinant GM-CSF + IL-4	Neonates	↓	↑	↓	n.d.	n.d.	[119]
Neutralization IL-1R	Reconstituted SCID	↓	↓	↓	n.d.	n.d.	[193]
Neutralization IL-6	Reconstituted SCID	Ø	↑	↑	n.d.	n.d.	[194]
Neutralization IL-17	Wild-type	↑	↓	n.d.	n.d.	n.d.	[209]
Neutralization IL-23	Wild-type	↑	↓	n.d.	n.d.	n.d.	[209]
Neutralization TNF- α	CD4+ T-cell depleted/reconstituted SCID	↑	n.d.	n.d.	n.d.	n.d.	[190]
Neutralization IFN- γ	Bone marrow transplant/SCID	↓↓	n.d.	↑	n.d.	↑	[178,179]

↑ Significant increase;

↓ Significant decrease;

↓↓ Clearance;

Ø No difference; GM-CSF: Granulocyte-macrophage colony-stimulating factor; i-TNF: TNF inhibitor; IRD: Immune reconstitution disease; n.d.: Not done; SCID: Severe combined immunodeficiency.

Table 2Immunomodulation of *Pneumocystis* infection: vaccination strategies.

Immunization	Animal model	Fungal burden	Antibody production	Lymphoproliferation	Mortality	IRD	Ref.
Passive							
Hyperimmune serum	SCID	↓	n.d.	n.d.	↓	↓	[165]
anti- <i>Pneumocystis</i> pool	SCID	↓	n.d.	n.d.	n.d.	n.d.	[164]
anti-gpA (intraperitoneal)	Steroid treated	↓	n.d.	n.d.	n.d.	n.d.	[163]
anti-gpA pool (IN)	SCID	↓	n.d.	n.d.	n.d.	n.d.	[164]
anti-Kex1, IgM (IN)	SCID	↓	n.d.	n.d.	n.d.	n.d.	[164]
anti-Kex1, IgG1 (IN)	SCID	↓	n.d.	n.d.	n.d.	n.d.	[164,166,167]
Serum/B cells from pCD40L/kexin	SCID	↓	n.d.	n.d.	n.d.	n.d.	[175]
Active							
Mult. PC (IT)	CD4+ T-cell depleted	↓↓	↑	n.d.	n.d.	n.d.	[168]
Mult. PC (IT)	CD4+ T-cell depleted + anti-IFN- γ / anti-IL-4	↓↓	↑	n.d.	n.d.	n.d.	[170]
Mult. PC-CTB (IN)	CD4+ T-cell depleted	↓↓	↑	n.d.	n.d.	n.d.	[169]
Mult. pCD40L/kexin (IM)	CD4+ T-cell depleted	↓	↑	n.d.	n.d.	n.d.	[175]
Mult. A12-thioredoxin (SC)	CD4+ T-cell depleted	↓	↑	n.d.	n.d.	n.d.	[171]

↑ Significant increase;

↓ Significant decrease;

↓↓ Clearance;

∅ No difference; CTB: GM-CSF: Granulocyte-macrophage colony-stimulating factor; IM: Intramuscular; IN: Intranasal; IRD: Immune reconstitution disease; IT: Intratracheal; Mult.: Multiple dose; n.d.: Not done; PC: *Pneumocystis*; PC-CTB: PC antigens plus cholera toxin fraction B; SC: Subcutaneous; SCID: Severe combined immunodeficiency.