Published in final edited form as:

Curr Opin Microbiol. 2017 December ; 40: 65–71. doi:10.1016/j.mib.2017.10.019.

New advances in understanding the host immune response to *Pneumocystis*

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Abstract

Pneumocystis jirovecii causes clinical pneumonia in immunocompromised hosts. Despite this, the inability to cultivate this organism *in vitro* has likely hindered the field in ascertaining the true impact of *Pneumocystis* in human infection. However the recent release of the genome as well as in advances in understanding host genetics, and other risk factors for infection and robust experimental models of disease have shed new light on the impact of this fungal pathogen as to better define populations at risk. This review will highlight these recent advances as well as highlight future needed areas of research.

Introduction

Pneumocystis is an unusual, fascinating, opportunistic fungal pathogen and a member of the Ascomycota division of fungi. There are two major life forms of the organism. The ascus (cyst) stains positively with Gomori-Methenamine-Silver (GMS) and is the most commonly assessed for pathologic diagnosis in respiratory specimens (induced sputum or bronchoalveolar lavage). The trophic form stains with Wright-Giemsa stain but due to its small size and non-specific staining pattern this form is generally not used for diagnosis. Given the opportunity, *Pneumocystis* causes pneumonia (PCP) in susceptible hosts. It is important to note that *Pneumocystis* is host specific with *Pneumocystis jirovecii* found in humans, *Pneumocystis carinii* in rats and *Pneumocystis murina* in mice. Recent genomic analysis suggests that, while the kinome of these species is conserved (unpublished observations) the major surface glycoproteins (MSG) recognised by the host are quite divergent. It has been postulated that humans serve as the reservoir for *Pneumocystis jirovecii* and this is supported by a high prevalence in childhood autopsy studies where high

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Conflict of interest: none

incidences of colonization have been reported [1]. This has important implications when designing diagnostic tools and new treatment strategies for infected patients. While PCP is recognised as an AIDS-defining illness, the increased incidence in non-HIV patients is alarming and warrants the need for improved diagnostic and treatment strategies for affected patients [2, 3].

Current Epidemiology of *Pneumocystis* infection

Initially, *Pneumocystis pneumonia* (PCP) was commonly reported in malnourished children in post-World War II Europe. In the 1970's *Pneumocystis* infection was a significant complication of cytotoxic chemotherapy regimens for hematologic malignancy. It was in this patient population that the combination antibiotic trimethoprim-sulfisoxazole (TMP/SMX) showed both prophylactic and therapeutic benefit [4]. Subsequent to this was a large increase in cases in HIV-infected individuals, particularly those with a circulating CD4⁺ T-cell count of < 200/mm³. Again in this population the use of TMP/SMX became the primary method of prophylaxis in patients at risk. Considering *Pneumocystis* is resistant to most anti-fungal therapies, there are very limited treatment choices and TMP/SMX remains the first-line therapy. However, recent studies demonstrate the efficacy of combination therapy using lower doses of TMP/SMX combined with the echinocandin class of anti-fungals, with treatment blocking transmission of the infection in mice [5]. However, echinocandin treatment was shown to selectively target the ascus form. Mice infected and treated were cleared of the ascus form but still had infection with trophic forms [5, 6].

TMP/SMX treatment combined with the successful control of HIV with combination anti-retroviral therapies has led to a shift in case prevalence from HIV+ cases to HIV-negative cases [7]. Among HIV-negative cases, haematological malignancies represent nearly one-third of PCP cases. This is along with prolonged and high dose glucocorticoid use as part of a regimen for leukaemia, organ transplantation and/or to manage cerebral oedema. The Mayo Clinic has recently reviewed their data and found that the median dose of prednisolone (or equivalent) was 30 mg/day with a median duration of 12 weeks, increasing the risk of PCP significantly [8]. Additional patient groups at risk are patients with connective tissue disease with vasculitis or polymyositis/dermatomyositis. In addition treatments targeting B-cells in cancer or in autoimmune inflammation with anti-CD20 have been associated with *Pneumocystis* infection [9, 10]. This has been modelled in the mouse where anti-CD20 could perturb CD4⁺ T-cell priming and enhance susceptibility to infection [11]. Furthermore, in western paediatric populations, primary immunodeficiency affecting T-cell function have been implicated, including mutations in genes that result in severe combined immunodeficiency (SCID) syndrome such as IL2RG and adenosine deaminase deficiency (ADA) mutations. Cases have also been reported in hyper IgE syndrome with autosomal dominant mutations in STAT3 [12] as well as in mutations in IL21R which can signal via STAT3 [13, 14]. A list of reported mutations in humans and supportive studies in murine models of infection are listed in Table I.

The impact of PCP on healthcare was demonstrated in a query of the Health Cost and Utilization Project (HCUP) National Inpatient Sample (NIS) database which shows there were approximately 10,590 hospitalizations for *Pneumocystis* in the US in 2014. Medical

lengths of stay were estimated at 8 to 9 days in HIV+ cases [15], which translated into an estimated annual healthcare burden of over \$1B in the US alone. Moreover there are likely sub-clinical cases that were not captured in these types of data. For example, autopsy data show a high frequency of detection of *Pneumocystis* in the lung of infants which is associated with mucous gene expression [1, 16]. Since testing is not routinely performed it is difficult to estimate the true incidence of disease. Thus the disease burden of *Pneumocystis* in the developing world is difficult to ascertain. Recent data suggests that using molecular testing; PCP may be as prevalent as 6.8% of all pneumonias in children under the age of five in Mozambique where there is also a high rate of HIV infection [17]. Thus there is a clear need to develop non-invasive diagnostics to obtain more accurate incidence and prevalence data.

The role of humoral immunity

Pneumocystis infection elicits both humoral and cellular immune responses in the host. *Pneumocystis* surface proteins (MSG) contain T and B cell epitopes critical in initiating host responses. Recent studies in both man and mice highlight the important role of B cells during PCP [11, 18]. Furthermore, the role for humoral immunity was suggested by the fact that mice depleted of CD4⁺ T-cells after recovery of a primary infection, were still protected against a secondary fungal challenge [19, 20]. However depletion of CD8⁺ T-cells or macrophages, which can mediate opsonic phagocytosis and killing of the fungus, abrogated protection in this secondary challenge model [21]. To this end, passive transfer of immune sera has been shown to prevent infection in mice that were susceptible to mutations perturbing T-cell immunity [22]. The Gigliotti lab raised a series of monoclonal antibodies against *Pneumocystis* and they identified a clone that recognized a conserved protein, Kexin. This antibody, 4F11 when administered via intranasal instillation to mice could prevent transmission of pneumonia in animals co-housed with infected animals [23]. Immunization with Kexin is protective in a *Pneumocystis* challenge model in mice [24] and protective in acquisition of spontaneous infection in immunosuppressed non-human primates [25]. MSG antigens have extensively been used for epidemiologic studies and correlate with exposure [26] but are not thought to be good vaccine targets due to their heterogeneity in expression. This is supported by recent genomic data that shows variation of these genes across species [27, 28]. Thus conserved antigens such as the recently described PCA1 [29] that is homologous between murine and human *Pneumocystis* species appear to be better targets. This is a promising approach considering HIV infection impairs the humoral response to MSG antigens, compared with HIV-uninfected PCP cases, as shown in a South African cohort of children hospitalized with PCP [30].

The role of cell-mediated adaptive immunity

Asci have a cell wall containing β -glucan which is recognised by alveolar macrophages (AMs), dendritic cells (DCs) and lung epithelial cells and prime T-cell responses (Figure 1). These cells play a crucial role in recognising, binding and initiating the CD4⁺ T cell host response to *Pneumocystis*. While the type of cell that primes a T-cell in response to *Pneumocystis* is important, a recent study has also highlighted that the form of organism affects the host response to infection [31]. In contrast to the ascus, the trophic form lacks a

cell wall and therefore innate cells respond poorly with reduced CD4⁺ T-cell recruitment and IFN γ production [32]. Furthermore, the trophic form was recently shown to actively suppress the pro-inflammatory response initiated by the asci, thereby potentially enhancing their survival. Here, trophic form-loaded DCs had a reduced capacity to stimulate CD4⁺ T-cell proliferation and polarization [32]. Convincing evidence points to macrophages as the cell type responsible for killing *Pneumocystis*. However, the type of macrophage response seems to be critical contributing factor to clearance. Recent studies have dissected these responses and shown that while classical macrophages (M1) were predominant in the absence of an intact immune system, such as in immunosuppressed rats, these M1 cells were defective in clearing *P. carinii*. In contrast, rats with a competent immune response had a more prominent alternative macrophage (M2) response and cleared infection. Treatment of immunosuppressed rats with M2 cells reverted rats to a protected phenotype [33, 34].

The complex interaction between innate immune cells, such as AMs and DCs with CD4⁺ T-cells is important for an effective host adaptive response, essential for *Pneumocystis* clearance [35]. The loss of CD4⁺ T-cells either in immunocompromised patients, such as AIDS patients, or in animal models have clearly demonstrated the importance of CD4⁺ T-cells in mediating the protective adaptive response to infection. In immunocompromised hosts that lack CD4⁺ T-cells and the absence of T-cell priming by innate immune cells leaves the host vulnerable to opportunistic fungal infections such as *Pneumocystis*. Previous work has sought to determine the mechanism of T-cell-mediated protection and have contributed to understanding the role T-cell priming plays in response to *Pneumocystis*.

Pneumocystis has been shown to induce Th1, Th2, Th17, or T regulatory responses, none of which have been conclusively associated with disease resolution (Figure 2). Evidence supporting the role of Th1 is shown in patients receiving anti-TNF therapy (Infliximab), in which PCP is the most frequent fungal infection. In rats, treatment with aerosolized IFN γ , which promotes a Th1 response and the production of TNF- α by macrophages reduces the intensity of *P. carinii* infection [36]. Furthermore, immunizing macaques with the recombinant subunit of the *P. jirovecii* protease kexin (KEX1) correlated with protection from *P. jirovecii* colonization and pneumonia and was associated with an increase in the frequency of peripheral blood Th1 cells [25]. However, while STAT4^{-/-} BALB/c mice were susceptible to infection, IFN- γ ^{-/-} and Tbx21^{-/-} mice maintain their ability to clear *P. murina* [37], disputing the role of Th1 in controlling infection. Th17 responses have been linked to mediating the host response to *Pneumocystis*, however the exact mechanism required for clearance remains to be determined. Mice deficient in IL-23p19, lack Th17 immunity and have a delayed clearance of *P. murina* but ultimately clear the infection [38], suggesting a role for compensatory mechanisms.

For Th2, IL-5 and recruited eosinophils were shown to play a role in enhancing clearance [39]. Here RAG-1-deficient mice treated with IL-5 presented with enhanced *P. murina* clearance. In an immunocompromised host, components of a Th2-driven immune response have proven to be beneficial. In contrast, Th2 responses to *Pneumocystis* drive inflammation and asthma-like pathology in healthy individuals comparable to that of the common allergen, house dust mite [9]. Therefore, a consequence of chronic exposure to *Pneumocystis* could indeed be asthma. A recent study has also linked the *Pneumocystis*-driven Th2 response to

inducible bronchus associated lymphoid tissue (iBALT) [40]. iBALT, is a lymphoid structure which forms in response to infectious stimuli and often associated with chronic diseases, such as rheumatoid arthritis, tuberculosis and chronic obstructive pulmonary disease (COPD). Here, the Th2 role was combined with a Th17 response as dual stimulation of IL-13 and IL-17A was required for the development of pulmonary lymphoid follicles. These two studies provide evidence for the ability of *Pneumocystis* to drive an exaggerated inflammatory response and pathology in healthy individuals.

Currently, there is limited data describing the T-cell intrinsic requirements during *Pneumocystis* clearance. Previous work has suggested the role of STAT4 [37] and as mentioned above there are case reports of disease in patients with mutations in STAT3 [12] and IL21R [13]. Considering the apparent dispensable contribution of Th1, Th2 and Th17 in *Pneumocystis* clearance, it is likely that multiple intrinsic pathways are required for clearance. Experiments targeting a combination of multiple pathways using gene-deficient mice could provide valuable insight into the mechanism. Taken together, the above evidence warrants further investigation into the mechanism of CD4⁺ T-cell intrinsic pathways which contribute to *Pneumocystis* clearance.

Conclusion

Significant progress has been made in understanding how the host responds to and clears *Pneumocystis*, however key basic information about the organism and disease are lacking. The reservoir of infection has not yet been identified and the mechanism of infection is poorly understood. Progress on the host immune response includes the fact that *Pneumocystis* seems to induce multiple T-cell-mediated responses including Th1, Th2 and Th17 however individually, these appear to be dispensable. This suggests that the T-cell intrinsic pathway is likely to include multiple components leading to a mixed cytokine milieu with compensatory mechanisms in gene-deficient animals potentially able to drive clearance. Future work in dissecting this pathway would significantly progress the field. Interestingly, the trophic form of *Pneumocystis* was shown to actively suppress the pro-inflammatory response initiated by the ascus form. This reduces the effectiveness of host defence and could create an environment to promote active disease from colonizing organisms in immunocompromised patients. Further studies investigating the effect of the life stage form in immunocompromised patients would be valuable in establishing an appropriate treatment strategy. In contrast and considering the high frequency of exposure to *Pneumocystis*, an interesting concept is that *Pneumocystis* is a potent allergen capable of inducing asthma, similar to house dust mite and that *Pneumocystis* is linked to COPD severity. Perhaps therapy targeting *Pneumocystis* could prove to be a promising treatment strategy in these patients. Lastly, one of the biggest limiting factors in developing new drugs against *Pneumocystis* remains, the lack of a continuous *in vitro* culture system, a task challenging researchers for decades. Identifying such a system would provide essential tools to determine drug susceptibility.

Acknowledgments

This work was supported in part by the Medical Research Council (RSA). JCH is supported by a Carnegie Corporation DEAL fellowship and the National Research Foundation of South Africa. JKK is supported by the

following PHS research grants: R01 HL-062052 and R01 AI-120033. We apologize to all our colleagues whose important contributions we could not cite due to space constraints.

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Highlights

- Trends for increased *Pneumocystis jirovecii* prevalence in developed countries
- Host mutations and susceptibility to *pneumocystis* infection
- *Pneumocystis*, a common colonizing organism with the potential to induce asthma

Figure 1. New developments in the host response to *Pneumocystis*

Recent human and animal studies have provided key information about the host's immune response to *Pneumocystis* exposure. T cell priming by APCs is an essential part in the immune response to infection. Priming is affected by the form of *Pneumocystis*. T helper cells are activated including Th1 and Th17 but are dispensable in clearing infection. Th2 responses to *Pneumocystis* drive an allergic reaction in healthy hosts. APCs = antigen presenting cells, Th = T helper, M1 = classically activated macrophages, M2 = alternatively activated macrophages [9, 10, 32, 34, 39].

Figure 2. CD4⁺ T helper cell responses to *Pneumocystis*

CD4⁺ T cells are essential in controlling *Pneumocystis* infection. The mechanisms by which these cells contribute to *Pneumocystis* clearance remains to be defined. Previous studies have demonstrated various T helper cell responses to infection, however many of these responses appear to be dispensable or compensated for by other mechanisms when deleted. Here we summarise some of the known CD4⁺ T cell mediated responses to *Pneumocystis*. APC - antigen presenting cell; Th - T helper; T reg - regulatory T cell [13, 37, 38, 41–46].

Table I

List of human mutations and orthologous genetic knockout mice and susceptibility to *Pneumocystis* infection. TBD- to be determined.

Symbol	Gene	Humans	Mice
Rag1	Recombination activating gene 1	+	+
Rag2	Recombination activating gene 2	+	+
Cd40/Cd40L	Cluster of differentiation 40/ligand	+	+
Prkdc	Protein kinase, DNA-activated, catalytic polypeptide	+	+
Il2rg	Interleukin-2 receptor subunit gamma	+	+
Cd4	Cluster of differentiation 4	+	+
Hla/MhcII	Human leukocyte antigen/major histocompatibility complex class II	+	+
Nemo	NF-kappa-B essential modulator	+	TBD
Icos	Inducible T-Cell co-stimulator	+	TBD
Btk	Bruton tyrosine kinase	+/-	TBD
Il21r	Interleukin-21 receptor	+	+
Stat3	Signal transducer and activator of transcription 3	+/-	+
Rorc	RAR related orphan receptor C	-	-
Il17ra/rc	Interleukin-17 receptor A/C	-	-