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BOOK OF ABSTRACTS

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POSTERS

TALKS (the author in italic is presenting)

T 01 **Demystifying the NIH grant application process for international investigators**

Dr. Dona Love [1]

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The National Institute of Allergy and Infectious Diseases (NIAID) funds one of the largest medical mycology research portfolios. The portfolio includes the major human fungal pathogens, including microsporidia and Pneumocystis spp., and covers basic fungal biology and translational areas of therapeutics, vaccines, and diagnostics. NIAID utilizes many granting mechanisms that are open to US and international researchers. These include investigator-initiated applications (R01, R21, and R03s) and targeted announcements for fungal research. Additionally, NIAID has a suite of preclinical services supporting therapeutic, diagnostic and vaccine development. These services are free and available to investigators in academia, not-for-profit organizations, industry, or governments worldwide. The NIAID granting mechanism can be complicated. Tips and tricks for navigating the NIAID application process and preclinical services will be discussed.

T 02 Centromere evolution during fungal pathogens *Pneumocystis* adaption to mammals

Dr. Ousmane Cisse [1], **Dr. Shelly Curran** [1], **Dr. H. Diego Folco** [2], **Dr. Yueqin Liu** [2], **Ms. Lisa Bishop** [1], **Dr. Honghui Wang** [1], **Dr. Elizabeth Fischer** [3], **Dr. A. Sally Davis** [4], **Ms. Spenser Babb-Biernacki** [5], **Dr. Vinson Doyle** [5], **Dr. Jonathan Richards** [5], **Dr. Sergio Hassan** [3], **Dr. John Dekker** [3], **Dr. Pavel Khil** [3], **Dr. Jason Brenchley** [3], **Dr. Shiv Grewal** [2], **Prof. Melanie Cushion** [6], **Dr. Liang Ma** [1], **Dr. Joseph A Kovacs** [1]

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Pneumocystis species offer a suitable genetic system to study centromere evolution in pathogens during host adaptation because of their unique specificity for mammals, and their phylogenetic proximity with the nonpathogenic yeast *Schizosaccharomyces pombe*, a popular model for cell biology. We used this system to explore how centromeres have evolved after divergence of the two clades ~460 million years ago. To address this question, we established a protocol combining short-term culture and ChIP-seq to characterize centromeres in multiple *Pneumocystis* species. We show that *Pneumocystis* have short epigenetic centromeres that function differently from those in *S. pombe* and exhibit similarities to centromeres in more distantly related host adapted fungal pathogens.

T 03* Genome analysis of Microsporidia MB: a malaria-transmissionblocking symbiont of the *Anopheles mosquito*

Ms. Lilian Ang'ang'o [1], **Dr. Jeremy Herren** [2], **Prof. Ozlem Tastan Bishop** [1], **Mr. Edward Makhulu** [3], **Ms. Jacqueline Wahura** [3], **Ms. Anne Wairimu** [3], **Dr. Thomas Onchuru** [2]

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Microsporidia are a unique set of fungi-like microorganisms found in very diverse environments. Moreover, symbiotic microsporidians are often involved in host protection against other pathogens through immune priming, competition for nutrients, and increased host development rate. A maternally transmitted microsporidian species, *Microsporidia MB, was* previously shown to completely block malaria development in the mosquito and is, therefore, a promising prospective candidate for malaria control. This study, therefore, aimed to provide insights into the genetic makeup of *MB* and investigate its genome content to further understand its biology of infection. This important element will inform its future application in field studies.
The genome of *Microsporidia MB* was sequenced from pooled infected *Anopheles arabiensis* ovaries using short paired-end Illumina sequencing. Through the *de novo* assembly of this genome, we identified that *MB* has a relatively average size of 13 Mb and a total of 1374 predicted genes. Notably, the bulk of the predicted genes were found to be of regulatory and transport function. In addition, we found that the genome of *MB* is phylogenetically closely related to *Vittaforma corneae* and other clade IV microsporidians, separate from the mosquito-microsporidians. Overall, our study highlights the first assembly of the *Microsporidia MB* genome and comparatively characterizes its gene content to other known microsporidians. The results identified here could be used in future studies of strain variation among samples from different regions and host-symbiont interactions to further understand the exact blocking mechanism of this unique microorganism.

*Student presentation

T 04 Diversity and phylogeny of mitochondrial genomes of *Pneumocystis* species from 11 mammalian species

Dr. Liang Ma [1], **Dr. Li Peng** [2], **Ms. Regan Halverson** [3], **Ms. Isabella Lin** [3], **Dr. Shelly Curran** [3], **Dr. Jason Brenchley** [4], **Dr. Patrizia Danesi** [5], **Dr. A. Sally Davis** [6], **Dr. Evan Dessasau** [7], **Ms. Summer Hunter** [8], **Dr. Jamie Rothenburge** [8], **Dr. Antti Sukura** [9], **Dr. Terry Wright** [10], **Prof. Melanie Cushion** [11], **Dr. Francis Gigliotti** [10], **Dr. Sanjeeev Gumber** [7], **Dr. Ousmane Cisse** [3], **Dr. Joseph A Kovacs** [3]

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Background
Accurate elucidation of genetic diversity and phylogeny of *Pneumocystis* is challenging due to difficulties in obtaining high-quality DNA samples for extensive genetic analysis, particularly whole nuclear genome sequencing. Mitochondrial large- and small-subunit rRNA genes have been widely used to identify and analyze genetic diversity and phylogeny of *Pneumocystis* owing to their multiple copies per cell, allowing easier PCR amplication than nuclear genes. The value of the complete mitochondrial genome (mtDNA) for such analysis remains unclear. We aimed to examine the diversity and phylogeny of *Pneumocystis* through comparative mtDNA analysis.

Methods
Complete or nearly complete mtDNA sequences were obtained by next-generation sequencing of total genomic DNA or PCR products of *Pneumocystis* samples from 6 different mammalian species including the prairie dog, squirrel, rat, hare, ferret and chimpanzee. mtDNA assembly, annotation and comparative analysis (including previously reported *Pneumocystis* mitogenomes from 6 additional mammalian species) were performed using our in-house bioinformactic pipeline.

Results
mtDNA of primate *Pneumocystis* share an identical number and order of genes in a circular configuration while all the remaining species have a linear configuration with the same set of genes but in different order compared to primate *Pneumocystis.* G+C content varies between 25.5-33.3%. Noncoding regions vary between 8.6-55.2%. The entire coding regions (~18kb) exhibt a nucleotide divergence ranging from 15.9% between *Pneumocystis* from rabbits and hares to 35.4% between *P. jirovecii* and *P. carinii*. Phylogeny inferred from mtDNA is generally consistent with that inferred from nuclear genome for species with both mtDNA and nuclear genomes available.

Conclusions
Pneumocystis
mtDNA from different animal species has remarkable diversity in sequence but minimum variation in gene organization except for the distinct rearrangement in gene order and structural configuration between primate and non-primate *Pneumocystis*. Our findings support mtDNA as a valuable complementary tool for diversity and phylogenetic analysis of the genus *Pneumocystis.*

T 05 Genetic polymorphisms and systemic inflammatory response in patients with chronic obstructive pulmonary disease (COPD) colonized by *Pneumocystis jirovecii*

Mrs. Rocio Salsoso[1], Dr. Vicente Friaza[1], Ms. Sara Martínez-Rodriguez[1], Dr. Carmen de la Horra[1], Ms. Mar Navarro[1], Prof. Ruben Morilla[1], Mr. Luis Giménez-Miranda[2], Prof. Francisco J. Medrano[1], *Prof. Enrique Calderon*[2]

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Objectives
The inflammatory response plays an important role in the pathogenesis of COPD. Our group has previously demonstrated that colonization by *Pneumocystis jirovecii* in patients with COPD produces an increase in systemic levels of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). On the other hand, polymorphisms have been identified in the genes that encode these cytokines related to their production capacity. This study aims to clarify whether the systemic response of IL-6 and TNF-α associated to *P. jirovecii* colonization in patients with COPD is conditioned by genetic factors.

Methods
48 unselected patients diagnosed with COPD (GOLD criteria) were included. Serum and respiratory samples (sputum/ oropharyngeal washing) were obtained in all cases. Identification of *P. jirovecii* was performed by real-time PCR (mtLSU-rRNA, region). Identification of the IL-6 and TNF genetic polymorphisms was performed by PCR and subsequent Restriction Fragment Length Polymorphism (RFLP) using protocols developed in our laboratory. IL-6 and was assessed by commercial EIA.

Results
The subjects studied were 46 men and 2 women, mean age of 71 ± 9.6 years, 81.3% smokers. The mean serum levels of IL-6 (pg/ml) and TNF (pg/ml) were significantly higher in colonized when comparing with non-colonized patients: 14.5 vs. 8.5 (p= 0.002) and 13.1 vs. 4.3 (p=0.05), respectively. There were no association between the *P. jirovecii* colonized status and IL-6 (GG, GC, CC) and TNF- α (GG, GA and AA) polymorphisms.

Conclusions
This study confirms that colonization by *P. jirovecii* increases the systemic response of IL-6 and TNF in patients with COPD and that the response is not related to the genetic polymorphisms of these cytokines.
The study was supported by the PAIDI, Consejería de Economía Conocimiento, Empresas y Universidad de la Junta de Andalucía (grant number PS20_00894).

T 06 Antibody-mediated modulation of *Pneumocystis* host response is dependent on specific Fc receptor effector functions

***Prof. Andrew Limper*[1], Dr. Ted Kottom, Dr. Eva Carmona[1]**

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Background

Pneumocystis jirovecii pneumonia (PJP) remains a significant cause of mortality during AIDS. In AIDS, the absence of CD4 lymphocytic immunity results in severe PJP. In addition to CD4 cells, organism clearance requires balanced macrophage responses since excessive inflammation promotes lung injury. New strategies to promote killing and clearance of *Pneumocystis* while balancing lung inflammation are required for patients with PJP who are refractory to current therapies. Our studies show that innate immunity to *Pneumocystis* is mediated by C-Type Lectin Receptors (CLRs) on macrophages and involves CARD9 activation. Additional data support roles for antibody mediated organism clearance. We therefore sought to determine the respective roles of CLR mediated innate immunity and antibody mediated humoral immunity in host responses during PCP.

Methods

We obtained mice with targeted defects in innate and humoral immune responses. We studied CARD9 deficient mice (impaired CLR function), as well as double knockout CARD9/FcγR mice (deficient in both CLR and antibody mediated functions). Murine pneumocystis models studied included CD-4 depleted mice with *P. murina* pneumonia. Organism burdens were determined by silver staining (GMS) and qPCR.

Results

We demonstrate that CARD9 and FcγR act together contributing to the macrophage immune response and both are important in organism control during PCP. Analysis by qPCR and GMS staining demonstrate significantly greater organism burden in double knockout CARD9/FcγR mice compared to CARD9-/- single KO mice alone. Strikingly, we observed that mice deficient in both Dectin-1 and Fcer1g (lacking the FcγR gamma chain) demonstrated markedly reduced organism clearance compared to *Card9-/-* infected animals. These mice possess deficiencies in immunoglobulin (Ig) Fc receptors directly mediating antibody responses, implicating humoral responses in *Pneumocystis* clearance.

Conclusions

Our data demonstrate that innate immune responses through the CLR-CARD9 axis and humoral response act together to mediate effective responses resulting in optimal organism killing and host inflammatory responses.

T 07 On the tolerance to labile copper by *Pneumocystis murina*

Dr. Alexey Porollo [1], **Mrs. Sandra Rebholz** [1], **Mr. Steven Sayson** [2], **Mr. Alan Ashbaugh** [2], **Prof. Melanie Cushion** [2]

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Pneumocystis species are pathogenic fungi, which are extracellular obligate organisms adapted to live in mammalian lungs. As part of innate immunity, mammals protect their lungs from invading microbes by secreting high levels of free labile copper. While being cytotoxic at elevated levels for most microorganisms, the excess of Cu does not appear to affect *Pneumocystis*. In this work, we challenged the *Pneumocystis murina* (mouse infecting species) organisms with CuSO4 to identify genes over-expressed upon Cu insult using RNA-seq. The Cu-induced expression of select genes (PNEG_00014, PNEG_01009, PNEG_01236, PNEG_01350, PNEG_01675, PNEG_01730, PNEG_02322, and PNEG_02609) has been validated by qPCR.
Cytotoxic effect by free labile copper is in part due to redox reactions that generate reactive oxygen species (ROS). Generation of the highly reactive hydroxyl radicals (HO·) in the presence of free Cu ions can cause oxidative stress by attacking cellular components and disrupting their normal function, leading to cell death or dysfunction. Glutathione (GSH) plays a major cellular antioxidant role in most phyla. Consequently, we conducted viability assays (ATP content by bioluminescence) with GSH inhibitors: buthionine sulphoximine (BSO) and methionine sulfoximine (MSO) that deplete intracellular levels of GSH by inhibiting γ-glutamylcysteine synthetase and glutamine synthetase respectively. Results showed that 1uM of CuSO4 reduced ATP by 50% vs untreated controls after 3 days of exposure; BSO at 4 concentrations ranging from 2.5mM to 0.1mM consistently reduced the ATP levels by ~30%; and MSO at 25-50- and 75uM reduced ATP by ~36%. These inhibitors will be used in combination to evaluate the potentiating effect of the GSH inhibitors and copper.

T 09 Effect of roflumilast on the immune response induced by *Pneumocystis* colonization in COPD patients

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Introduction
Our group has shown that colonization by *Pneumocystis jirovecii* in patients with COPD produces an increase in the systemic inflammatory response. Roflumilast is a phosphodiesterase 4 (PDE4) inhibitor with anti-inflammatory properties that is used in patients with COPD and exacerbations. However, it remains speculative whether roflumilast can reduce systemic inflammation in COPD patients. The objective of this study was to verify the effect of Roflumilast on the immune response induced by *Pneumocystis* in COPD patients.

Methods
We studied 24 patients aged 69.9 ± 7.8 years with severe COPD who started treatment with 500 mg of roflumilast once daily. Serum samples were collected before and after 12 weeks of treatment. Immune response markers were studied in serum using a multiplex ELISA matrix (SearchLight, Billerica, MA, USA). The presence of *P. jirovecii* was determined in sputum samples by specific PCR. For statistical analysis, the Wilcoxon test for paired samples was used.

Results
Roflumilast significantly reduced interferon gamma, IL1b, IL6, IL-8, and TNF levels in patients with COPD. Of the subjects included in the study, 10 were colonized by *P. jirovecii* at the beginning of the treatment and in them the levels of interferon gamma, IL1b, IL6, IL-8 and TNF decreased significantly [3.8 *vs* 1.08 pg/ml (p=0.038); 1.3 *vs*. 0.3 pg/ml (p=0.015); 20.4 *vs*. 9.1 pg/ml (p=0.005); 23.3 *vs*. 16.5 pg/ml p=0.017); 5.1 *vs* 1.7 pg/ml (p=0.012)], while in non-colonized subjects only gamma interferon decreased significantly (9.2 vs 1.4 pg/ml; p=0.011) and TNF (11.3 vs 5.7 pg/ml; p=0.001).

Conclusions
Roflumilast has a greater effect in reducing the innate immune response and IL8 levels in COPD patients who are colonized with *P. jirovecii* than in those who are not, suggesting that *Pneumocystis* may be a specific cause of activation of the innate immunity in subjects with COPD.

T 10 The significance of the WNT/β-catenin signaling pathway in *Pneumocystis* pneumonia

Dr. Eva Carmona [1], **Dr. Ted Kottom** [1], **Prof. Andrew Limper** [1]

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Background
Immune mechanisms against *Pneumocystis*, are in part mediated by myeloid cells, including alveolar macrophages (AMs). Pneumocystis jirovecii pneumonia results in an exuberant host proinflammatory response, attributable to the organism's abundant cell wall β-glucans. AMs promote clearance of organisms but are also major sources of proinflammatory mediators contributing to the profound pulmonary inflammation during *Pneumocystis* pneumonia (PCP). Little is known currently about the mechanisms modulating AM proliferative and inflammatory fate decisions *in vivo*, and the downstream effects on tissue inflammation and recovery following PCP. In this study, we hypothesize that the Wnt/β-catenin pathway has novel immune functions that help modulate host AM inflammatory activity and organism killing and burden control in the PCP lung.

Methods
We studied mice with targeted defects in β-catenin (*Ctnnb1-/-* β-catenin deficient) compared to wildtype mice. Murine pneumocystis models studied included CD-4 depleted mice (GK 1.5 Ab), infected with *P. murina* pneumonia. Organism burdens were determined both by silver staining (GMS) and by specific qPCR assays. In addition, inflammatory responses for TNFa, IL-6 and IL-1b were determined by ELISA and by H&E staining.

Results
Analysis by qPCR and GMS staining demonstrate significantly greater organism burden in *Ctnnb1-/-* mice as well as enhanced inflammation as measured by proinflammatory TNFα ELISA and H&E staining compared to controls. Finally, staining of *Pneumocystis jirovecii*-infected human lung samples noted decreased expression in these samples as compared to healthy controls. Findings of the Wnt/β-catenin pathway importance in the AM host inflammatory response and organism burden control in PCP in the lung represents a newly emerging mechanism modulating host defense during infection.

Conclusions
Data presented here initially evaluated the role of Wnt/β-catenin pathway in *Pneumocystis* pneumonia (PCP) lung inflammation and organism killing. These observations seek to inform novel therapeutic approaches for PCP, in patients who are refractory or fail standard antibiotic therapy.

T 11* **Inflammasome activation and neutrophil extracellular traps during *Pneumocystis* pneumonia**

Mr. Steven Sayson [1], **Mr. Alan Ashbaugh** [1], **Prof. Melanie Cushion** [2]

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Pneumocystis spp. are host-obligate fungal pathogens that reside in the lung alveoli of mammalian hosts. These fungi cause disease in immunocompromised individuals and can cause a lethal pneumonia called *Pneumocystis jirovecii* pneumonia (PjP). While PjP remains the most common opportunistic infection in patients with HIV/AIDS, incidence of PjP has risen in association with increased use of immunosuppressive therapies for malignancies. PjP elicits profound inflammation, leading to considerable morbidity and mortality within these patients.

The NLRP3 inflammasome is a multimeric protein complex of the innate immune system that is activated in response to microbes and pathogen-associated molecular patterns These inflammasomes activate caspase-1 for the maturation of the pro-inflammatory cytokine, IL-1β. The pore forming protein, gasdermin D, facilitates the release of IL-1β to further propagate a proinflammatory response. However, uncontrolled NLRP3 inflammasome activation drives excess inflammation and disease.

The purpose of this study was to understand of the contribution of neutrophils, inflammasome activation, and NETosis during the inflammatory response in *Pneumocystis* pneumonia. Immunosuppressed mice were cohoused with *Pneumocystis murina* (Pm)-infected mice. Uninfected control and Pm-infected mice were then sacrificed after 5- and 7- weeks to correspond to moderate and high infections, respectively (n = 6). Lungs were collected to quantify organism burden and for RNA isolation.

RNAseq revealed increased expression of NLRP3 inflammasome-related genes within the lungs of Pm-infected mice. Furthermore, increased expression of peptidylarginine deiminase 4 and histones suggest NETosis, a regulated cell death that releases extracellular DNA with citrullinated histones to ensnare and kill pathogens. Preliminary microscopic studies demonstrate the expression of neutrophil elastase and citrullinated histone H3 within the lungs of Pm-infected mice. Contradictory to previous reports, these data suggest that neutrophils and neutrophil extracellular traps (NETs) may be produced during PmP and contribute to the inflammatory damage seen within the lungs.

* Student presentation

T 12 *Pneumocystis jirovecii* and systemic inflammatory response in patients with chronic obstructive pulmonary disease (COPD)

Ms. Sonia Gutierrez [1], Dr. Carmen de la Horra [2], *Prof. Enrique Calderon [1],* Prof. Ruben Morilla [2], Ms. Mar Navarro [2], Ms. Sara Martínez-Rodriguez [2], Dr. Juan Delgado [2], Prof. Francisco J. Medrano [2]

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Objectives
(i) To know the dynamics of Pneumocystis jirovecii colonization in stable COPD patients; (ii) To evaluate the effect of clearing the colonization on the systemic inflammatory response in patients with COPD.

Methods
Prospective cohort study with quarterly visits and one-year follow-up in which 53 patients with a previous diagnosis of COPD were included consecutively after informed consent. Those with other concomitant lung disease, neoplasia, immunosuppression and/or COPD exacerbation in the previous month were excluded. Infection by respiratory pathogens was ruled out using conventional microbiological techniques. At each visit, clinical-biological characteristics were evaluated. Identification of *P. jirovecii* was performed by real-time PCR (mtLSU-rRNA, region) in sputum samples. Serum levels of proinflammatory cytokines and surfactant proteins (SP-A and SP-D) in serum were assessed by EIA.

Results
Of the 53 included patients, 94.4% (50/53) were men, mean age of 71±9.5 years, 86.8% ex-smokers, 41,5% in GOLD stage IV. At the beginning 41.5% were colonized by *P. jirovecii*; 14 (63.3%) of these 22 cleared the infection during follow-up. Subjects colonized by P. jirovecii presented higher levels of IL-6, IL-8 and MCP-1 ($p<0.001$). During the follow-up, 4 patterns of colonization were observed: (i) clearance (n=14); (ii) new colonizations (n=3); (iii) persistent negatives (n=28); and (iv) persistent positives (n=8). The comparative results of the observed patterns will be present in the meeting.

Conclusions
(i) In our environment, more than 40% of patients with stable COPD are colonized by *P. jirovecii*, although in half of the cases clears up the infection over time; (ii) Colonization is associated with increased systemic inflammatory activity; (iii) The inflammatory activation appears after colonization, but does not seem to revert after clearance, since cytokines remain elevated after clearance.
The study was supported by the PAIDI, Consejería de Economía Conocimiento, Empresas y Universidad de la Junta de Andalucía (grant number PS20_00894).

T 13 **In vitro studies of *Pneumocystis* using Precision Cut Lung Slices**

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Pneumocystis pneumonia remains the number one cause of fungal pneumonia in infancy and is a common infection in immunocompromised individuals. Research in the field has been hampered by the inability to culture this fungus leading to a delay in obtaining genomic/transcriptomic information on fungal life cycle, mechanisms of fungal attachment as well as a lack of in vitro antibiotic susceptibility. When placed in media, *Pneumocystis murina* dies within hours. However we have preliminary data that with *P. murina* is placed in culture lung tissue form precision cut lung slices (PCLS) that both the troph and ascus can survive up to 14 days. Moreover, we have observed evidence of fungal biofilms in this model. Additional preliminary data show that this model can be used for in vitro antibiotic susceptibility for both trimethoprim-sulfamethoxazole and echinocandins. Thus this model appears to be useful for in vitro anti-fungal testing as well to study fungal attachment and fungal clearance mediated by effector cells such as macrophages.

T 14* **Single cell RNA sequencing of the *Pneumocystis carinii* life cycle stages**

Mr. Aaron Albee [1], **Dr. Alexey Porollo** [2], **Mr. Steven Sayson** [1], **Mr. Alan Ashbaugh** [1], **Dr. Melanie Cushion** [1]

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Pneumocystis spp. (species) are host obligate fungal pathogens which can result in severe pneumonia for immunocompromised humans and other mammals. Infections resulting in Pneumocystis pneumonia have historically been related to chronic immunosuppressive diseases, in particular HIV/AIDS. More recently, patients with malignancies were identified as those with the greatest case numbers *Pneumocystis jirovecii* pneumonia. Research of these pathogens has been hampered by the lack of an *in vitro* culture system capable of continuously growing *Pneumocystis* spp. While a sexual cycle including mating forms and production of the apparent transmissive agent, the ascus, has been hypothesized, the field has been unable to investigate how individual stages develop and their related gene expression profiles. To overcome this problem, we developed a protocol to generate a viable single cell suspension of *Pneumocystis carinii* (Pc) which was examined by single cell RNA sequencing.
Using a method of gradient separation, Pc populations of trophic and ascus stages obtained from bronchial alveolar lavage fluid of infected rats were enriched, while host cell contamination and cell aggregation were reduced. The samples were successfully sequenced using the 10X Chromium X single cell 3' low throughput over two pilot studies. Improvements were shown in both the depth of sequencing and the quality of the transcriptome in the second pilot. These samples were able to be clustered by their differential expressions of genes with some specific to developmental stages such as meiosis, mitosis, and sporulation. A trajectory analysis of the second pilot suggests a potential order within the clusters.
The data from these pilot studies will now be used to optimize the sequencing conditions for greater transcriptomic coverage of the gene expression related to mating and ascus development. These data can be used to develop biomarkers to identify specific stages of development and strategies to improve treatment by targeting specific stages.

*Student presentation

T 15 **Studies on the composition of the *Encephalitozoon hellem* polar tube**

Ms. Elizabeth Weyer[1], Dr. Bing Han[2], Dr. Rebekah Guevara[1], Dr. Tadakimi Tomita[1], *Dr. Louis Weiss*[13]

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Microsporidia are obligate intracellular, unicellular, spore-forming eukaryotes related to the Cryptomycota. An important and characteristic feature of all microsporidia is the presence of an extrusion apparatus consisting of a polar tube (which is termed the polar filament when is it within the spore) that coils around the sporoplasm in the spore and is that attached to the anterior end of the spore by the anchoring disk. When exposed to specific environmental conditions, such as passage though the host's gastrointestinal track, spore germination occurs, and the polar tube rapidly everts forming a hollow tube that acts as a conduit to deliver the sporoplasm to the host cell. The formation and composition of the polar tube as well as the mechanism of invasion remain active areas of scientific investigation. Currently, there are six polar tube proteins (EhPTP1 to EhPTP6) which have been identified in *Encephalitozoon hellem*. We have been utilizing proteomic approaches to further define the composition of the polar tube and to identify previously unrecognized PTPs and spore wall proteins (SWPs). This approach is ongoing but has resulted in the identification of a new PTP (EhPTP7) for *E. hellem* and a new *E. hellem* SWP (EhSWP6). We are also optimizing purification methods for polar tubes. In addition, we have been using cross linking mass spectrometry approaches (XL-MS) to define protein-protein interactions among the various PTPs. Preliminary results are promising, and this procedure is being optimized. In addition, we have examined PTP interactions using yeast two hybrid methods. [Supported by NIH AI124753].

T 16 Structural cell biology of Microsporidia using cryo-ET and volume EM

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Microsporidia are highly diverged from commonly studied model cells such as yeast, and possess a rich diversity of unusual organelles. Perhaps the most famous of these organelles in the polar tube, a harpoon-like projectile that mediates host cell invasion. However, many other fascinating structures have been described in these parasites, including complex membranous stacks, such as the polaroplast. How the polar tube is built, and how the polar tube and other organelles are organized within the parasite has been challenging to understand in 3D, because most imaging methods yield 2D information (e.g., imaging thin sections by TEM) or have limited spatial resolution (e.g., confocal microscopy). Recent developments in 3D imaging, including cryo electron tomography (cryo-ET) and serial block face scanning electron microscopy (SBF-SEM), have enabled the reconstruction of cells and sub-cellular structures in 3D at much higher resolution. Using these imaging approaches, we have begun to decipher the 3D organization of the polar tube and other organelles in the microsporidian parasite, *Encephalitozoon intestinalis*, as well as investigate the interactions between the parasite and the host cell. In parallel, we are using scRNA-seq to profile the transcriptional landscape of the parasite replicating within human host cells, gaining insight into both the parasite life cycle as well as host responses to infection. I will present our recent work in these areas, which are beginning to provide insights into polar tube assembly and structure, as well as the underlying transcriptional program that coordinates the complex processes of polar tube production and parasite maturation.

T 17 Multilocus genetic diversity of distinct *Pneumocystis spp.* Variants in pigs from Austrian farms

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Pneumocystis spp. in pigs (referred to as *P. suis*) has been described since the late 1950s. However, its epidemiology remains poorly understood. The few studies available show varying prevalence of this fungus, between 6.5 and 51%. Yet, there is a lack of information about genotypes of *P. suis* and their genetic diversity.
Starting from 2018, we have collected 502 pig lung samples from Austrian farms and screened them via *in situ* hybridization (ISH) targeting the 18S rRNA gene of *Pneumocystis* spp. We identified a total of 156 positive samples (31%). In most of them, *Pneumocystis* was present with a low organism load (score 1, n=100), followed by score 2 (n= 41) and 3 (n=15). A multilocus sequence typing (MLST) system was used to characterize a cohort of 52 samples (ISH score 2 and 3). Consistent with preliminary next generation sequencing data and previous literature showing the existence of two variants of *P. suis*, we observed the co-infection with both variants in all these samples.
The loci used for the scheme are mitochondrial small and large subunits (mtSSU and mtLSU), rRNA operon including internal transcribed spacers and 5.8S (ITS1-5.8S-ITS2), dihydropteroate synthase (*dhps*), dihydrofolate reductase (*dhfr*), and cytochrome B (*cob*). Different loci showed varying amplification rate and typeability. The two main variants showed substantial nucleotide divergence (10-20%) in all six loci. Additionally, the level of allelic polymorphisms for each variant also differed among the six loci, with *dhfr* gene showing the highest number of subvariants.
Our MLST analysis confirms the presence of two distinct variants of *P. suis*, which may represent two separate species of *Pneumocystis* in pigs as recently hypothesized, and also reveals substantial polymorphisms within both variants. To our knowledge, this is the first multilocus genotyping study of *P. suis*.

T 19* ***Pneumocystis jirovecii*** **antigenic variation uses reassortment of subtelomeric genes' repertoires**

Mrs. Caroline Meier [1], **Dr. Marco Pagni** [2], **Ms. Sophie Richard** [1], **Dr. Konrad Mühlethaler** [3], **Dr. Joao Almeida** [4], **Prof. Gilles Nevez** [5], **Prof. Melanie Cushion** [6], **Prof. Enrique Calderon** [7], **Dr. Philippe Hauser** [1]

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Surface antigenic variation is crucial for major pathogens that infect humans, e.g. *Plasmodium*, *Trypanosoma*, *Giardia*. In order to escape the immune system, they exploit various mechanisms in order to modify or exchange the protein that is exposed on the cell surface, at the genetic, expressional, and/or epigenetic level. Understanding these mechanisms is important to better prevent and fight the deadly diseases caused. However, those used by the fungus *Pneumocystis jirovecii* that causes life-threatening pneumonia in immunocompromised individuals remain poorly understood. Here, though this fungus is currently not cultivable, our detailed analysis of the subtelomeric sequence motifs and genes encoding six families of major surface glycoproteins suggest that the system involves homologous recombinations during meiosis. This leads to the reassortment of the repertoire of ca. 80 non-expressed genes of family I present in each strain, from which single genes are retrieved for mutually exclusive expression within subpopulations of cells. The recombinations generates also constantly new mosaic genes. Dispersion of the new alleles and repertoires, supposedly by immunocompetent carrier individuals, appears very efficient because identical alleles are observed in patients from all over the world. Our observations suggest a unique strategy of antigenic variation allowing colonization of the non-sterile niche corresponding to lungs of immunocompetent humans.

*Student presentation

T 20 *Pneumocystis jirovecii* antigenic variation may be mediated by DNA triplexes

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Like other major microbial pathogens, *Pneumocystis jirovecii* possesses a system to vary its cell surface that is believed to allow escaping the human immunity. This system would rely on repertoires reassortment, mutually exclusive expression, and mosaicism of the subtelomeric genes encoding six families of major surface glycoproteins (Meier et al. submitted). We analysed the similarities and motifs present around these genes within a set of subtelomeres of a single strain. The 33 bps conserved at the beginning of each of the ca. 80 genes of family I are presumably the site where a recombination occurs and exchanges the single allele expressed. It proved to be a polypurine-polypyrimidine stretch including an imperfect mirror repeat that could form a DNA triplex. Such mirror sequences are widespread in eukaryotic genomes, but remain poorly understood so far. Nevertheless, circumstantial evidence suggests that they are involved in a number of genetic processes such as transcription, replication, rearrangements, translocations, and homologous recombination. Mediation of homologous recombination is the function that was most recurrently mentioned because mirror repeats have often been reported close to recombination sites. We propose a model of the mechanisms involved in the antigenic variation system of *P. jirovecii* including mediation by DNA triplexes.

T 21 Determination of the mitochondrial genetic code in fission yeasts and *Pneumocystis* through mass spectrometry

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Background
Despite their closer phylogenetic relationship to classic yeasts than mold and prototozoa, and the absence of experimental evidence, fission yeasts and *Pneumocystis* have been assigned with translation table 4 (originally for mold and protozoa) instead of table 3 (originally for classic yeasts) for their mitogenomes. We aimed to determine the mitochondrial genetic code in representative species of fission yeasts and *Pneumocystis* through mass-spectrometry.

Methods
Mitochondria proteins were extracted from *S. pombe* culture and partially purified *P. carinii* and *P. macacae* cell pellets, digested with trypsin and/or chymotrypsin, and analyzed by liquid chromatography–mass spectrometry (LC-MS).

Results
For each species, LC-MS yielded a total of 31,343-49,279 unique peptide groups matching 2,269-3,590 proteins, mainly from nuclear genes (also including host genes for *P. carinii* and *P. macacae*). There were 16-21 unique peptides mapped to 6-11 mitogenome-encoded proteins in regions with sequences predicted using translation table 4 or table 1 while there was no peptide consistent with sequence predicted with table 3. Based on mitogenome sequence, an in-frame TGA condon is present once in *S. pombe*, five times in *P. carinii* and absent in *P. macacae*. This codon is expected to encode Trp by tables 4 and 3 rather than a stop signal by table 1, which is expected to produce a truncated protein with aberrant function. No peptide related to this codon was detected by LC-MS due to the low coverage of the data.

Conclusions
Our findings provide the first experimental evidence at the amino acid level to support the use of translation table 4 for mitogenomes of fission yeasts and *Pneumocystis.*

T 22 *Pneumocystis pneumonia* outbreaks among transplant recipients: unraveling the mystery

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Mycophenolic acid (MPA) targets inosine 5′-monophosphate dehydrogenase (IMPDH) of human lymphocytes. It is used as an immunosuppressant in solid organ transplant (SOT) recipients who are moreover at risk for *Pneumocystis* pneumonia (PCP). Numerous PCP outbreaks involving mostly SOT recipients have been reported worldwide. This event may be related to specific *Pneumocystis* organisms. Through a single center study, we have identified a mutation G1020A (Ala261Thr) in *impdh* gene of *Pneumocystis* isolates from 10 SOT recipients treated with MPA. Considering that other fungi known to be in vitro resistant to MPA harbor an analogous mutation (e.g. *C. albicans* strain, EC3), it was assumed that it represented a marker of MPA selection pressure. In this context, the objective of the present study was to test the hypothesis that, beyond the presence of IMPDH mutants, specific *Pneumocystis* strains infect SOT recipients.

Methods

The study was conducted in 27 centers in France and Switzerland; 117 PCP patients were retrospectively included. *Pneumocystis* isolates from 58 SOT recipients (44 of whom were exposed to MPA) and 59 control patients (non-SOT recipients, not exposed to MPA) were genotyped using MLST combined with *impdh* gene analysis.
Results. The G1020A mutation (Ala261Thr) was detected in *Pneumocystis* isolates from 40 SOT recipients (37 exposed to MPA) and none from the control patients. The mutation was not detected among the remaining *Pneumocystis* isolates from 18 SOT patients (7 were exposed to MPA and 11 were not). This mutation was associated with MPA exposure ($\chi 2$, $p < 0.001$). A specific multilocus genotype associated with the aforementioned mutation was detected in 38 SOT patients and none of the control patients ($\chi 2$, $p < 0.001$).

Conclusions

A specific *Pneumocystis* strain with a selective advantage effectively infects SOT recipients. MPA selection pressure explains its maintenance, circulation, and therefore involvement in PCP outbreaks within this patient population.

T 23* Clusters of patients with *Pneumocystis jirovecii* pneumonia harboring similar repertoires of genes encoding the major surface glycoproteins

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The surface antigenic variation system of the pathogenic fungus *Pneumocystis jirovecii* uses reassortment of the repertoires of ca. 80 allelic genes encoding the major surface glycoproteins of family I (*msg*-I; Meier et al. submitted). The characterization of the repertoires present in 29 patients with *Pneumocystis* pneumonia from five different cities evidenced one cluster of three patients with identical repertoires, one cluster of four patients with similar ones, and one cluster of two patients with weakly similar ones. A novel genotyping technique consisting in sequencing the ITS1-5.8S-ITS2 region with PacBio circular consensus sequence confirmed only the former and the last clusters. The former cluster presented the peculiarity that one patient was diagnosed seven years after the two other ones. The transmission of the strain to this third patient may result from two not mutually exclusive hypotheses : (i) a chain of transmission involving unknown patients, suggesting a slow dynamics of reassortment of the *msg*-I repertoires, and (ii) acquisition of dormant *P. jirovecii* cells that were in the environment. The cluster with four patients was difficult to interpret. It might result from several transmission events of the fungus between the patients that led to the accumulation in each patient of numerous strains, coupled to an enrichment with specific *msg*-I alleles in the geographical area. The last cluster with weakly similar repertoires might reflect interhuman transmission followed by relatively fast dynamics of reassortment of the *msg*-I repertoires.

*Student presentation

T 24 **Shelter dogs are important reservoir of opportunistic protists**

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Dogs are one of the most popular companion animals living in close contact with humans. While most owners are aware of helminths infecting their pets, protists are less known but just as significant.
We tested shelter dogs from Poland and Czech Republic for the presence of the most common opportunistic protists: *Cryptosporidium* spp., *Giardia intestinalis*, *Encephalitozoon* spp. and *Enterocytozoon bieneusi*. Fresh fecal samples were collected as soon as possible after defecation. Molecular detection was based on the nested-PCR protocols amplifying ITS regions of *E. bieneusi* or *Encephalitozoon* spp. and the SSU rRNA regions of *Cryptosporidium* spp. and *Giardia intestinalis*.
Among 187 samples tested, pathogens were detected in 51 in total (27 %), with higher prevalence observed in Polish than Czech animals (30 % vs. 24.5 %). The highest prevalence was observed for *G. intestinalis* (15.5 %), followed by *E. bieneusi* (10.2 %), while *Encephalitozoon* spp. and *Cryptosporidium* spp. were detected in 2 and 1 samples, respectively. A significant difference in species prevalence was observed depending on the region: *G. intestinalis* was more frequent in Polish (21.8 %) versus Czech animals (8.1%), while *E. bieneusi* was seen more often in Czech dogs (16.3 %) than in Polish ones (4.9 %). Both animal-specific and potentially zoonotic protists' species and genotypes were found. None of the animal had an apparent diarrheal symptom at the time of sampling.
Our study indicates the need for routine testing of shelter dogs for opportunistic protists as they can serve as an source of the environment contamination with dispersive forms of these pathogens. Considering that tested pathogens might cause a life-threatening infection in humans from risk groups, it is also crucial to improve the awareness of new dog owners on the relevance of diagnosing and treating zoonotic diseases.
Funding: National Science Centre, Poland [2020/39/O/NZ6/02313]; Wroclaw Medical University [SUBK.A060.23.027];

T 25 Meta-analysis of published data on *Pneumocystis* in 248 different mammal species

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Pneumocystis spp. have been described in a large variety of mammal species. The susceptibility of different mammals to *Pneumocystis* infection is unknown and influence of the host has only been sparsely investigated.
A total of 113 publications on *Pneumocystis* in pet, farm, zoo, and wild animals was analyzed. *Pneumocystis* prevalence, detection method, level of infection, and immunological status were evaluated. For wild animals, social structure, habitat, and lifestyle parameters were considered additionally.
Since 1909, *Pneumocystis* has been investigated in 248 mammal species from 37 countries belonging to the orders Afrosoricida, Artiodactyla, Carnivora, Chiroptera, Didelphimorphia, Eulipotyphla, Hyracoidea, Lagomorpha, Perissodactyla, Pilosa, Primates, and Rodentia. Prevalence ranged between 1-93%. In 47% of the citations, a prevalence of ≤25%, in 26% a 26-50% prevalence, and in 5% a prevalence of >75% was documented. High prevalence was reported in Carnivora, Eulipotyphla, and Rodentia. The prevalence level was significantly depending on the detection method ($p<0.001$), but not on the sample size ($p=0.239$). In Carnivora, significantly more high-grade infection levels occurred, while in Artiodactyla, Chiroptera, Eulipotyphla, Lagomorpha, and Rodentia low-grade infection levels dominated ($p<0.001$). Compared to pet animals, only low fungal loads occurred in zoo and wild animals ($p<0.001$). Immunosuppression was only proven in dogs and horses. Compared to animals living in groups, loners showed significantly more high-grade infection levels ($p=0.014$). Group size, preferred habitat, diet, activity phase, lifestyle, hibernation, or migratory behavior were not significantly associated with the level of infection.
Pneumocystis spp. seem to adapt to the lungs of potentially all mammals, but low prevalence data dominate. Domesticated species generally show higher proportions of high-grade infection levels compared to wild animals. Occasionally, however, high-grade infections are also described in wild animals. Potential factors impairing immunity should be considered in future studies. Finally, the effect of habitat or climate changes on *Pneumocystis* epidemiology should be illuminated.

POSTERS

P 04 Can *Pneumocystis jirovecii* be transmitted via human breast milk?

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Milk from the Human Milk Bank is considered as the optimal feeding alternative for infants and the gold standard for the preterm newborn's nutrition. However, apart from a multitude of biologically active compounds and human cells, human milk contains microorganisms, including pathogenic ones. Thus, the detailed safety assessment of milk deposited in Human Milk Banks is a priority, also in the context of the presence of pathogens, especially those dangerous to the health and life of both mother and child.

One of the pathogens relatively often diagnosed in infants is *Pneumocystis jirovecii,* which localize in human lungs and may lead to the respiratory failure and lung dysfunction, known as *Pneumocystis* pneumonia (PcP). Based on previous studies, it is known that infants acquire the primary *Pneumocystis* infection very early in life and it is highly common in premature infants, contributing to the higher severity of respiratory distress syndrome among them.

Our current study is designed to screen human milk samples collected by the Regional Bank of Breast Milk at Wroclaw Medical University for the presence of *P. jirovecii*. Here, we show for the first time *P. jirovecii* human milk infection confirmed by molecular methods.

This research may constitute the confirmation of the transmammary route of *P. jirovecii* infection in humans and may bring us closer to understanding the phenomenon of primary *Pneumocystis* infection in infants and preterm newborns.

Funding: National Science Centre, Poland [2020/39/O/NZ6/02313]; Wroclaw Medical University [SUBK.A060.23.027].

P 05 Tracking the ability of echinocandin treated mice to transmit *Pneumocystis* infection after re-initiation of the sexual cycle

Mr. Alan Ashbaugh [1], **Prof. Melanie Cushion** [1]

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The life cycle of *Pneumocystis* has not been definitively described but recent data strongly suggests that the sexual cycle is needed for proliferation and transmission. Results from our lab have shown that *Pneumocystis* (Pm) infected mice treated with echinocandins (ECH) deplete the asci containing β-1,3-D-glucan (BG), blocking proliferation and transmission We have also shown that withdrawal of the echinocandin treatment allows the sexual cycle to return and infection to re-populate the lungs. In this study, we determine when mice can transmit infection after withdrawal of echinocandin treatment and to identify any quantifiable thresholds for transmission.

Pm infected mice were treated with ECH for 3 weeks. After the 3 week period, the ECH treatment was withdrawn and mice were sacrificed at day 10, 14 and 21 to determine organism burden, BG content, obtain Pm organisms for inoculations and perform gene sequencing analysis. Additionally, mice at these timepoints were used to seed naïve immunosuppressed mice to determine at what the point the infection can be transmitted.

Asci from treated mice reappeared after cessation of the ECH treatment by day 10 and there was a significant increase between day 10 and day 21. ECH-treated seed mice were unable to transmit infection to naïve mice until the day 21 timepoint. BG content in the lungs of the ECH-treated mice was significantly reduced from the control mice and did not significantly change at any timepoint.

These results indicate a delayed ability to transmit Pm infection after re-initiation of the sexual cycle until a threshold of asci is reached. On-going gene sequencing analysis may provide more answers as to what genes are expressed as the fungi regain the ability to sexually replicate and could provide new targets for treatment.

P 08 Characterization of the fluorescent probe, monochlorobimate, for rapid assessment of glutathione inhibitors in *Pneumocystis murina*

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Glutathione (γ-L-glutamyl-L-cysteinylglycine, **GSH**) is a low molecular weight thiol that occurs in 2 free forms, the reduced form (GSH) and glutathione disulfide. The reactive thiol group (-SH) makes it a major cellular antioxidant and an emerging antifungal target. The synthesis of GSH involves two ATP-requiring enzymatic steps: formation of γ-glutamylcysteine from glutamate and cysteine and formation of GSH from γ-glutamylcysteine and glycine. Two enzymes are involved, glutamate cysteine ligase and GSH synthase, both of which are found in the sequenced Pneumocystis genomes.
We sought to establish a rapid screening method to evaluate the effects of GSH inhibitors on intracellular pools of GSH in *P. murina*. The preferred methods for GSH measurement are fluorometric assays because of their high sensitivity and specificity. The only probe able to penetrate cells and react directly with cellular thiols is monochlorobimate (**MCB**). Previous studies used microscopy for quantification in yeast and mammalian cells. For a more efficient system, a multi-well platform for rapid assessment of GSH levels was adapted. To optimize and test the system, 3 concentrations of MCB, 10-40- and 50μM, were reacted with 3 *P. murina* densities at 5x106, 1x107, and 5x107/mL. Controls included no MCB and 1 mg/mL pentamidine. After 24 hr of incubation in 48 well plates at 37oC, 5% CO2, 200μL from each of the triplicate wells/group were transferred to Corning 96-well black polystyrene plates, the MCB concentrations were added, and read continuously over 60 minutes using a BioTek Synergy/HTX (360/40, 460/40). The Vmax and Km were calculated, and 10μM MCB and 0.5-1.0x107/mL *P. murina* were determined as optimal. We have established a new assay for the rapid assessment of agents targeting GSH synthesis and are currently conducting studies using the GSH irreversible synthesis inhibitors, methionine- and buthionine sulfoximine, and 3-bromopyruvate, which has been shown to reduce GSH in yeast.

P 09 Role of EphA2 tyrosine kinase receptor-signaling pathway in *Pneumocystis* pneumonia (PCP)

Dr. Ted Kottom [1], **Dr. Eva Carmona** [1], **Prof. Andrew Limper** [1]

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Background

Pneumocystis jirovecii is one of the most common fungal pathogens in immunocompromised patients. Pneumocystis jirovecii pneumonia (PJP) results in an exuberant host proinflammatory response, attributable to the organism's abundant cell wall β-glucans. Myeloid cells, including alveolar macrophages (AMs) are critical for anti-PJP innate immunity. AMs promote clearance of organisms but are also major sources of proinflammatory mediators contributing to the profound pulmonary inflammation during PCP. Currently little is known about inflammatory and killing potential in vivo of AMs, and their downstream effects on tissue inflammation during PCP. Until the recent discovery of the importance of the EPH-EphrinA1 (EphA2) tyrosine kinase receptor-signaling pathway in *Candida albicans* and *Cryptococcus neoformans* fungal infections, this pathway was largely considered mainly in the context of carcinogenesis. We hypothesize that the EphA2 receptor kinase pathway has important immune functions that help modulate host AM inflammatory activity and organism killing and burden control in the PCP lung. Previously we published that in lung epithelial cells, EphA2 receptor binds *Pneumocystis* organisms, and that engagement of *Pneumocystis* β-glucans with this receptor results in receptor phosphorylation and downstream proinflammatory IL-6 release in this cell type.

Methods

We provide preliminary data demonstrating for the first time that the EphA2 receptor-signaling pathway strongly contributes to the macrophage host immune response and is paramount in organism control during PCP. Analysis by qPCR and GMS staining demonstrate significantly more organism burden in *EphA2-/-* mice as well as significantly reduced inflammation as measured by proinflammatory ELISAs and H&E staining.

Results

These findings of this receptor's importance in the proinflammatory host response and organism burden control to a microbial pathogen in lung AMs is to our knowledge the first of its kind.

Conclusions

These highly novel findings are conceptually innovative and may provide completely new strategies to treat PCP and possibly other fungal diseases.

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