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Analysis of the intestinal microbial community and inferred functional capacities during the host response to *Pneumocystis* pneumonia

Derrick R. Samuelson¹, Tysheena P. Charles¹, Nicholas M. de la Rua¹, Christopher M. Taylor², Eugene E. Blanchard², Meng Luo², Judd E. Shellito^{1,2}, and David A. Welsh^{1,2,*}

¹Department of Medicine, Section of Pulmonary/Critical Care & Allergy/Immunology, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA, 70112

²Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA, 70112

Abstract

Background—*Pneumocystis* pneumonia is a major cause of morbidity and mortality in patients infected with HIV/AIDS. In this study, we evaluated the intestinal microbial communities associated with the development of experimental *Pneumocystis* pneumonia, as there is growing evidence that the intestinal microbiota is critical for host defense against fungal pathogens.

Methods—C57BL/6 mice were infected with live *Pneumocystis murina* via intratracheal inoculation and sacrificed 7 and 14 days post infection for microbiota analysis. In addition, we evaluated the intestinal microbiota from CD4+ T cell depleted mice infected with *P. murina*.

Results—We found that the diversity of the intestinal microbial community was significantly altered by respiratory infection with *P. murina*. Specifically, mice infected with *P. murina* had altered microbial populations, as judged by changes in diversity metrics and relative taxa abundances. We also found that CD4+ T cell depleted mice infected with *P. murina* exhibited significantly altered intestinal microbiota that was distinct from immunocompetent mice infected with *P. murina*, suggesting that loss of CD4+ T cells may also affects the intestinal microbiota in the setting of *Pneumocystis* pneumonia. Finally, we employed a predictive metagenomics approach to evaluate various microbial features. We found that *Pneumocystis* pneumonia significantly alters the intestinal microbiota's inferred functional potential for carbohydrate, energy, and xenobiotic metabolism, as well as signal transduction pathways.

*Correspondence: David A. Welsh, Section of Pulmonary/Critical Care & Allergy/Immunology, Department of Medicine, Louisiana State University Health Sciences Center, 1901 Perdido Street, Suite 3205, New Orleans, Louisiana 70112. Phone: 504-568-3431. Fax: 504-5684295. dwelsh@lsuhsc.edu.

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Conclusions—Our study provides insight into specific-microbial clades and inferred microbial functional pathways associated with *Pneumocystis* pneumonia. Our data also suggest a role for the gut-lung axis in host defense in the lung.

Keywords

Microbiota; Pneumonia; *Pneumocystis*; Metagenomics

Introduction

Pneumonia, due to the opportunistic human fungal pathogen *Pneumocystis jirovecii*, is an AIDS-defining illness, and there is a direct inverse relationship between CD4+T cell counts in the blood and the risk for infection.¹ *Pneumocystis* is also a major cause of mortality in patients whose CD4+ T cell are significantly depressed due to malignancy, chemotherapy, or other immunosuppression.^{1, 2} Animal models of immunodeficiency demonstrate that the loss of CD4+ T cells renders mammals susceptible to *Pneumocystis* lung infection.² In spite of current treatment strategies for HIV infection *Pneumocystis* pneumonia remains a common clinical problem.³ While Highly Active Antiretroviral Therapy (HAART) has reduced the incidence of *Pneumocystis* infections in HIV+ individuals, the reduction is not as dramatic as is observed with other opportunistic infections.³ Subpopulations of HIV-infected patients remain at risk despite receiving HAART therapy.³⁻⁵ Furthermore, an increasing number of patients are receiving immunosuppressive medical regimens, making them vulnerable to pneumonia.⁶ These data indicate that there is a need for increased understanding of *Pneumocystis* infections.

The intestinal microbiome and mucosal tissues broadly interact in both health and disease. Prokaryotic and eukaryotic members of the microbiota influence the host in several ways, such as the supply of nutrients and regulation of the host immune system. How the commensal microbial communities influence the host immune system is poorly understood, but it appears that the microbiota is a major regulator of the immune system and that bacterial signals have profound influences on antibacterial defenses in the GI tract, as well as, in other distal organs.⁷ The influence of the GI microbiota on lung immunity (i.e., the gut-lung axis) is an area of great interest, but the underlying mechanisms remain incompletely understood. Commensal microbes of the GI tract contribute to host defense in *Escherichia coli* pneumonia via toll-like receptor signaling,⁸ and germ-free mice have a strikingly higher mortality rate following *Pseudomonas aeruginosa* pneumonia compared to conventional mice.⁹ There is a growing appreciation of the role specific microbes and/or communities play to modulate and promote pulmonary immunity. Several recent studies have shown that the intestinal microbiota is critical for maintenance of T cell subsets that are important for systemic immunity. For example, colonization of germ-free mice with *Bacteroides fragilis* that synthesize PSA results in a higher number of circulating CD4+ T cells and levels of circulating Th1 cells.¹⁰ While, colonization with *Clostridial* strains directs the expansion of lamina propria and systemic regulatory T cells (Treg).¹¹ Finally, segmented filamentous bacteria (SFB) colonization has been associated with expansion of the Th17 cell population and a slight increase in Th1 cells.¹²⁻¹⁴ While, several studies have demonstrated that the gut microbiota promotes pulmonary immunity and resistance to pneumonia,^{8, 9, 15-17}

no studies have addressed the effects of lung pathogens and/or pneumonia on the gastrointestinal microbiota.

Interest in the microbiome specific to HIV infection has been growing exponentially, partly due to early findings which suggests that bacterial translocation from the intestines causes persistent immune activation.^{18, 19} In addition, individuals with HIV continue to have excess non-AIDS morbidity and mortality despite effective viral suppression with HAART, which appears to be driven, in part, by microbial translocation and the resultant immune activation.^{20, 21} Much work has gone into elucidating the mechanisms by which intestinal microbiota augment or disrupt intestinal barrier function, immune response to antigen and systemic immune activation. However, the role of the dysbiotic intestinal microbiome of HIV infected individuals during host defense, particularly in the lung, is ill-defined.

In this study we evaluated the diversity of the intestinal microbial community in mice with a *P. murina* respiratory infection. We found that mice infected with *P. murina* had altered gut microbial populations, as judged by changes in alpha and beta diversity, as well as, changes in taxa abundances. We also found that CD4+ T cell depleted mice infected with *P. murina* exhibited significantly altered intestinal microbiota that was distinct from immunocompetent mice infected with *P. murina*, suggesting that loss of CD4+ T cells also affects the intestinal microbiota. Finally, we found that *Pneumocystis* pneumonia significantly alters the intestinal microbiota's potential for carbohydrate, energy, and xenobiotic metabolism and bacterial pathogenesis, specifically cell motility and signal transduction pathways.

Materials and Methods

Mice

Female 6–8 week old specific-pathogen free C57BL/6 mice were obtained from Charles Rivers Breeding Laboratories (Wilmington, MA). Animals were housed in filter-topped cages and provided autoclaved water and chow (Teklad Sterilizable Rodent Diet cat #8656, Harlan Indianapolis, IN) *ad libitum*. Animals were acclimated in the animal care facility at the Louisiana State University Health Sciences Center (LSUHSC) for at least one week prior to beginning experimentation. All mice were free of *P. murina* prior to experimentation and SPF conditions were maintained throughout the course of the experiment. Each experimental group consisted of 10 mice housed in three separate cages to minimize the caging effects on the microbiota. All studies were approved by the LSUHSC Institutional Animal Care and Use Committee. Experiments were repeated two times and results were confirmed. Results from one experiment are presented and are representative of both experiments.

P. murina inoculation

P. murina organisms for inoculation were obtained from lung homogenates from chronically infected B6-Rag2^{-/-}Il2rg^{-/-} (C57BL/6 background) mice and purified as previously described.^{2, 22} Control mice received naïve lung homogenate, to control for non-specific inflammatory responses. All inoculum was normalized to the amount of initial lung tissue (*i.e.*, if one right lung was used for the *P. murina* inoculum one right lung was used as the naïve lung control). The number of *P. murina* cysts was quantified microscopically and the

inoculum concentration was adjusted to 2×10^6 cysts/ml. Recipient mice were lightly anesthetized with isoflurane (1–4% to effect). Animals were suspended by their front incisors, the tongue was gently extended out with forceps and 100 μ l inoculum (2×10^5 cysts) was injected into the trachea using a P200 pipette. After inhalation of inoculum was observed, the tongue was released and the animal was allowed to recover from anesthesia.

CD4+T cell Depletion

One group of mice were depleted of CD4+ T cells by intraperitoneal (i.p.) injection of 0.1 mg anti-CD4+ mAb (hybridoma GK1.5; National Cell Culture Center) in 100 μ l PBS 3 days prior to infection. Control mice were given IgG isotype control antibody via i.p. injections. Depletion was maintained by i.p. injection every 6 days. This treatment protocol results in >97% sustained depletion of CD4+ lymphocytes from blood and lymphoid tissue.²² CD4+ T cell depletion was also confirmed via flow cytometry for CD45+ CD3+ CD4+ cell populations in the lungs and spleen (Supplemental Figure 1).

RNA isolation and real-time PCR for *P. murina* rRNA quantification

Total RNA was isolated from lung tissue of infected mice by the TRIzol method (Invitrogen, Grand Island, NY), reverse transcribed, and real time quantitative PCR (mitochondrial small ribosomal subunit RNA) was used to determine *P. murina* lung burden, as previously described.^{23, 24} Briefly, upon sacrifice, the entire right lung of each mouse was removed. After the trachea, bronchi, and the surrounding connective tissue were removed, the lung was homogenized with TRIzol reagent to isolate total RNA. An RNA standard for the assay, a portion of *Pneumocystis murina* rRNA (GenBank accession number AF257179) was cloned into PCR 2.1 Vector (Invitrogen, Carlsbad, CA) and used as a standard control. TaqMan PCR primers 5'-ATG AGG TGA AAA GTC GAA AGG G-3' and 5'-TGA TTG TCT CAG ATG AAA AAC CTC TT-3' were then used to amplify mouse *Pneumocystis* rRNA. A reporter 6-carboxyfluorescein (FAM) probe 5'-6FAM-AACAGCCCAGAATAATGAATAAAGTTCCTCAATTGTTAC-TAMRA-3' (where TAMRA is 6-carboxytetramethylrhodamine) was employed for real time quantification. Quantitative PCR has been previously validated against microscopic enumeration.^{23, 24}

Determining the population structure of the microbiota

Sequencing and bioinformatics were performed by the Louisiana State University School of Medicine Microbial Genomics Resource Group (<http://metagenomics.lsuhsu.edu/mgrg>). The intestinal contents (luminal content from the duodenum to colon) were flash frozen and genomic DNA extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen Valencia, CA) modified to include bead-beating and 16S metagenomic sequencing was performed as previously described.^{25, 26} Briefly, 16S ribosomal DNA hypervariable regions V3 and V4 were amplified using gene-specific sequences, Illumina adaptors, and molecular barcodes primers. Precisely, the V3 Forward: 5'-CCTACGGGAGGCAGCAG-3' with a forward adapter (5'-AATGATACGGCGACCGAGATCTACAC-3'), a forward pad: (TATGGTAATT), and a forward linker (GG), and the V4 Reverse: 5'-GGACTACHVGGGTWTCTAAT-3' with a reverse adapter (5'-CAAGCAGAAGACGGCATACGAGAT-3'), a reverse pad (AGTCAGTCAG), and a reverse linker (CC) were used for sequencing of fecal DNA samples. Samples were then sequenced

on an Illumina MiSeq (Illumina, San Diego, CA) using the 2×300bp V3 sequencing kit. In addition, internal sequencing controls (negative water control and a positive mock community control) were included to test for contamination of the sequencing runs. The read files were processed through the UPARSE pipeline (drive5, Tiburon, California) merging forward and reverse reads and then truncating merged reads that covered the V3/V4 region to a uniform length of 400 bp, discarding reads with quality score less than 15 in the first 400 bp. Unique reads were clustered into operational taxonomic units (OTU) at 97% similarity. Chimeric OTUs were removed as identified by UCHIME by drive5. Finally, the original filtered reads were mapped to the OTUs using USEARCH by drive5 at 97% identity. QIIME 1.9.1 (open source, www.qiime.org) was used to pick and align a representative set.²⁷ Uclust by drive5 was used to assign a taxonomic classification to each read in the representative set via the RDP database.²⁸ Relative abundance of each OTU was examined at phylum, class, order, family, genus, and species levels. Beta (between communities) and alpha (within a community) diversity metrics, as well as, taxonomic community assessments were produced using QIIME 1.9.1 scripts.

Metagenome inference and metabolic pathway reconstruction

The IMG (Integrated Microbial Genomes and Metagenomes) reference genomes were used to infer the approximate gene content of detected phylotypes (OTUs) using PICRUSt v0.9.0 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved STates) (<http://picrust.github.io/picrust/>).²⁹ PICRUSt has been used to produce accurate metagenome predictions for both mammal gut samples and soil samples.²⁹ The FastTree Greengenes (GG) annotated genomes was used to pick closed reference OTUs from the de-multiplexed and USEARCH quality-filtered reads at 97% identity. In addition, procrustes analysis was performed to confirm the beta diversity conclusions generated from open and closed reference picking methods. No statistical differences were observed between picking methods (Supplemental Figure 2). The gene contents were then reconstructed across the GG tree and assigned to KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology copy numbers.³⁰ Metagenome prediction accuracy was calculated using the weighted Nearest Sequenced Taxon Index (weighted NSTI). Inferred relative gene abundances were subsequently binned into pathways and functional categories defined by the BRITE hierarchy. Non-microbial categories (*e.g.*, Organismal Systems and Human Diseases) were excluded from further analysis. Potential microbial functions associated with *P. murina* infection status were identified by LEfSe (Linear Discriminant Analysis Effect Size) (<http://huttenhower.sph.harvard.edu/lefse/>), as described elsewhere.³¹

Statistical analysis

Results are presented as mean ± S.E.M. Statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA, USA) and statistical significance was measured at $P = 0.05$. The non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) followed by post hoc Dunn's multiple comparisons of the means was used to assess fungal lung burden.

For sequencing data, alpha diversity rarefaction curves were produced by plotting several diversity metrics against the number of sequences considered from a sample. All subsequent analysis of diversity were performed on OTU tables rarefied to 1,000 sequences/sample to

account for variations in sequencing depth. Statistical significance was compared using a nonparametric permutation test for a pairwise comparison of categories. The p values were Bonferroni-corrected for multiple testing. Beta diversity, principal coordinates analysis plots were produced, using both weighted (considers abundance of each species) and unweighted (considers presence/absence of species) UniFrac metrics. Plots were visualized using the Emperor 3D Viewer (open source, <http://biocore.github.io/emperor/>). Statistical significance was assessed with a nonparametric permutation test to compare a chosen category. ANOVA was used to test for differences in relative abundance of specific OTUs for each group. An unweighted g-test was used to evaluate the statistical significance of the presence/absence of OTUs across categories. Additionally, two-way ANOVA with Bonferroni corrected comparisons of the means was used for differential representation of taxa between sham infected and *P. murina* infected mice and for *P. murina* infected CD4-depleted mice and *P. murina* infected mice.

For metagenomic data, statistical significance was measured via LEfSe as described elsewhere.³¹ Briefly, Kruskal-Wallis test with quantitative tests for biological consistency (Wilcoxon-rank sum test) was used for metagenomics analysis. The differentially abundant and biologically relevant features (functional categories) are ranked by effect size after undergoing linear discriminant analysis (LDA). All p-values were corrected for multiple hypothesis testing using Benjamini and Hochberg's false discovery rate correction. A q-value of 0.25 and an effect size threshold of 2 (on a log10 scale) were used for this study. Further, all categories with a significant LDA score were further analyzed using a two-way ANOVA with Bonferroni corrected comparisons of the means to determine the differential representation of inferred functional categories between sham infected and *P. murina* infected mice and for *P. murina* infected CD4-depleted mice and *P. murina* infected mice.

Nucleotide sequence accession number

All data generated from sequencing have been deposited in the National Center for Biotechnology Information (NCBI) of the National Institutes of Health via the Sequence Read Archive (SRA) with accession number SRP071074.

Results

Pneumocystis pneumonia alters the intestinal microbiota seven days post infection

We sought to determine if the intestinal microbiota is altered by *P. murina* infection using 16s rDNA deep sequencing. C57BL/6 mice were grouped into the following categories; uninfected animals (designated Sham Infected), *P. murina* infected immune intact animals (*P. murina* Infected), and mice depleted of CD4+ T cells and infected with *P. murina* (CD4+ T cell Depletion + *P. murina* Infection). CD4-depletion was maintained continuously throughout the experiment. The experimental design and timeline is described in Figure 1. We first evaluated the lung burden of mice infected with *P. murina* seven days post infection. We found that the burden of *P. murina* in the lung was $\sim 1 \times 10^5$ rRNA copies/lung and did not differ significantly between CD4-depleted and CD4-intact animals at this time point (Figure 2A). In addition, we evaluated the intestinal content by qPCR and found that there was no detectable *P. murina* organisms (data not shown). We then evaluated the cytokine

response (i.e., IFN- γ) in the lung (Figure 2B) and serum (Figure 2C) of *P. murina* infected mice. We found that immune intact mice had significantly higher levels of IFN- γ in the lung and serum compared to both sham infected and CD4 T cell depleted mice infected with *P. murina*. We also evaluated the levels of TNF- α in the lung and serum. Levels of TNF- α were not significantly different in either the lung or serum at this time point (Supplemental Figure 3A-B). We then assessed the differences in the intestinal microbiota of sham infected mice, *P. murina* infected mice, and CD4 T cell depleted mice infected with *P. murina*. We evaluated the beta diversity using both unweighted and weighted UniFrac metric via Bray-Curtis dissimilarity. This analysis revealed significant differences between the three experimental intestinal microbial populations ($P = 0.001$ and $P = 0.002$ for unweighted and weighted, respectively). We also found three distinct microbial communities associated with each treatment condition, as judged by principal coordinate analysis (PCoA) (Figure 2D and 2E). Analysis of the alpha diversity of the OTUs from *P. murina* infected mice and CD4-depleted mice infected with *P. murina* revealed a significant loss in the number of observed species present in the intestinal tract 7 days post respiratory infection (Figure 2F). However, no significant difference was observed between CD4-intact and CD4-depleted animals. Finally, we evaluated the taxonomic structure in each treatment condition. Mice infected with *P. murina* had significant changes in the relative abundances of the genera *Lactobacillus*, *Turicibacter*, *Oscillospira*, and *Ruminococcus bromii* and the family *Lachnospiraceae* (Figure 2G). Similarly, CD4-depleted mice exhibited significant changes in the abundance of *Lactobacillus*, *Turicibacter*, *Oscillospira*, and *Ruminococcus bromii* and the family *Lachnospiraceae* (Figure 2G). We also evaluated differences in taxonomic membership in CD4-intact and CD4-depleted animals and found that the abundance of *Lactobacillus* and *Ruminococcus bromii* were significantly different between the two groups (Figure 2G). These bacterial communities are commonly isolated from the murine intestinal tract, which suggest that changes in the composition and quantity of the microbial communities that correlate with changes in a given phenotype are a result of the perturbation and not likely due to the introduction of novel species. Additionally, microbial communities prior to infection/CD4 T cell depletion were similar between all three groups (Supplemental Figure 4).

We then assessed the core microbiota (defined as taxa present in all of the animals within an experimental group) from each treatment group and sought to evaluate the conserved and unique species present during each treatment condition. Analysis of the core microbiota from sham infected mice, *P. murina* infected mice, and CD4-depleted mice infected with *P. murina* revealed that 10 of the taxa were present in every animal regardless of CD4-depletion or *P. murina* infection (Figure 3A and 3B). We also found that each treatment condition had one unique taxa that was only detected in 100% of the animals in the respective group. Specifically, a member of the *Bacteroides* genus was only found in the sham infected group, a member of the *Aldercreutzia* genus was only found in *P. murina* infected mice, and *Lactobacillus ruminis* was only detected in 100% of the *P. murina* infected CD4-depleted mice (Figure 3A and 3B). Four taxa were shared between the sham infected and *P. murina* infected animals and five taxa were conserved between the sham infected and *P. murina* infected CD4-depleted mice. In addition, only one taxa

(*Parabacteroides*) was shared between *P. murina* infected and *P. murina* infected CD4-depleted mice (Figure 3A and 3B).

Finally, we sought to evaluate the metagenome inferred metabolic pathways of sham infected mice, *P. murina* infected mice, and CD4-depleted mice infected with *P. murina*. To investigate the intestinal microbiota functions associated with *P. murina* infection we used PICRUSt analysis to infer putative metagenomes from the 16s rRNA gene profiles. More precisely, a closed reference OTU picking method (97% similarity) using the available Greengenes as reference OTUs was used. PICRUSt then predicts counts of functional genes on each sample and then groups them into KEGG Orthology groups, based on the BRITE hierarchy. We then used LEfSe and Prism 5 to identify microbial relevant functions that exhibited significant differences in functional abundance associated with *P. murina* infection. We first evaluated seven functional classes (cell motility, signal transduction, biosynthesis of secondary metabolites, carbohydrate metabolism, energy metabolism, lipid metabolism, and xenobiotic metabolism and degradation). This analysis showed that mice infected with *P. murina* had significant increases in the abundance of genes associated with signal transduction, carbohydrate metabolism, energy metabolism, and xenobiotic metabolism and degradation (Figure 4A). No significant differences were observed between sham infected animals and *P. murina* infected CD4-depleted mice or between *P. murina* infected mice and *P. murina* infected CD4-depleted animals, which is consistent with the intermediate phylogenic phenotype in the CD4-depleted group (Figure 4A).

We then analyzed the individual pathways within each significantly different functional class. We found that mice infected with *P. murina* had a significant increase in the function category of carbohydrate metabolism, which specifically was associated with increased functional capacity for ascorbate/aldarate metabolism, C5-branched dibasic acid metabolism, TCA cycle genes, fructose/mannose metabolism, galactose metabolism, glycolysis and gluconeogenesis, pentose and glucuronate interconversions, pentose phosphate pathway, propanoate metabolism, pyruvate metabolism, and starch and sucrose metabolism (Figure 4B). We also observed a significant increase in the abundance of genes involved in phosphatidylinositol signal systems (signal transduction category, Figure 4C), carbon fixation and methane metabolism (energy metabolism category, Figure 4D) and of dioxin degradation, cytochrome P450 drug and xenobiotic metabolism, naphthalene degradation, and xylene degradation (xenobiotic metabolism and degradation category, Figure 4E) between *P. murina* infected mice and sham infected control animals. No significant differences were observed between sham infected animals and *P. murina* infected CD4-depleted mice or between *P. murina* infected mice and *P. murina* infected CD4-depleted animals (Figure 4B-E).

***Pneumocystis* pneumonia alters the intestinal microbiota fourteen days post infection**

We also evaluated the lung burden and composition of the intestinal microbiota of mice infected with *P. murina* fourteen days post infection to evaluate any potential temporal effects on the intestinal microbiota, as the immune intact animals began to clear the infection. We found that the burden of *P. murina* in the lung was $\sim 1 \times 10^5$ rRNA copies/lung, however there was a decrease in the number of animals infected with *P. murina* in the

CD4-intact animals compared to CD4-depleted mice (Figure 5A). Evaluation of the intestinal content by qPCR found that there were no detectable *P. murina* organisms (data not shown). We then evaluated the cytokine response (i.e., IFN- γ) in the lung (Figure 5B) and serum (Figure 5C) of *P. murina* infected mice. We found that immune intact mice had significantly higher levels of IFN- γ in the lung compared to sham infected mice, while levels of IFN- γ in the serum were decreased (although not significantly) compared to the sham infected and CD4 T cell depleted mice infected with *P. murina*. We also evaluated the levels of TNF- α in the lung and serum. Levels of TNF- α were not significantly different in either the lung or serum at this time point (Supplemental Figure 3C-D). We then assessed the differences in the intestinal microbiota of sham infected mice, *P. murina* infected mice, and CD4 T cell depleted mice infected with *P. murina*. We evaluated the beta diversity of unweighted and weighted UniFrac metrics via Bray-Curtis dissimilarity, which showed significant differences between the three groups ($P=0.001$ and $P=0.001$ for unweighted and weighted, respectively). PCoA visualization also demonstrated group effects (Figure 5D and 5E). However, the dissimilarity between the CD4-depleted and CD4-intact mice infected with *P. murina* was much less than was seen at seven days post infection. Analysis of the alpha diversity of the OTUs from *P. murina* infected mice and CD4-depleted mice infected with *P. murina* found that the number of observed species present in the intestinal tract 14 days post respiratory infection was no longer significantly different between any of the three groups of animals (Figure 5F). This suggests that diversity may be lost early during infection, but recovers at later time points. Upon evaluation of the taxonomic structure of each treatment condition, we found that mice infected with *P. murina* had significant changes in the relative abundances of the genus *Turicibacter* and the family *Clostridiales* and *Lachnospiraceae* compared to control animals (Figure 5G). Similarly, CD4-depleted mice exhibited significant changes in the abundance of *Lactobacillus*, *Turicibacter*, *Allobaculum*, and *Akkermansia muciniphila* and the family *Lachnospiraceae*, *S24-7*, and *Clostridiales* as compared to sham infected mice (Figure 5G). The abundance of *Lactobacillus*, *Turicibacter*, *Allobaculum*, and *Akkermansia muciniphila* and the family *Clostridiales* were significantly different between CD4-intact and CD4-depleted animals (Figure 5G).

Analysis of the core microbiota from sham infected mice, *P. murina* infected mice, and CD4-depleted mice infected with *P. murina* revealed that 15 of the taxa were present in every animal regardless of CD4-depletion or *P. murina* infection (Figure 6A and 6B). Each treatment condition had unique core microbes. Specifically, a member of the *Erysipelotrichaceae* family, and members of the *Dehalobacterium*, *Anaerostipes*, and *Defluviitalea saccharophilla* genus were only found in the sham infected group, a member of the *Clostridiaceae* family and *Roseburia* genus were only found in *P. murina* infected mice, and *Parabacteroides* was only detected in 100% of the *P. murina* infected CD4-depleted mice (Figure 6A and 6B). No taxa were shared between the sham infected and *P. murina* infected animals and one taxon was conserved between the sham infected and *P. murina* infected CD4-depleted mice. In addition no taxa were shared between *P. murina* infected and *P. murina* infected CD4-depleted mice (Figure 6A and 6B).

To investigate the intestinal microbiota functions associated with *P. murina* infection at 14 DPI we used PICRUSt analysis to infer putative metagenomes from the 16S rRNA gene profiles, as described for 7 DPI. This showed that mice infected with *P. murina* had

significant decreases in the abundance of genes associated with cell motility, signal transduction, carbohydrate metabolism, energy metabolism, lipid metabolism, and xenobiotic metabolism and degradation (Figure 7A). No significant differences were observed between sham infected animals and *P. murina* infected CD4-depleted mice. We also observed a significant increase in genes associated with biosynthesis of secondary metabolites and lipid metabolism between *P. murina* infected mice and *P. murina* infected CD4-depleted animals (Figure 7A).

Analyzing the individual pathways within each significantly different functional class, we found that mice infected with *P. murina* had a significant decrease in the abundance of genes involved in bacterial chemotaxis, bacterial motility proteins, and flagellar assembly (cell motility category Figure 7B), two-component systems (signal transduction category, Figure 7C), flavone/flavonol biosynthesis, flavonoid biosynthesis and stilbenoid diarylheptanoid and gingerol biosynthesis (biosynthesis of secondary metabolites, Figure 7D), carbon fixation and methane metabolism (energy metabolism category, Figure 7E), amino sugar metabolism, ascorbate/aldarate metabolism, C5-branched dibasic acid metabolism, galactose metabolism, glyoxylate/dicarboxylate metabolism, inositol phosphate metabolism, and pentose and glucuronate interconversions (carbohydrate metabolism category, Figure 7F), atrazine degradation, caprolactam degradation, chlorocyclohexane degradation, dioxin degradation, drug metabolism, flourobenzoate degradation, styrene degradation, and xylene degradation (xenobiotic metabolism and degradation category, Figure 7G), and ether lipid metabolism, fatty acid elongation, sphingolipid metabolism, steroid biosynthesis, and steroid hormone biosynthesis (lipid metabolism category, Figure 7H) between *P. murina* infected mice and sham infected control animals.

We also observed significantly increased gene abundances in *P. murina* infected CD4-depleted mice for caffeine metabolism, stilbenoid diarylheptanoid and gingerol biosynthesis, flourobenzoate degradation, fatty acid elongation, and steroid biosynthesis compared to sham infected animals. This was also accompanied by a significant loss of gene abundances in *P. murina* infected CD4-depleted mice for bacterial chemotaxis, bacterial motility proteins, flagellar assembly, two-component systems, flavone/flavonol biosynthesis, carbon fixation, and methane metabolism compared to sham infected animals. Further we observed a significant increased gene abundances in *P. murina* infected CD4-depleted mice for caffeine metabolism, stilbenoid diarylheptanoid and gingerol biosynthesis, flourobenzoate degradation, fatty acid elongation, and steroid biosynthesis compared to *P. murina* infected mice.

Limitations

There are several limitations to the current study. First, we did not assess the microbiome in the gut in CD4 T cell depleted mice without *Pneumocystis* infection. As such, the major factor (CD4 depletion or *Pneumocystis*) which modulates the composition of the intestinal microbiota cannot be determined. We believe both factors contribute to changes in the microbial composition, however we are primarily interested in how pneumonia is altering the gut microbiome in the context of two different infection models. Additionally, inferred

functional capacity data is meant to guide the development of future research as the data is based only on assigned functions and not on biochemical measurements.

Discussion

Immunological cross-talk occurs between the intestinal tract and the respiratory tract and disturbances in intestinal homeostasis affects systemic (e.g., lung) immune responses.^{7, 32-34} We sought to understand the effects of respiratory infection on the composition of the intestinal microbiota community and on the inferred metagenomics functions associated with the altered microbiota. Using a murine model of *Pneumocystis* pneumonia we found that respiratory infection significantly impacts the intestinal microbiota, as well as, the inferred metagenomics functions. These findings are consistent with a study in a murine model of allergy, in which the microbial community structure was significantly impacted by intranasal challenge with house dust mite.³⁵ These data are also consistent with recently published data, which demonstrate that the gut microbiota regulates Th17 CD4 T cell polarization during pulmonary fungal infections.¹⁷ Signals (fungal compounds, cytokines, chemokines, or immune cells) derived from *P. murina* lung infection could potentially facilitate interactions along the gut-lung axis by; (1) altering the gut pro- or anti-inflammatory bacterial molecule availability, (2) production of immune modulatory metabolites, (3) expansion of specific immune cell populations, or (4) changing the intestinal permeability. In our model it is likely that signals from the infected lung direct alterations in the intestinal bacterial community structure that support immune cell proliferation and enhance gut-mediated production of immune modulatory metabolites, as we observed shifts in the inferred metabolic profile of the microbiota after *P. murina* infection, which favor these responses. Our results may also suggest that the gut-lung axis is bidirectional with signals/products produced during lung infection inducing changes in the gut microbial populations that, in turn, provide feedback to the lung.

Mice infected with *P. murina* exhibit significant changes in the relative abundances of the genus *Lactobacillus* (increased at day 7 and the same at day 14), *Turicibacter* (decreased at day 7 and increased at day 14), *Oscillospira* (decreased at day 7 and the same at day 14), and *Ruminococcus bromii* (increased at day 7 and the same at day 14) and the family *Lachnospiraceae* (decreased both day 7 and 14) and *Clostridiales* (the same at day 7 same and decreased at day 14) compared to control mice. The changes in the taxonomic structure in *P. murina* infected mice were also accompanied by changes in the inferred functional profile of the intestinal microbiota. Specifically, mice infected with *P. murina* had significant increases in the inferred functional capacity for carbohydrate metabolism (increased at day 7 and decreased at day 14), energy metabolism (increased at day 7 and decreased at day 14), xenobiotic metabolism (increased at day 7 and decreased at day 14), and signal transduction (increased at day 7 and decreased at day 14).

These changes in the taxonomic structure and the inferred functional capacity of the microbiota could influence the host's response to infection. For example, the ability to metabolize carbohydrates such as lactose, galactose, and ascorbate (all of which increase during *P. murina* infection) could have direct effects on host immune response. The metabolism of lactose can occur in two distinct pathways. One pathway utilizes enzymes

common to both host and gut microbes, while the other path is exclusive to enzymes found in *Firmicutes* (the phylum most affected by *P. murina* infection).³⁶ The pathway through *Firmicutes* leads to the conversion of lactose to tagatose, which is catalyzed by *Lactobacillus rhamnosus*, *L. gasseri*, and *L. casei*. This is consistent with our findings that the relative abundance of *Lactobacillus* significantly increases 7 days post infection. Tagatose can also be generated by the conversion of galactose to tagatose, which can be facilitated by *Lactobacillus*, as well. The production of tagatose, a monosaccharide, is mainly considered as an anti-hyperglycemic molecule, however unabsorbed tagatose can be converted to short-chain fatty acids (SCFA), by fermentation from gut bacteria. We also observe increased inferred capacity for glycolysis and pentose phosphate pathways, which could also be contributing to the production of SCFA. Intestinal microbiota-mediated production of various SCFAs has been shown to be important for host systemic immunity (i.e., the lung), as SCFA can be absorbed into the bloodstream, via transporters in the intestinal lumen.³⁷⁻³⁹ More precisely, SCFAs, especially butyrate, seem to exert broad anti-inflammatory activities by affecting immune cell migration, adhesion, cytokine expression, as well as, cellular proliferation, activation, and apoptosis through the activation of signaling pathways (NF- κ B) and inhibition of histone deacetylase. This is illustrated by two recent studies which demonstrated that short-chain fatty acids directly regulate and/or prime the size and function of the regulatory T cell (Treg) pool in the colon.^{40, 41} Increased levels of butyrate also induce the expression of IL-10, which influences the balance between Th1, cytotoxic CD8+ T cells and Treg cells.^{40, 41} These data support our results, which indicate that signals from the infected lung direct alterations in the intestinal bacterial community structure, which may further support immune cell proliferation (Tregs) and enhance gut-mediated production of immune modulatory metabolites (SCFA).

These results could be correlated with the normal host immune response to *P. murina* infection. Specifically, animals infected with *P. murina* have significantly elevated levels of neutrophils, lymphocytes (CD4+ and CD8+ T cells), macrophages, and eosinophils seven days post infection.⁴² However, only neutrophils remained significantly elevated 14 days post infection suggesting that the host immune response is changing by 14 days post infection, which is consistent with our taxonomic and metagenomics findings. In addition to cellular responses, TNF-alpha, IFN-gamma, IL-1, IL-6, and RANTES message are increased throughout the course of *Pneumocystis* infection.⁴² This is similar to our observed results showing that IFN-gamma is significantly increased in the lungs and the serum of immune-intact mice infected with *P. murina* 7 days post infection when compared to sham or CD4 T cell depleted mice. We also observe a decreased level of serum IFN-gamma in immune-intact mice infected with *P. murina* 14 days post infection. These data could suggest that as mice begin to clear *Pneumocystis* circulating levels of IFN-gamma decrease as the intestinal microbial communities begin to transition to a “normal” community composition. Increased abundances of bacterial populations that, in turn, upregulate SCFA (anti-inflammatory) production may be an additional mechanism for the host to control the inflammatory response to *P. murina* infection. These results also strongly suggest that communication along the gut-lung axis is part of the normal host response to *P. murina* infection.

We also examined how the host's immune status during *Pneumocystis* pneumonia effects the gut microbial population, by evaluating the differences between CD4-intact and CD4-

depleted mice infected with *P. murina*. We found that the inferred functional capacity of the gut microbiota from CD4 T cell depleted animals infected with *P. murina* differed from that of immune-intact mice infected with *P. murina*. We believe this is due to several factors: First, immune-intact mice infected with *P. murina* develop a self-limiting infection, while CD4-depleted mice develop a persistent infection, which ultimately leads to death.² Therefore, as immune-intact *P. murina* infected animals respond and clear the pathogen the composition of the intestinal microbiota and associated functional capacity may initially contribute to the control of infection, through pro-inflammatory signaling, followed by further changes in the microbiota as the host's immune response returns to a baseline level. On the other hand, CD4-depleted animals cannot develop a normal host response or clear the pathogen, so the functional capacity slightly increases initially. However the response never normalizes and several metabolic pathways remain activated, which may be part of the host's attempt to control the infection. These data may also suggest that in the context of CD4-depletion the differential changes in the microbiota limits the “superorganism” ability to respond to infection. Regardless, the temporal changes that we observed both at a taxonomic and metagenomic level suggest that the composition of the intestinal microbiota throughout the course of infection could influence the hosts' ability to combat infection, both in the intestinal tract and at distal mucosal sites.

Conclusions

In summary, our analyses delineate features of the intestinal microbiota that are associated with *Pneumocystis* pneumonia. While the current data demonstrates an associative change in the microbiota with *P. murina* infection and causative studies are needed to completely understand the role of the gut-lung axis during *Pneumocystis* infection. This research strongly warrants further studies to fully appreciate the role and effects of the intestinal microbiome on respiratory disease. This data also provides a foundation for future studies involving transcriptional analysis to better establish the metabolomics profiles associated with respiratory disease. In future studies, it will be interesting to dissect whether or not the microbiota changes contribute to the control of respiratory infection or participate in the induction of *P. murina* immune memory. Furthermore, this data warrants further investigation of the biogeographical and spatial distribution of the microbiota within the intestinal tract. To our knowledge this is the first report of respiratory infection induced changes to the intestinal microbiota.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic outline of the experimental protocol used in this study

C57BL/6 mice were infected intratracheally with live *P. murina* ($\sim 2 \times 10^5$ cysts in 100 μ l of PBS) and sacrificed at 7 and 14 days post infection. Mice were grouped into the following categories; uninfected animals (designated Sham Infected), *P. murina* infected immune intact animals (*P. murina* Infected), and mice depleted of CD4+ T cells and infected with *P. murina* (CD4+ T cell Depletion + *P. murina* Infection). CD4-depletion started three days prior to infection with *P. murina* and was maintained continuously throughout the experiment.

Figure 2. *Pneumocystis pneumonia* significantly alters the intestinal microbiota community 7 days post infection

Immune intact mice and CD4-depleted mice were infected intratracheally with live *P. murina* ($\sim 2 \times 10^5$ cysts in 100 μ l of PBS), control animals were given a naïve lung homogenate. Animals were then sacrificed seven days post infection and *P. murina* lung burden and microbiota community structures were assessed. (A) *P. murina* lung burden 7 days post infection. (B) IFN- γ levels in the lung and (C) IFN- γ levels in the serum 7 days post infection. Principal coordinate analysis of the Unifrac metric (D unweighted and E weighted). (F) Observed species alpha diversity and (G) taxonomy classification were analyzed using QIIME software to determine differences in microbial community structures. The sequences from 10 animals per treatment are shown in each analysis. The taxonomy summary (G) represents taxa with greater than 1% total representation. Each dot represents an individual mouse, bars represent mean $\pm$ SEM, N=10. For taxonomic classifications * indicates $P < 0.05$ for control infected v. *P. murina* infected animals, + indicates $P < 0.05$ for control infected animals v. *P. murina* infected CD4-depleted mice, and # indicates $P < 0.05$ for *P. murina* infected v. *P. murina* infected CD4-depleted mice by two-way ANOVA with Bonferroni multiple comparisons corrected posttest. Mice with no detectable *P. murina* in the lung were given a count of 1 for visualization on a log scale.

Figure 3. Core microbiome of sham infected mice, *P. murina* infected mice, and CD4-depleted mice infected with *P. murina* 7 days post infection
Core taxa present in 100% of each treatment condition were compared. Taxa in gray were found in all of the animals, taxa in red were only found in *P. murina* infected animals, taxa in green were only found in sham infected animals, taxa in blue were only found in CD4-depleted animals infected with *P. murina*, taxa in yellow were only found in *P. murina* infected animals and sham infected mice, taxa in purple were found in *P. murina* infected animals and CD4-depleted animals infected with *P. murina*, and taxa in teal were found in sham infected animals and CD4-depleted animals infected with *P. murina*. Taxa found in each section are shown in the breakout tables.

Figure 4. *Pneumocystis pneumonia* significantly alters the inferred functional capacity of the intestinal microbiota community 7 days post infection

Immune intact mice and CD4-depleted mice were infected intratracheally with live *P. murina* ($\sim 2 \times 10^5$ cysts in 100 μ l of PBS), control animals were given a naïve lung homogenate. Animals were then sacrificed seven days post infection and *P. murina* lung burden and inferred functional capacity of the microbiota communities were assessed. (A) The relative abundance of seven functional categories during *P. murina* infection. (B) Relative abundance of specific pathways involved in carbohydrate metabolism. (C) Relative abundance of specific pathways involved in signal transduction. (D) Relative abundance of specific pathways involved in energy metabolism. (E) Relative abundance of specific pathways involved in xenobiotic metabolism. Boxplots denote top quartile and bottom quartile, whiskers are plotted by min to max, N=10, * indicates P < 0.05 for control infected v. *P. murina* infected animals, by two-way ANOVA with Bonferroni multiple comparisons corrected posttest.

Figure 5. *Pneumocystis pneumonia* significantly alters the intestinal microbiota community 14 days post infection

Immune intact mice and CD4-depleted mice were infected intratracheally with live *P. murina* ($\sim 2 \times 10^5$ cysts in 100 μ l of PBS), control animals were given a naïve lung homogenate. Animals were then sacrificed fourteen days post infection and *P. murina* lung burden and microbiota community structures were assessed. (A) *P. murina* lung burden 14 days post infection. (B) IFN- γ levels in the lung and (C) IFN- γ levels in the serum 14 days post infection. Principal coordinate analysis of the Unifrac metric (D unweighted and E weighted). (F) Observed species alpha diversity and (G) taxonomy classification were analyzed using QIIME software to determine differences in microbial community structures. The sequences from 10 animals per treatment are shown in each analysis. The taxonomy summary (G) represents taxa with greater than 1% total representation. Each dot represents an individual mouse, bars represent mean $\pm$ SEM, N=10. For taxonomic classifications * indicates $P < 0.05$ for control infected v. *P. murina* infected animals, + indicates $P < 0.05$ for control infected animals v. *P. murina* infected CD4-depleted mice, and # indicates $P < 0.05$ for *P. murina* infected v. *P. murina* infected CD4-depleted mice by two-way ANOVA with Bonferroni multiple comparisons corrected posttest. Mice with no detectable *P. murina* in the lung were given a count of 1 for visualization on a log scale.

Figure 6. Core microbiome of sham infected mice, *P. murina* infected mice, and CD4-depleted mice infected with *P. murina* 14 days post infection

Core taxa present in 100% of each treatment condition were compared. Taxa in gray were found in all of the animals, taxa in red were only found in *P. murina* infected animals, taxa in green were only found in sham infected animals, taxa in blue were only found in CD4-depleted animals infected with *P. murina*, taxa in yellow were only found in *P. murina* infected animals and sham infected mice, taxa in purple were found in *P. murina* infected animals and CD4-depleted animals infected with *P. murina*, and taxa in teal were found in sham infected animals and CD4-depleted animals infected with *P. murina*. Taxa found in each section are shown in the breakout tables.

Figure 7. *Pneumocystis pneumonia* significantly alters the inferred functional capacity of the intestinal microbiota community 14 days post infection

Immune intact mice and CD4-depleted mice were infected intratracheally with live *P. murina* ($\sim 2 \times 10^5$ cysts in 100 μ l of PBS), control animals were given a naïve lung homogenate. Animals were then sacrificed fourteen days post infection and *P. murina* lung burden and inferred functional capacity of the microbiota communities were assessed. (A) The relative abundance of seven functional categories during *P. murina* infection. (B) Relative abundance of specific pathways involved in cell motility. (C) Relative abundance of specific pathways involved in signal transduction. (D) Relative abundance of specific pathways involved in biosynthesis of secondary metabolites. (E) Relative abundance of specific pathways involved in energy metabolism. (F) Relative abundance of specific pathways involved in carbohydrate metabolism. (G) Relative abundance of specific pathways involved in xenobiotic metabolism. (H) Relative abundance of specific pathways involved in lipid metabolism. Boxplots denote top quartile and bottom quartile, whiskers are plotted by min to max, N=10, * indicates $P < 0.05$ for control infected v. *P. murina* infected animals or for *P. murina* infected v. *P. murina* infected CD4-depleted mice. # indicates $P < 0.05$ for Sham infected v. *P. murina* infected CD4-depleted mice by two-way ANOVA with Bonferroni multiple comparisons corrected posttest.