Published in final edited form as:

Fungal Genet Biol. 2019 June ; 127: 45–49. doi:10.1016/j.fgb.2019.03.001.

Profound mycobiome differences between segregated mouse colonies do not influence Th17 responses to a newly introduced gut fungal commensal

Itai Doron^{1,2,#}, Irina Leonardi^{1,2,#}, Iliyan D. Iliev^{1,2,3,4,*}

¹Gastroenterology and Hepatology Division, Joan and Sanford I. Weill Department of Medicine, Weill Cornell Medicine, Cornell University, New York, NY 10021, USA.

²The Jill Roberts Institute for Research in Inflammatory Bowel Disease, Weill Cornell Medicine, Cornell University, New York, NY 10021, USA.

³Department of Microbiology and Immunology, Weill Cornell Medicine, Cornell University, New York, NY 10065, USA

⁴Immunology and Microbial Pathogenesis Program, Weill Cornell Graduate School of Medical Sciences, Weill Cornell Medicine, Cornell University, New York, NY 10065, USA.

Abstract

Gut mycobiota dysbiosis can negatively impact the outcome of several diseases of inflammatory origin, suggesting a role of the mycobiota in influencing the host immunity. However, it is unknown whether the gut mycobiota composition can create an immune environment that would influence the immune response to a newly introduced intestinal fungus. Using ITS1 deep sequencing, we evaluated the mycobiome structure of C57BL/6J mice acquired from Jackson (JAX) or bred in a controlled environment at our own mouse facility (WCM-CE) for several generations. We found that C57BL/6J mice from these segregated mouse colonies harbor dramatically different mycobiota. To assess whether the mycobiota make up can influence immune responses to colonization with a fungus foreign to the murine GI tract, we colonized JAX and WCM-CE mice with the human commensal *C. albicans* and measured Th17 responses in the gut. We found that independent of mycobiota composition, mice produced strong Th17 responses to gastrointestinal *C. albicans* colonization. Our data suggest that different mouse colonies can carry dramatically different mycobiota. Nevertheless, strong Th17 responses to a newly introduced opportunistic commensal fungus are potentially induced independent of the mycobiota background in this experimental setting.

Introduction

The microbiome has a powerful effect on host immunity and disease outcomes. Multiple experimental studies suggest that the microbiome is a stronger driver of immune and disease phenotypes than the genetic makeup (1). TLR2^{-/-} mice are highly susceptible to chemically

*Correspondence to: iliev@med.cornell.edu.

#These authors contributed equally to the study

induced colitis in one facility while inflammation is histologically undetectable in another facility (2, 3). The deletion of the fractalkine receptor CX3CR1 in mice has been shown to confer both susceptibility and protection to DSS-induced colitis between different facilities (4, 5). Controlled experiments using mice from two different facilities showed that the Nlrp6-ASC inflammasome, previously regarded as a hallmark host innate immune axis controlling gut microbiota, has a minimal impact on microbiota composition (6). It has been further shown that genetically identical mice from different facilities have different ability to mount Th17 responses (7). Such dramatic changes in phenotypes between genotypically similar mice have thus been attributed to dissimilarities in their microbiomes and several controlled studies have identified microbes or microbial factors responsible for these effects (7–9).

In addition to bacteria, fungi are a key component of the microbiome and can be influenced by diet, antibiotic treatment, environment or disease status (10–13). Similar to what has been observed for commensal bacteria, fluctuations in the mycobiota can influence immunity and disease phenotypes. The presence of the murine gut commensal *Candida tropicalis* can aggravate experimental colitis in genetically susceptible mice, while its absence leads to less severe disease and even protective outcome associated with the outgrowth of *Lactobacillus murinus* and the expansion of T_{REG} cells (13, 14). We have recently shown that gut mycobiota dysbiosis induced by prolonged treatment with antifungal drugs can negatively influence the outcome of airway allergic disease (15, 16). These findings, suggest a role of the mycobiota in influencing the host immunity. However, it is unknown whether the gut mycobiota composition can create an immune environment that would influence the immune response to a newly introduced intestinal fungus.

To assess this question, using ITS1 deep sequencing approach we evaluated the mycobiome structure of C57BL/6J mice acquired from Jackson or bred in a controlled negative pressure environment at our own facility (WCM-CE) for several generations. We found that C57BL/6J mice from this segregated mouse colony harbor dramatically different mycobiota. JAX mice had a more diverse mycobiota predominantly driven by the Basidiomycota phylum while the mycobiota of mice bred in our facility was mostly composed of Ascomycota. To assess whether such dramatically different mycobiota make up can influence immune responses to colonization with a fungus foreign to the murine GI tract, we colonized mice with the human commensal *C. albicans* and measured Th17 responses in the gut. We found that independent of mycobiota composition, mice produced strong Th17 responses to gastrointestinal *C. albicans* colonization. Our data suggest that different mouse colonies can carry dramatically different mycobiota. Nevertheless, strong Th17 responses to a newly introduced opportunistic fungal commensal are potentially induced independent of the mycobiota background.

Results

Commercially available Jackson C57BL/6J mice and C57BL/6J mice bred in a controlled environment at WCM facility carry dramatically different gut mycobiota.

To investigate the variability of the mycobiota between mice of different facilities we used high-throughput sequencing of the internal transcribed spacer 1 (ITS1) region of fungal

ribosomal DNA. Using this approach, we characterized the intestinal fungi of C57BL/6J mice bred in a controlled negatively pressurized environment at a dedicated room in our facilities at Weill Cornell Medicine (WCM-CE) and C57BL/6J mice obtained from the Jackson Laboratory's Mice & Services (JAX). We observed substantial differences between mycobiota β - and α -diversity of WCM-CE and JAX mice (Figure 1A–C). Further analysis revealed that differences are driven by Ascomycota phyla that was highly abundant in WCM-CE mice and Basidiomycota that was more abundantly represented in JAX mice (Figure 1C). Consistent with these findings, we observed considerable differences between WCM-CE and JAX mice at the genus level (Figure 1D). This was most notable in the relative abundance of *Candida* species, which make up the majority of the WCM-CE mycobiota, while only constituting ~25% of the JAX mycobiota (Figure 1D). Similar comparisons of several most abundant genera among mice from both facilities revealed that the relative abundance of most other genera was higher in JAX mice, including mostly Basidiomycota genera (*Cryptococcus*, *Wallemia*, *Sporobolomyces*, and *Guehomyces*; Figure 1D), as well as several Ascomycota genera (*Phoma*, *Galactomyces*, *Saccharomyces*, and *Alternaria*; Figure 1D). Nevertheless the total fungal load in JAX mice was significantly lower (13). Interestingly, JAX mice mycobiota composition remained highly dissimilar to WCM-CE mycobiota even 3 weeks after being housed in the WCM-CE room under the same diet as the WCM-CE mice (Figure 1E). Altogether, this data show that mice from segregated colonies carry dramatically different gut mycobiota.

Th17 responses to a newly introduced opportunistic fungus are not depended to gut mycobiota composition.

Since we have previously shown that changes in mouse native mycobiota can have dramatic effects on host immunity (15, 16), we wondered whether immunity to an unrelated exogenous fungus can be influenced by original microbiota composition. To assess the functional outcome of mycobiota composition on immunity to a newly introduced fungal pathogen, we colonized JAX and WCM-CE mice with the human commensal *C. albicans*. Since *C. albicans* does not naturally colonize the murine gastrointestinal tract, mice are not naturally exposed to this human commensal. Yet, *C. albicans* can colonize and persist in the murine GI tract upon antibiotic treatment (16). Colonization of mice with *C. albicans* by oral gavage induces strong IL-17 production by CD4⁺ T cells in the murine gut (13, 17) allowing us to reliably track the induction of T cell responses upon fungal colonization. To this end, we colonized WCM-CE and JAX mice with *C. albicans* for 10 days with concomitant administration of the antibiotic cefoperazone. Interestingly, the baseline levels of Th17 polarization were lower (although not significantly) in Jackson mice as indicated by IL-17 production and ROR γ t expression among CD4⁺ T cells. *C. albicans* colonization induced the expression of ROR γ t in the colonic lamina propria CD4⁺ T cells of both WCM-CE and JAX mice (Figure 2A, B). Accordingly, CD4⁺ T cells from the cLP of WCM-CE and JAX mice both showed increased production of IL-17 following *C. albicans* colonization (Figure 2C, D). This data suggest that Th17 immune responses to a newly introduced opportunistic fungus to which mice are naïve do not depended on gut mycobiota composition.

Discussion

Our findings suggest that intestinal immunity to a newly introduced fungal opportunistic commensal can overcome the effect of gut microbiota on the host immunity and argue towards the importance of Th17 responses to gut opportunistic fungi. Further studies should elucidate whether such a prompt response to opportunistic fungal colonization has benefit to the host and whether it would influence other non-Th17 immune responses.

Previous studies have documented that, similar to what has been described for bacteria, healthy laboratory mice show a high degree of temporal fungal variation (10, 18). Our study explored the variation of mycobiota between two highly controlled barrier facility environments. We found that in addition to previously described variations (10), facility environment can dramatically influence the mycobiota composition. It is yet unclear whether the *Basidiomycota* domination identified in JAX mice represent true intestinal colonizers or is rather due to environmental and dietary fungi transiently passing through the GI tract from the air or diet (19). Nonetheless, compositional differences did not influence Th17 responses to newly introduced *C. albicans*. We further show that it is possible to maintain a “high Ascomycota” mycobiota in a controlled setting. Given the recently reported importance of fungi in mouse models of colitis, colon cancer, liver disease and lung allergy (13, 16, 20–23), increased presence in species of phylum Basidiomycota, but most notably high Ascomycota (mostly driven by *Candida spp.*) content in the gut of IBD patients (24, 25), our results suggest that maintenance of “high Ascomycota” mouse colonies is possible. Such mice can provide better options for modeling human diseases.

Materials and Methods

Mice

C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). WCM-CE mice were generated after C57BL/6J mice were subsequently bred for at least four generations, housed under specific pathogen-free (SPF) conditions in a negative pressure controlled environment at Weill Cornell Medicine. All experiments were conducted with at least four mice per group with prior approval from the Institutional Care and Use Committee of Weill Cornell Medicine.

DNA isolation and fungal gene quantitative analysis

2–3 fecal pellets were collected per mouse between 8 and 12 weeks of age. Total DNA was isolated following lyticase treatment, bead beating with 0.1mm and 0.5 mm beads and processing using Qiagen’s QIAmp DNA mini kit as in (13). Fungal DNA was validated in each sample by RT-qPCR.

Mycobiota library generation, sequencing, and analysis

Fungal ITS1–2 regions were amplified by PCR using the following primers: ITS1F CTTGGTCATTTAGAGGAAGTAA; ITS2R GCTGCGTTCTTCATCGATGC. Primers were modified to include forward (5’ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]) and reverse (5’

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]) sequencing adaptors. ITS1–2 amplicons were generated with 35 cycles using Invitrogen AccuPrime PCR reagents (Carlsbad, CA). In a second PCR reaction, amplicons from each sample were uniquely indexed using Illumina Nextera XT v2 barcoded primers (San Diego, CA). 2×300 paired end sequencing was performed on an Illumina MiSeq platform (Illumina, CA) using the following PCR protocol: Initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 2 min, followed by an elongation step at 72°C for 30 min. Quality control of all libraries were conducted by qPCR, DNA 1000 Bioanalyzer (Agilent), and Qubit (Life Technologies) to validate and quantify library construction prior to preparing a Paired End flow cell. Analysis was performed as in our recent studies (13, 16).

Growth and administration of *Candida albicans* strain

Candida albicans (SC5314) was obtained from the American Type Culture Collection (Manassas, VA). *Candida* was cultured in aerobic conditions on Sabouraud Dextrose Broth (SDB; EMD Chemicals) at 37°C. Cefoperazone (0.4 mg/ml; Sigma-Aldrich, St. Louis, MO) was provided to all mice ad libitum in drinking water for the whole length of the colonization experiments. Mice were administered via oral gavage with $5 \cdot 10^7$ *C. albicans* cells at days 1, 4 and 7 after the start of the cefoperazone water. Mice were sacrificed at day 10 and sampled as described below.

Isolation of large intestine lamina propria cells

Mice were sacrificed and mesenteric lymph nodes (mLN) and large intestine (colon and cecum) were removed by dissection and placed in cold PBS until further processing. mLN were mashed onto a nylon screen (70µm), and the cells obtained were pooled, washed twice and resuspended in cold PBS. Intestinal LP cells were isolated as previously described (13). The cell suspensions were filtered through a mesh, overlaid on percoll or used directly for immunophenotyping of LP cells.

Antibodies and flow cytometry

Cell suspensions were prepared as described above, blocked with CD16/CD32 (Mouse BD Fc Block™, 2.4G2, BD Biosciences) and stained with antibodies against CD4 (GK1.5, eBioscience), CD45 (30-F11, Tonbo). For intracellular staining of transcription factors, cells were stained with surface markers, fixed permeabilized and stained with FoxP3 (FJK-16s, eBioscience) and RORgt (B2D, eBioscience). For intracellular cytokine detection, cells were incubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich) and 10 µg/ml Brefeldin A (BFA; Sigma-Aldrich) in complete RPMI media at 37° C for 6 h. After surface staining with CD45, CD4 cells were fixed, permeabilized and staining was performed using PE-labeled anti-IL-17 mAb (eBio17B7; eBiosciences) according to the manufacturer's instructions. Flow cytometry was performed using a LSRFortessa (BD Biosciences) and data were analyzed with FlowJo software (TreeStar Inc.).

Acknowledgments

This work was funded by the US National Institutes of Health (grants DK113136 and AI137157 to I.D.I.), Crohn's and Colitis Foundation Senior Research Award and Irma T. Hirschl Career Scientist awards to I.D.I., Crohn's and Colitis Foundation research fellowship to I.L. and support from the Jill Roberts Institute for Research in IBD.

References

- Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Liston A, and Raes J. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol* 2013;14(1):R4. [PubMed: 23347395]
- Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, and Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 2004;118(2):229–41. [PubMed: 15260992]
- Cario E, Gerken G, and Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology* 2007;132(4):1359–74. [PubMed: 17408640]
- Medina-Contreras O, Geem D, Laur O, Williams IR, Lira SA, Nusrat A, Parkos CA, and Denning TL. CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. *The Journal of clinical investigation* 2011;121(12):4787–95. [PubMed: 22045567]
- Kostadinova FI, Baba T, Ishida Y, Kondo T, Popivanova BK, and Mukaida N. Crucial involvement of the CX3CR1-CX3CL1 axis in dextran sulfate sodium-mediated acute colitis in mice. *Journal of leukocyte biology* 2010;88(1):133–43. [PubMed: 20335311]
- Mamantopoulos M, Ronchi F, Van Hauwermeiren F, Vieira-Silva S, Yilmaz B, Martens L, Saeys Y, Drexler SK, Yazdi AS, Raes J, et al. Nlrp6- and ASC-Dependent Inflammasomes Do Not Shape the Commensal Gut Microbiota Composition. *Immunity* 2017;47(2):339–48 e4. [PubMed: 28801232]
- Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, and Littman DR. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell host & microbe* 2008;4(4):337–49. [PubMed: 18854238]
- Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* 2011;331(6015):337–41. [PubMed: 21205640]
- Round JL, and Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009;9(5):313–23. [PubMed: 19343057]
- Dollive S, Chen YY, Grunberg S, Bittinger K, Hoffmann C, Vandivier L, Cuff C, Lewis JD, Wu GD, and Bushman FD. Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. *PLoS One* 2013;8(8):e71806. [PubMed: 23977147]
- Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, Lewis JD, and Bushman FD. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PLoS One* 2013;8(6):e66019. [PubMed: 23799070]
- Erb Downward JR, Falkowski NR, Mason KL, Muraglia R, and Huffnagle GB. Modulation of post-antibiotic bacterial community reassembly and host response by *Candida albicans*. *Sci Rep* 2013;3(2191).
- Leonardi I, Li X, Semon A, Li D, Doron I, Putzel G, Bar A, Prieto D, Rescigno M, McGovern DPB, et al. CX3CR1(+) mononuclear phagocytes control immunity to intestinal fungi. *Science* 2018;359(6372):232–6. [PubMed: 29326275]
- Tang C, Kamiya T, Liu Y, Kadoki M, Kakuta S, Oshima K, Hattori M, Takeshita K, Kanai T, Saijo S, et al. Inhibition of Dectin-1 Signaling Ameliorates Colitis by Inducing *Lactobacillus*-Mediated Regulatory T Cell Expansion in the Intestine. *Cell host & microbe* 2015;18(2):183–97. [PubMed: 26269954]
- Wheeler ML, Limon JJ, Bar AS, Leal CA, Gargus M, Tang J, Brown J, Funari VA, Wang HL, Crother TR, et al. Immunological Consequences of Intestinal Fungal Dysbiosis. *Cell host & microbe* 2016;19(6):865–73. [PubMed: 27237365]

16. Li X, Leonardi I, Semon A, Doron I, Gao H, Putzel H, Kim Y, Kabata H, Artis D, Fiers W, Ramer-Tait A, Iliev ID Response to Fungal Dysbiosis by Gut Resident CX3CR1+ Mononuclear Phagocytes Aggravates Allergic Airway Disease. *Cell Host and Microbes* 2018;In Press(
17. Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, Suda W, Imaoka A, Setoyama H, Nagamori T, et al. Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell* 2015;163(2):367–80. [PubMed: 26411289]
18. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009;139(3):485–98. [PubMed: 19836068]
19. Suhr MJ, and Hallen-Adams HE. The human gut mycobiome: pitfalls and potentials--a mycologist's perspective. *Mycologia* 2015;107(6):1057–73. [PubMed: 26354806]
20. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, Brown J, Becker CA, Fleshner PR, Dubinsky M, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* 2012;336(6086):1314–7. [PubMed: 22674328]
21. Wang T, Fan C, Yao A, Xu X, Zheng G, You Y, Jiang C, Zhao X, Hou Y, Hung MC, et al. The Adaptor Protein CARD9 Protects against Colon Cancer by Restricting Mycobiota-Mediated Expansion of Myeloid-Derived Suppressor Cells. *Immunity* 2018;49(3):504–14 e4. [PubMed: 30231984]
22. Malik A, Sharma D, Malireddi RKS, Guy CS, Chang TC, Olsen SR, Neale G, Vogel P, and Kanneganti TD. SYK-CARD9 Signaling Axis Promotes Gut Fungi-Mediated Inflammasome Activation to Restrict Colitis and Colon Cancer. *Immunity* 2018;49(3):515–30 e5. [PubMed: 30231985]
23. Yang AM, Inamine T, Hochrath K, Chen P, Wang L, Llorente C, Bluemel S, Hartmann P, Xu J, Koyama Y, et al. Intestinal fungi contribute to development of alcoholic liver disease. *The Journal of clinical investigation* 2017.
24. Lewis JD, Chen EZ, Baldassano RN, Otley AR, Griffiths AM, Lee D, Bittinger K, Bailey A, Friedman ES, Hoffmann C, et al. Inflammation, Antibiotics, and Diet as Environmental Stressors of the Gut Microbiome in Pediatric Crohn's Disease. *Cell host & microbe* 2015;18(4):489–500. [PubMed: 26468751]
25. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, Cohen D, Liguori G, Bourrier A, Nion-Larmurier I, et al. Fungal microbiota dysbiosis in IBD. *Gut* 2017;66(6):1039–48. [PubMed: 26843508]

Figure 1. Gut mycobiota differs between mice housed at two segregated facilities. 6–8 week old C57Bl/6J mice were purchased by Jackson (JAX) or bred mice in a helicobacter-negative controlled environment room at WCM (WCM-CE). **A.** Nonmetric multidimensional scaling (NMDS) plot of distance ordination based on the Bray-Curtis dissimilarities in the colon of WCM-CE and Jackson facility mice for fungal OTUs. **B.** α diversity (Shannon and Simpson diversity indices) among the fungal mycobiota of mice from each facility, each dot represents an individual mouse. **C.** NMDS plot of distance ordination between Ascomycota (left) and Basidiomycota (right) phyla in the colons of WCM-CE and JAX mice. **D.** Bar graphs representing the composition of the fecal mycobiota of WCM-CE and JAX mice. **E.** NMDS plot of distance ordination between JAX mice at day 0 (blue) or day 21 (green) in WCM-CE room, and WCM-CE mice (red). Legend represents main fungal genera found in the mice with fungi belonging to Ascomycota phylum in black and Basidiomycota phylum in purple. Bar graph values represent relative abundances by

genus; each bar represents an individual mouse. Statistical analysis: * $P < 0.05$, ** $P < 0.01$ (Unpaired T-test).

Figure 2. *Candida albicans* colonization induces Th17 responses in cLP of Jackson and WCM-CE mice.

C57Bl/6J mice purchased by Jackson (JAX) or bred in WCM helicobacter-negative controlled environment room (WCM-CE) were sacrificed 10 days post colonization (+ *C. a.*) or not (NT) with 5×10^7 *C. albicans* at day 10. **A.** Representative flow cytometry plot and quantification of the expression of RORγt and IL-17 by CD4⁺ T cells in the colonic lamina propria (cLP) of WCM-CE Mice. **B.** Representative flow cytometry plot and quantification of the expression of RORγt by CD4⁺ T in the colonic cLP of JAX Mice. **C.** Representative flow cytometry plot and quantification of the production of IL-17 by CD4⁺ T in the colonic cLP of WCM-CE Mice. **D.** Representative flow cytometry plot and quantification of the expression of IL-17 by CD4⁺ T in the colonic cLP of JAX Mice. **C.** Each dot represents an individual mouse. Statistical analysis: * $P < 0.05$, ** $P < 0.01$ (two tailed, Mann-Whitney U test).