PEARLS

Into the wild: How exposure to wild or domesticated fungi shapes immune responses in mice

Ying-Han Chen[1], Ken Cadwell [1,2,3]*

1 Kimmel Center for Biology and Medicine at the Skirball Institute, New York University Grossman School of Medicine, New York, New York, United States of America, 2 Department of Microbiology, New York University Grossman School of Medicine, New York, New York, United States of America, 3 Division of Gastroenterology and Hepatology, Department of Medicine, New York University Grossman School of Medicine, New York, New York, United States of America

* Ken.Cadwell@med.nyu.edu

Competing interests: I have read the journal's policy and have the following conflictsKC has received research support or consulting fees from Pfizer, Takeda, Pacific Biosciences, Puretech Health, Genentech, and Abbvie. KC is an inventor

What is the gut mycobiota and why should we care?

Throughout our lives, humans are exposed to diverse microbes that impact the development and function of our immune system. As an immense and absorptive mucosal surface exposed to the external environment, the gastrointestinal (GI) tract is a major interface in which these immunogenic interactions occur. Human subject studies have linked disruption of the intestinal bacterial community to a variety of disease conditions, and experimental models have helped us interpret these findings by elucidating mechanisms by which bacteria evoke pro- or anti-inflammatory responses through host cells and receptors. More recent studies are beginning to define the impact of nonbacterial agents in the gut such as fungi.

The collection of fungi present in the GI tract is referred to as the gut mycobiota and can be considered part of the larger community of microbiota and meiofauna inhabitants consisting of bacteria, archaea, viruses, protozoa, and helminths. Although relatively low in abundance compared with bacteria, fungi are ubiquitous colonizers with a large role in the developmental succession of the infant microbiota [1]. Genera of fungi in the human gut include *Candida*, *Saccharomyces*, *Aspergillus*, and *Malassezia*. However, at least some of the fungi routinely detected in the human gut are transient passengers ingested with food [2]. Stable colonization may require adaptation to the host GI tract, such as evasion of antibody responses and the ability to switch between yeast and hyphal cell morphology [3–5].

Early-life exposure to fungi is associated with reduced prevalence of wheezing and asthma later in childhood [6]. Also, alterations in the abundance of fungi and colonization by particular strains, such as those that produce toxins at high levels, are linked with susceptibility to immune-mediated disorders, most notably inflammatory bowel disease (IBD) [7–10]. Medical interventions that disrupt the balanced coexistence of intestinal fungi and bacteria, such as allogeneic hematopoietic cell transplantation, can lead to disastrous health consequences for the host [11]. These observations reveal the existence of a consequential symbiotic relationship with a community of intestinal fungi.

What local responses are triggered during intestinal colonization by fungi?

Numerous mechanisms restrict the ability of fungi to establish invasive infections or diseases in the GI tract. In addition to Toll-like receptors (TLRs) and nucleotide oligomerization

domain (NOD)-like receptors (NLRs) well known in the innate immunity field, pattern-recognition C-type lectin receptors (CLRs) play a central role in fungal recognition. CLRs Dectin-1, Dectin-2, and Mincle activate Spleen tyrosine kinase (Syk) upon binding carbohydrate polymers of the fungal cell wall such as β-glucan, mannans, and chitin, trigger signaling pathways leading to cytokine production, phagocytosis, and other effector functions. In the gut, CX3CR1+ mononuclear phagocytes (MNPs) highly express CLRs and downstream signaling mediators and, following recognition of fungi, migrate to the draining lymph nodes to induce the differentiation of naïve T cells into T helper 17 (Th17) cells and production of antifungal antibodies [12]. Some fungi produce gliotoxin and other metabolic by-products that are toxic to host cells. Macrophages in the colon extend balloon-like protrusions into the lumen that absorb fluids containing these mycotoxins to prevent their hazardous uptake by the epithelium [13]. These processes act in concert to limit fungal overgrowth, avoid mycobiota dysbiosis, and prevent inflammatory damage.

Does colonization by intestinal fungi contribute to the normal development of the immune system?

Laboratory mice are broadly used for studying how the microbiota contributes to the maturation and function of the immune system. However, there is growing evidence that the limited microbial exposure experienced by laboratory mice kept in ultra-hygienic specific pathogen-free (SPF) facilities leads to a less mature immune system when compared with free-living mammals such as humans. Laboratory mice exposed to feral or pet store mice, also referred to as "dirty" mice, better recapitulate observations made in humans in models of infectious disease, colorectal cancer, and pharmaceutical interventions [14–17]. In these experiments, immune maturation is associated with exposure to disease-causing transmissible agents (i.e., pathogens), especially viruses and parasites. Do fungi contribute to maturation of the immune system?

Laboratory mice that experience a seminatural environment through release into an outdoor enclosure facility, a process referred to as "rewilding," display >100-fold increases in fungal burden [18]. In this outdoor enclosure, the zinced iron walls with electric fencing excludes predators and rodents harboring pathogens, while allowing exposure to natural soil, vegetation, insects, and weather (Fig 1). As such, rewilded laboratory mice are exposed to environmental microbes (i.e., microbes acquired from the environment rather than through horizontal transfer from another rodent) but remain seronegative for pathogens excluded from SPF facilities. The fungal mycobiota of rewilded mice resembles wild mice [16] and is characterized by increased diversity and acquisition of Ascomycota phylum members, especially *Aspergillus* species. In addition to exhibiting hallmarks of increased lymphocyte differentiation and cytokine production, rewilded mice display a striking expansion of granulocytes, a group of myeloid lineage white blood cells such as neutrophils [18,19]. Granulocyte numbers in the blood correlate with fungal burden, and transferring fungi isolated from the feces of rewilded mice into laboratory mice reproduces the increase in peripheral granulocytes. Similarly, colonizing the intestines of laboratory mice with *Candida albicans* leads to an increase in circulating granulocytes, and reducing fungal burden with the antifungal drug fluconazole leads to a corresponding decrease in granulocytes. Hence, fungi in the natural environment contribute to the composition of circulating immune cells.

It is possible that rewilded mice represent an intermediary condition of microbial exposure occupying a state in between SPF and feral mice. Rather than a binary status of "clean versus dirty" or "naïve versus exposed," immune maturation induced by microbes likely exists in a continuum. Humans display interindividual differences in their history of infections.

Fig 1. Free-range laboratory mice. (**A**) A close-up view of a wedge in the Stony Ford outdoor enclosure facility. Individual wedges contain 2 watering stations and a feeding station (yellow star) supplied with the same mouse chow that laboratory mice receive in the institutional vivarium. (**B**) During the process of rewilding, adult C57BL/6J mice raised in a specific pathogen-free (SPF) facility are introduced into the enclosure where they are exposed to fungi in the environment as they explore their surroundings. *Photo courtesy of Christina B. Hansen and Andrea Graham, Princeton University.*

https://doi.org/10.1371/journal.ppat.1010841.g001

Incorporating mouse models with different degrees of microbial exposure including fungi may help capture some of this diversity.

In what other ways does intestinal colonization by fungi affect physiology?

Human Th17 cells reactive to gut-resident *C. albicans* are cross-reactive with airborne fungi [20], and in mice, this Th17 response protects against invasive bacterial and fungal infections [21,22]. However, *C. albicans* strains recovered from the intestines of IBD patients display high cytotoxicity and induce greater production of the inflammatory cytokine IL-1β from macrophages compared with non-IBD isolates [7], highlighting the fine line between beneficial and adverse immune reactions to fungal colonization. Adverse consequences of an altered gut mycobiota are not limited to the GI tract. In mouse models of allergic airway inflammation, administration of antimicrobials that cause overgrowth of intestinal fungi can exacerbate disease through multiple mechanisms, including increasing prostaglandin E2 (PGE2) levels that alter the polarity of lung macrophages and local activation of CX3CR1+ MNPs that promote long-distance eosinophil infiltration [23,24]. Alteration of gut mycobiota composition and burden are also linked to alcoholic hepatitis through the translocation of β-glucan into systemic circulation [25]. Another study found a positive correlation between alcoholic hepatitis and fecal candidalysin, an exotoxin secreted by *C. albicans* [26]. Lastly, systemic IL-17A induced by mucosal fungi acts on neurons to regulate social behavior in mice, demonstrating how the gut mycobiota can contribute to the gut–brain axis [27].

Can we improve disease models by incorporating intestinal fungi?

How the gut mycobiota influences the host may be useful to consider when attempting to model disease events in laboratory mice because intestinal fungi are generally low in abundance or undetectable by culturing methods in mice bred in SPF facilities. For example, blood neutrophil levels are lower in laboratory mice than in humans. As a major phagocyte

population and first line defenders during infections, this discrepancy may confound attempts to fully utilize the mouse model to study immunity. Recent evidence highlights a previously unappreciated heterogeneity of neutrophils, especially mature neutrophils in the periphery, which may be key for their function during chronic conditions, such as cardiovascular inflammation [28]. The diversity of neutrophils was revealed by single-cell RNA sequencing analysis, which identified an expanded subset enriched in the expression of interferon (IFN)-stimulated genes during bacterial infection [29]. In addition to increasing the number of circulating neutrophils, does GI colonization by fungi impact neutrophil maturation and function?

While colonizing laboratory mice with a genetically tractable model fungus represents an accessible reductionist approach to address these types of questions, it would be highly informative to fully characterize rewilded mice, which display alterations in abundance and composition of both the gut mycobiota and bacterial microbiota. Other experimental systems add another level of complexity by introducing viruses, which have broad effects on the mucosal immune system [30,31]. Knowledge gained from comparing different gradations of microbial exposure will better define the relative contribution of intestinal fungi to immunity and may help us improve the mouse model for investigating the biology of free-living mammals.

References

1. Rao C, Coyte KZ, Bainter W, Geha RS, Martin CR, Rakoff-Nahoum S. Multi-kingdom ecological drivers of microbiota assembly in preterm infants. Nature. 2021; 591(7851):633–8. Epub 2021/02/26. https://doi.org/10.1038/s41586-021-03241-8 PMID: 33627867; PubMed Central PMCID: PMC7990694.

2. Auchtung TA, Fofanova TY, Stewart CJ, Nash AK, Wong MC, Gesell JR, et al. Investigating Colonization of the Healthy Adult Gastrointestinal Tract by Fungi. mSphere. 2018; 3(2). Epub 20180328. https://doi.org/10.1128/mSphere.00092-18 PMID: 29600282; PubMed Central PMCID: PMC5874442.

3. Ost KS O'Meara TR, Stephens WZ, Chiaro T, Zhou H, Penman J, et al. Adaptive immunity induces mutualism between commensal eukaryotes. Nature. 2021. https://doi.org/10.1038/s41586-021-03722-w PMID: 34262174

4. Witchley JN, Penumetcha P, Abon NV, Woolford CA, Mitchell AP, Noble SM. Candida albicans Morphogenesis Programs Control the Balance between Gut Commensalism and Invasive Infection. Cell Host Microbe. 2019; 25(3):432–43 e6. Epub 2019/03/15. https://doi.org/10.1016/j.chom.2019.02.008 PMID: 30870623; PubMed Central PMCID: PMC6581065.

5. Shao TY, Kakade P, Witchley JN, Frazer C, Murray KL, Ene IV, et al. Candida albicans oscillating UME6 expression during intestinal colonization primes systemic Th17 protective immunity. Cell Rep. 2022; 39(7):110837. https://doi.org/10.1016/j.celrep.2022.110837 PMID: 35584674; PubMed Central PMCID: PMC9196946.

6. Behbod B, Sordillo JE, Hoffman EB, Datta S, Webb TE, Kwan DL, et al. Asthma and allergy development: contrasting influences of yeasts and other fungal exposures. Clin Exp Allergy. 2015; 45(1):154–63. https://doi.org/10.1111/cea.12401 PMID: 25200568; PubMed Central PMCID: PMC4733530.

7. Li XV, Leonardi I, Putzel GG, Semon A, Fiers WD, Kusakabe T, et al. Immune regulation by fungal strain diversity in inflammatory bowel disease. Nature. 2022; 603(7902):672–8. Epub 20220316. https://doi.org/10.1038/s41586-022-04502-w PMID: 35296857.

8. Iliev ID, Cadwell K. Effects of Intestinal Fungi and Viruses on Immune Responses and Inflammatory Bowel Diseases. Gastroenterology. 2020. Epub 2020/12/22. https://doi.org/10.1053/j.gastro.2020.06.100 PMID: 33347881.

9. Jain U, Ver Heul AM, Xiong S, Gregory MH, Demers EG, Kern JT, et al. Debaryomyces is enriched in Crohn's disease intestinal tissue and impairs healing in mice. Science. 2021; 371(6534):1154–9. Epub 2021/03/13. https://doi.org/10.1126/science.abd0919 PMID: 33707263.

10. Limon JJ, Tang J, Li D, Wolf AJ, Michelsen KS, Funari V, et al. Malassezia Is Associated with Crohn's Disease and Exacerbates Colitis in Mouse Models. Cell Host Microbe. 2019; 25(3):377–88 e6. Epub 20190305. https://doi.org/10.1016/j.chom.2019.01.007 PMID: 30850233; PubMed Central PMCID: PMC6417942.

11. Rolling T, Zhai B, Gjonbalaj M, Tosini N, Yasuma-Mitobe K, Fontana E, et al. Haematopoietic cell transplantation outcomes are linked to intestinal mycobiota dynamics and an expansion of Candida parapsilosis complex species. Nat Microbiol. 2021; 6(12):1505–15. Epub 20211111. https://doi.org/10.1038/s41564-021-00989-7 PMID: 34764444; PubMed Central PMCID: PMC8939874.

12. Iliev ID, Cadwell K. Effects of Intestinal Fungi and Viruses on Immune Responses and Inflammatory Bowel Diseases. Gastroenterology. 2021; 160(4):1050–66. Epub 20201219. https://doi.org/10.1053/j.gastro.2020.06.100 PMID: 33347881; PubMed Central PMCID: PMC7956156.

13. Chikina AS, Nadalin F, Maurin M, San-Roman M, Thomas-Bonafos T, Li XV, et al. Macrophages Maintain Epithelium Integrity by Limiting Fungal Product Absorption. Cell. 2020; 183(2):411–28 e16. Epub 2020/09/25. https://doi.org/10.1016/j.cell.2020.08.048 PMID: 32970988; PubMed Central PMCID: PMC7646275.

14. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. Nature. 2016; 532(7600):512–6. https://doi.org/10.1038/nature17655 PMID: 27096360; PubMed Central PMCID: PMC4871315.

15. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. Cell. 2017; 171(5):1015–28 e13. https://doi.org/10.1016/j.cell.2017.09.016 PMID: 29056339.

16. Rosshart SP, Herz J, Vassallo BG, Hunter A, Wall MK, Badger JH, et al. Laboratory mice born to wild mice have natural microbiota and model human immune responses. Science. 2019; 365(6452). https://doi.org/10.1126/science.aaw4361 PMID: 31371577.

17. Fiege JK, Block KE, Pierson MJ, Nanda H, Shepherd FK, Mickelson CK, et al. Mice with diverse microbial exposure histories as a model for preclinical vaccine testing. Cell Host Microbe. 2021; 29 (12):1815–27 e6. Epub 20211102. https://doi.org/10.1016/j.chom.2021.10.001 PMID: 34731647; PubMed Central PMCID: PMC8665115.

18. Yeung F, Chen YH, Lin JD, Leung JM, McCauley C, Devlin JC, et al. Altered Immunity of Laboratory Mice in the Natural Environment Is Associated with Fungal Colonization. Cell Host Microbe. 2020; 27 (5):809–22 e6. Epub 2020/03/27. https://doi.org/10.1016/j.chom.2020.02.015 PMID: 32209432.

19. Lin JD, Devlin JC, Yeung F, McCauley C, Leung JM, Chen YH, et al. Rewilding Nod2 and Atg16l1 Mutant Mice Uncovers Genetic and Environmental Contributions to Microbial Responses and Immune Cell Composition. Cell Host Microbe. 2020; 27(5):830–40 e4. Epub 2020/03/27. https://doi.org/10.1016/j.chom.2020.03.001 PMID: 32209431; PubMed Central PMCID: PMC7228860.

20. Bacher P, Hohnstein T, Beerbaum E, Rocker M, Blango MG, Kaufmann S, et al. Human Anti-fungal Th17 Immunity and Pathology Rely on Cross-Reactivity against Candida albicans. Cell. 2019; 176 (6):1340–55 e15. Epub 20190221. https://doi.org/10.1016/j.cell.2019.01.041 PMID: 30799037.

21. Shao TY, Ang WXG, Jiang TT, Huang FS, Andersen H, Kinder JM, et al. Commensal Candida albicans Positively Calibrates Systemic Th17 Immunological Responses. Cell Host Microbe. 2019; 25(3):404–17 e6. Epub 2019/03/15. https://doi.org/10.1016/j.chom.2019.02.004 PMID: 30870622; PubMed Central PMCID: PMC6419754.

22. Tso GHW, Reales-Calderon JA, Tan ASM, Sem X, Le GTT, Tan TG, et al. Experimental evolution of a fungal pathogen into a gut symbiont. Science. 2018; 362(6414):589–95. Epub 2018/11/06. https://doi.org/10.1126/science.aat0537 PMID: 30385579.

23. Kim YG, Udayanga KG, Totsuka N, Weinberg JB, Nunez G, Shibuya A. Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE(2). Cell Host Microbe. 2014; 15(1):95–102. https://doi.org/10.1016/j.chom.2013.12.010 PMID: 24439901; PubMed Central PMCID: PMC3957200.

24. Li X, Leonardi I, Semon A, Doron I, Gao IH, Putzel GG, et al. Response to Fungal Dysbiosis by Gut-Resident CX3CR1(+) Mononuclear Phagocytes Aggravates Allergic Airway Disease. Cell Host Microbe. 2018; 24(6):847–56 e4. Epub 20181129. https://doi.org/10.1016/j.chom.2018.11.003 PMID: 30503509; PubMed Central PMCID: PMC6292739.

25. Yang AM, Inamine T, Hochrath K, Chen P, Wang L, Llorente C, et al. Intestinal fungi contribute to development of alcoholic liver disease. J Clin Invest. 2017; 127(7):2829–41. Epub 2017/05/23. https://doi.org/10.1172/JCI90562 PMID: 28530644; PubMed Central PMCID: PMC5490775.

26. Chu H, Duan Y, Lang S, Jiang L, Wang Y, Llorente C, et al. The Candida albicans exotoxin candidalysin promotes alcohol-associated liver disease. J Hepatol. 2020; 72(3):391–400. Epub 20191010. https://doi.org/10.1016/j.jhep.2019.09.029 PMID: 31606552; PubMed Central PMCID: PMC7031049.

27. Leonardi I, Gao IH, Lin WY, Allen M, Li XV, Fiers WD, et al. Mucosal fungi promote gut barrier function and social behavior via Type 17 immunity. Cell. 2022; 185(5):831–46 e14. Epub 20220216. https://doi.org/10.1016/j.cell.2022.01.017 PMID: 35176228; PubMed Central PMCID: PMC8897247.

28. Silvestre-Roig C, Braster Q, Ortega-Gomez A, Soehnlein O. Neutrophils as regulators of cardiovascular inflammation. Nat Rev Cardiol. 2020; 17(6):327–40. Epub 20200129. https://doi.org/10.1038/s41569-019-0326-7 PMID: 31996800.

29. Xie X, Shi Q, Wu P, Zhang X, Kambara H, Su J, et al. Single-cell transcriptome profiling reveals neutrophil heterogeneity in homeostasis and infection. Nat Immunol. 2020; 21(9):1119–33. Epub 20200727. https://doi.org/10.1038/s41590-020-0736-z PMID: 32719519; PubMed Central PMCID: PMC7442692.

30. Dallari S, Heaney T, Rosas-Villegas A, Neil JA, Wong SY, Brown JJ, et al. Enteric viruses evoke broad host immune responses resembling those elicited by the bacterial microbiome. Cell Host Microbe. 2021; 29(6):1014–29 e8. Epub 20210423. https://doi.org/10.1016/j.chom.2021.03.015 PMID: 33894129; PubMed Central PMCID: PMC8192460.

31. Fiege JK, Langlois RA. Embracing the heterogeneity of natural viruses in mouse studies. J Gen Virol. 2022; 103(6). https://doi.org/10.1099/jgv.0.001758 PMID: 35737518.