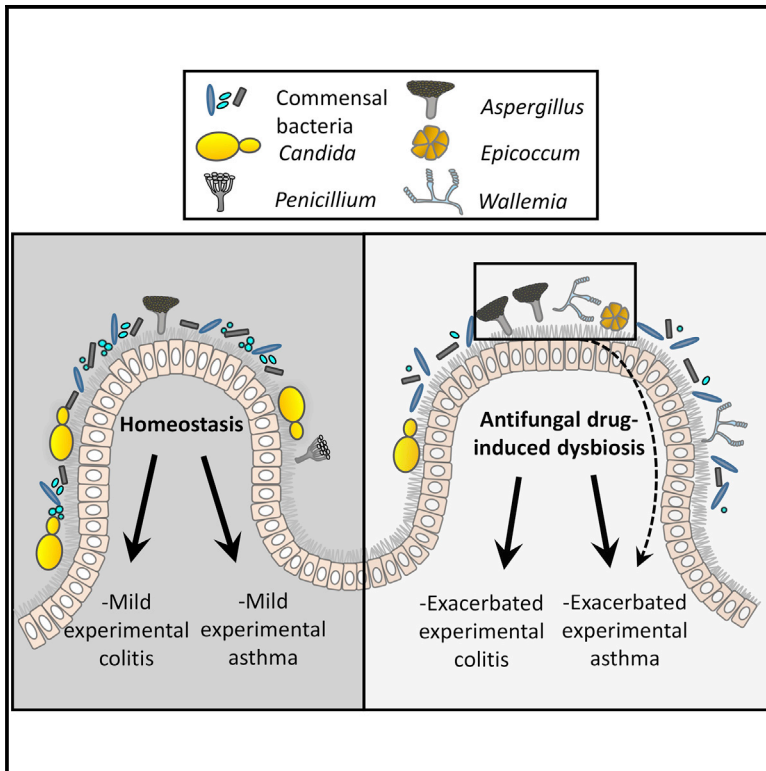


Cell Host & Microbe

Immunological Consequences of Intestinal Fungal Dysbiosis

Graphical Abstract



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In Brief

Compared to bacteria, the role of fungi within the intestinal microbiota is poorly understood. Using oral antifungal drugs to disrupt the intestinal fungal microbiota in mice, Wheeler et al. (2016) find that fungal community dysbiosis can worsen the outcome of colitis and allergic airway disease.

Highlights

- Oral treatment of mice with antifungal drugs exacerbates disease severity of colitis
- Oral antifungals increase the severity of allergic airway disease in mice
- Antifungal drugs induce dysbiosis in which some fungi are reduced and others expand
- Three identified fungi are sufficient to increase severity of allergic airway disease



Immunological Consequences of Intestinal Fungal Dysbiosis

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SUMMARY

Compared to bacteria, the role of fungi within the intestinal microbiota is poorly understood. In this study we investigated whether the presence of a “healthy” fungal community in the gut is important for modulating immune function. Prolonged oral treatment of mice with antifungal drugs resulted in increased disease severity in acute and chronic models of colitis, and also exacerbated the development of allergic airway disease. Microbiota profiling revealed restructuring of fungal and bacterial communities. Specifically, representation of *Candida* spp. was reduced, while *Aspergillus*, *Wallemia*, and *Epicoccum* spp. were increased. Oral supplementation with a mixture of three fungi found to expand during antifungal treatment (*Aspergillus amstelodami*, *Epicoccum nigrum*, and *Wallemia sebi*) was sufficient to recapitulate the exacerbating effects of antifungal drugs on allergic airway disease. Taken together, these results indicate that disruption of commensal fungal populations can influence local and peripheral immune responses and enhance relevant disease states.

INTRODUCTION

Microbial communities associated with mucosal surfaces of the body play essential roles in diverse biological processes ranging from digestion to behavior and are increasingly recognized as integral components of normal physiology (Clemente et al., 2012; Collins et al., 2012). Research over the last several decades has revealed that intestinal bacteria are critical for regulating homeostatic and protective immune responses; however, in recent years it has become evident that additional players such as fungi and viruses also have the potential to influence these processes (Underhill and Iliev, 2014; Virgin, 2014). We and others have utilized culture-independent sequencing approaches to define the composition of fungal populations associated with mucosal sur-

faces including the intestinal tract, oral cavity, skin, and lungs (Huffnagle and Noverr, 2013; Mukherjee et al., 2015; Underhill and Iliev, 2014). We previously showed that deficiency in the antifungal innate immune receptor Dectin-1 leads to more severe disease in an experimental model of colitis and that this is accompanied by fungal invasion of the colonic mucosa and expansion of opportunistic fungi such as *Candida* and *Trichosporon* spp. (Iliev et al., 2012). In support of this, pathogenic *Candida* spp. have been reported to be enriched in the intestines of some IBD patients (Ott et al., 2008), and a polymorphism in the gene for Dectin-1 is associated with increased disease severity in ulcerative colitis patients (Iliev et al., 2012). These observations suggest that fungal dysbiosis could be an important factor in development or progression of inflammatory diseases. However, little is known about the importance of a healthy fungal microbiota in regulation of immune responses.

Intestinal commensal bacteria are integral to immune homeostasis within the intestines as well as at distant sites such as the lungs (Molloy et al., 2012). Studies with antibiotic-treated and germ-free mice have demonstrated that lack of innate immune stimulation by commensal bacteria predisposes mice to allergic diseases such as asthma, owing to a general Th2 skewing of the immune system (Huffnagle, 2010; Marsland and Salami, 2015), and also results in more severe disease in chemically induced models of colitis due to compromised intestinal epithelial barrier integrity (Rakoff-Nahoum et al., 2004). In humans, epidemiological data have indicated that early-life treatment with broad-spectrum antibiotics is associated with an increased risk of developing allergic diseases such as asthma (Murk et al., 2011; Semic-Jusufagic et al., 2014) and may also contribute to the development of inflammatory bowel disease (Ungaro et al., 2014). Little is presently known about the immunological effects of disrupting the healthy intestinal fungal community and whether this could have beneficial or detrimental effects on inflammatory disease development.

Here we provide evidence for the role of a healthy intestinal fungal community in regulation of immune responses in mice. Disruption of intestinal fungi by antifungal drug treatment increases disease severity in chemically induced and T cell transfer-mediated models of experimental colitis. It also exacerbates development of allergic airway disease induced by house dust

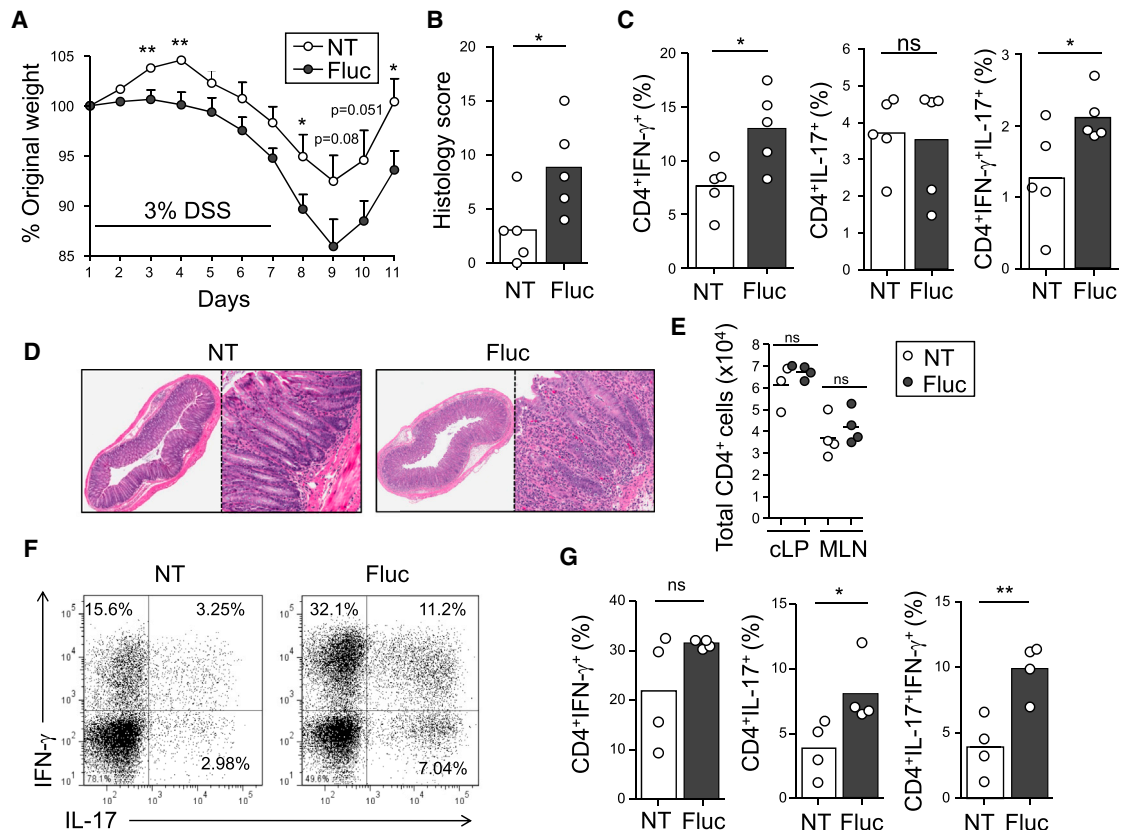


Figure 1. Antifungal Treatment Exacerbates Colitis

(A–C) Mice were given fluconazole in their drinking water for 3 weeks, followed by treatment with DSS for 7 days to induce acute colitis. Body weight (A), histology score on H&E-stained colon sections (B), and the percentage of IL-17- and IFN- γ -producing colonic lamina propria CD4⁺ T cells (C) were determined upon sacrifice.

(D) H&E-stained colonic sections from *Rag1*^{-/-} mice adoptively transferred with CD4⁺CD45RB^{high} T cells and treated with fluconazole for 6 weeks.

(E) Total CD4⁺ T cell numbers in colonic LP and MLNs.

(F and G) Dot plots (F) and bar graphs (G) show the percentage of IL-17- and IFN- γ -producing CD4⁺ T cells isolated from colonic lamina propria 6 weeks after naive T cell transfer. Each symbol represents a different mouse. One of several independent experiments with similar outcome is shown. Error bars, SD; *p < 0.05, **p < 0.01, Student's t test.

mite (HDM) challenge. High-throughput rDNA sequencing analysis of fecal material demonstrated that antifungal treatment resulted in restructuring of both the fungal and bacterial commensal communities. Surprisingly, antifungal treatment led to a relative increase in several specific fungal species that are present at low levels or absent in healthy untreated mice. Oral supplementation of mice with a mixture of three of these fungi (*A. amstelodami*, *E. nigrum*, and *W. sebi*) was sufficient to recapitulate the exacerbated features of allergic airway disease that were observed with antifungal drug treatment. These results suggest that in addition to bacteria, a healthy fungal microbiome is important for regulation of immune responses.

RESULTS

Disruption of Commensal Fungi by Antifungal Treatment Exacerbates Chemically Induced and T Cell Transfer-Mediated Colitis in Mice

To determine if disruption of the healthy intestinal fungal community impacts the response to immune challenge, mice were

treated for 3 weeks with the commonly used antifungal agent fluconazole and were subsequently given DSS in their drinking water to induce acute colitis. We have previously shown that fluconazole is protective in situations where antifungal immunity is genetically compromised and pathogenic fungi have expanded (Iliev et al., 2012); however, we were surprised to find that prolonged treatment of healthy wild-type mice with fluconazole promoted more severe colitis. This was measured by failure to recover weights and histology (Figures 1A and 1B) and correlated with higher frequencies of inflammatory CD4⁺ T cells in the intestinal lamina propria (Figure 1C). These results are consistent with a recent study showing similar effects of fluconazole treatment on the severity of DSS colitis (Qiu et al., 2015). We next examined whether disruption of gut fungal communities would also affect intestinal disease in the T cell transfer-mediated model of chronic colitis. *Rag1*-deficient mice were adoptively transferred with naive CD4⁺CD45RB^{high} T cells and treated or not with fluconazole for the duration of the experiment. Consistent with our results in the DSS-induced model of colitis, prolonged fluconazole treatment led to exacerbated disease characterized by increased mucosal

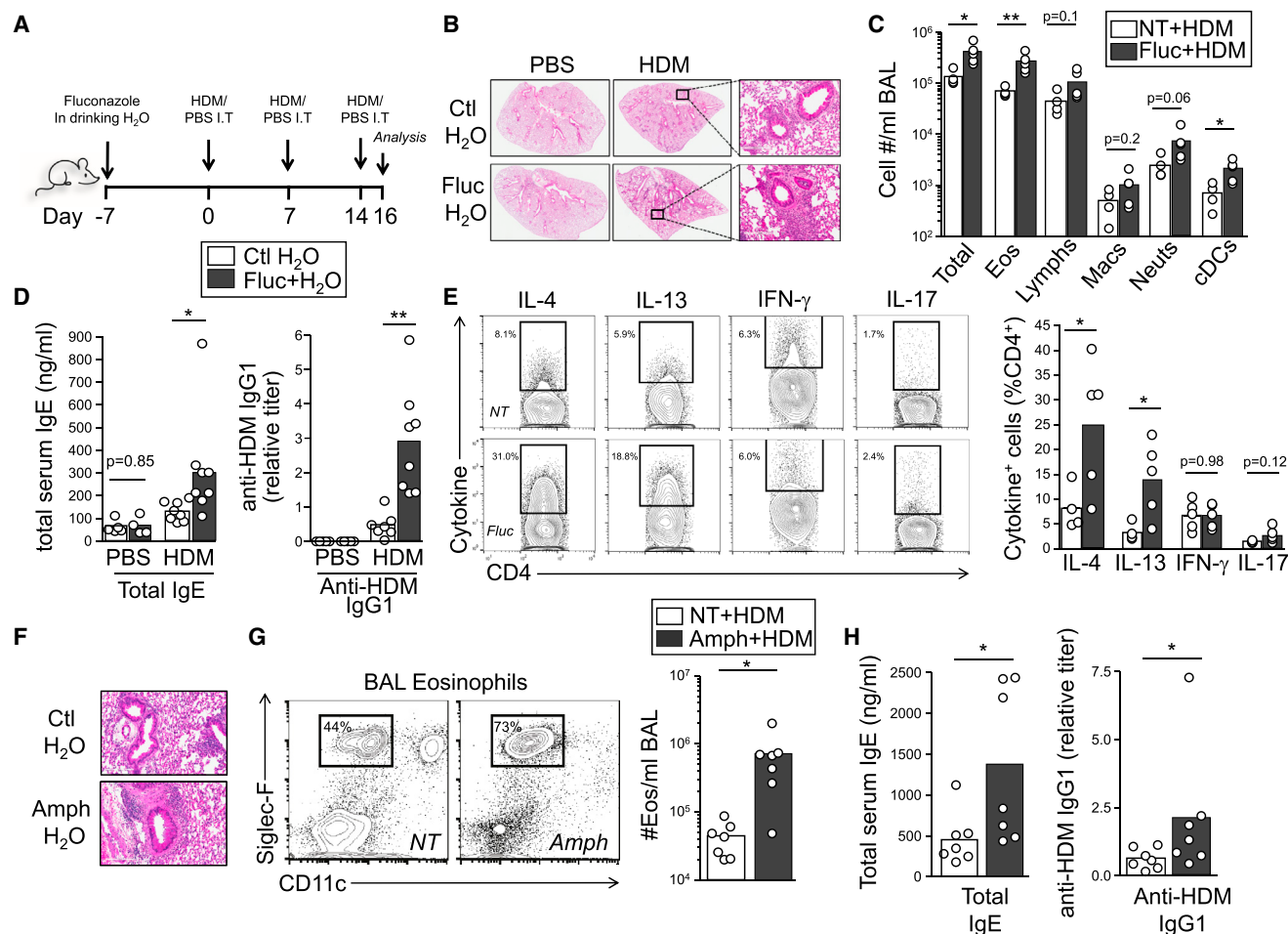


Figure 2. Antifungal Treatment Exacerbates Allergic Airway Disease

(A–D) (A) Experimental setup for antifungal treatment and induction of allergic airway disease. Lung histology (H&E) (B), BAL cell counts (C), and serum antibody titers (D) from mice treated with (filled black bars) and without (open bars) fluconazole and immunized with HDM or not (PBS).

(E) Representative intracellular cytokine staining (left panel) and quantification of cytokine-producing CD4⁺ T cells (right panel) from HDM restimulated mediastinal lymph nodes from control and fluconazole-treated mice.

(F–H) Allergic airway disease in amphotericin-B-treated mice. H&E lung histology (F), representative FACS gating strategy showing eosinophil frequency (left panel) and quantification of total eosinophils (right panel) in the BAL (G), and serum antibody titers (H) in control (open bars) and amphotericin-treated (filled black bars) mice immunized with HDM.

Data are representative of four (A–E) and two (F–H) independent experiments. Each dot represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, Student's *t* test. See also Figure S1.

erosion, crypt destruction, and infiltration of inflammatory cells in the colons of fluconazole-treated mice (Figure 1D). Although fluconazole treatment did not affect the total number of CD4⁺ T cells infiltrating the colon and MLNs of adoptively transferred mice (Figure 1E), the frequencies of inflammatory Th1 and Th17 cells significantly increased during fluconazole-induced gut fungal community dysbiosis (Figures 1F and 1G). Together, these data suggest that a healthy gut fungal community is important in resistance to intestinal inflammatory disease.

Disruption of Commensal Fungi in Mice by Antifungal Treatment Exacerbates Development of Allergic Airway Disease

It is well-established that bacterial populations in the gut can have systemic effects on the immune system, and this has

been largely investigated in the context of allergic diseases such as asthma (Marsland and Salami, 2015). We therefore wondered if disruption of the fungal community in the gut might also impact immune responses at distant sites in the body. To test this, wild-type mice were treated with fluconazole in the drinking water followed by induction of allergic airway disease by intratracheal HDM immunization (Figure 2A). Several fungi are known to exacerbate symptoms in a subset of severe asthma patients (Denning et al., 2006), so we anticipated that antifungal treatment might be protective in this model. Surprisingly, fluconazole-treated mice displayed exacerbated disease, characterized by increased inflammatory cellular infiltration (mainly eosinophils) into the lungs (Figures 2B and 2C). Consistent with this observation, mRNA levels of the eosinophil chemoattractants CCL11 and CCL21 were elevated in lung tissue of

fluconazole-treated mice immunized with HDM (see Figure S1A available online). Fluconazole treatment also resulted in elevation of serum Th2-associated antibodies, IgE and HDM-specific IgG1, in response to HDM immunization (Figure 2D), as well as increases in Th2 cytokines (IL-4, IL-5, IL-10) measured in the supernatants of HDM-restimulated mediastinal lymph node cultures (Figure S1B). Furthermore, the percentage of CD4⁺ T cells producing IL-4 and IL-13 from restimulated lymph node cultures was elevated in fluconazole-treated mice, whereas IL-17- and IFN- γ -producing CD4⁺ T cells were unchanged compared to control mice immunized with HDM (Figure 2E). These findings indicate that the exacerbating effect of fluconazole treatment is restricted to Th2-mediated inflammation in this model.

To determine if fluconazole might have nonspecific effects that could influence the response, we monitored weight change, intestinal permeability, and levels of the liver enzyme alanine transaminase (ALT) in mice treated for 3 weeks with fluconazole and did not observe differences (Figures S1C–S1E). We also assessed the induction of allergic airway disease in mice treated with two additional antifungal agents, amphotericin-B and 5-fluorocytosine (5-FC), which target fungi through different mechanisms compared to fluconazole (Gallis et al., 1990). Consistent with the fluconazole observations, mice treated with amphotericin-B (Figures 2F–2H and S1G) or 5-FC (Figures S1F and S1G) displayed exacerbated disease following HDM immunization characterized by severe eosinophilia in the lungs and increased Th2 polarization of CD4⁺ T cells from restimulated mediastinal lymph node cultures compared to control mice. Elevation in Th2-associated antibodies was also observed in serum from amphotericin-B-treated mice immunized with HDM (Figure 2H). Amphotericin-B is poorly absorbed through the gastrointestinal tract (Gallis et al., 1990), suggesting that the observed effect on lung inflammatory disease is likely mediated by disruption of fungal populations in the gut rather than an extraintestinal site such as the lungs or skin. Together, these results suggest that the enhanced response observed with fluconazole was not due to an off-target effect of the drug but was likely a specific effect of disrupting the fungal microbiota in the intestine.

Treatment of Mice with Antifungal Drugs Results in Intestinal Dysbiosis of Commensal Fungal and Bacterial Populations

The effect of antifungal drug treatment on intestinal and lung disease was initially predicted to be due to depletion of commensal fungal populations; however, we consistently observed only a 3- to 5-fold reduction in the total amount of fungal 18S rDNA in the feces after several weeks of fluconazole treatment (Figure 3A), indicating that antifungals might only target certain populations of fungi in the gut. To better understand the changes that occur within the intestinal microbiota following fluconazole treatment, we utilized high-throughput rDNA sequencing to determine if there was a general reduction of all fungal species or if the fungal community was restructured in a way that could potentially contribute to the effects observed in the lung and gut models. We isolated DNA from stool samples, amplified and sequenced fungal ITS1 (internal transcribed spacer 1) rDNA regions, and assigned operational taxonomic units using a custom-curated ITS database (Tang et al., 2015b). The approach mapped an average of 80% of all sequences at the fungal genera and species level,

identifying 57 genera of fungi. While fluconazole treatment consistently led to a relative decrease in detection of *Candida* spp., other fungal genera, such as *Aspergillus*, *Wallemia*, and *Epicoccum*, expanded upon this treatment (Figures 3B and 3C). We next asked whether amphotericin-B affected gut fungal communities in a similar way. Principle coordinates analysis confirmed that fluconazole and amphotericin treatment led to changes in gut fungal communities that were distinct from non-treated controls (Figure 3D). Furthermore, using an algorithm for high-dimensional biomarker discovery, LEfSe (Segata et al., 2011), we observed that relative detection of specific fungi including *Penicillium brevicompactum* and *Candida tropicalis* was significantly decreased upon antifungal treatment (Figures 3E and 3F and S2A), while this treatment led to relative expansion of *Aspergillus amstelodami*, *Epicoccum nigrum*, and *Wallemia sebi* (Figures 3E, 3G, and S2A).

Fungal and bacterial populations occupy similar spaces in the gut and can influence each other (Erb Downward et al., 2013; Fan et al., 2015; Mason et al., 2012). We therefore wondered if disruption of commensal fungi could have an effect on bacterial populations that could impact disease. We sequenced bacterial 16S rDNA regions and found that antifungal treatment did not affect overall bacterial diversity, but did affect specific bacterial taxa (Figures S2B–S2D). We observed decreased relative detection of *Bacteroides*, *Allobaculum*, *Clostridium*, *Desulfovibrio*, and *Lactobacillus* spp., while relative detection of *Anaerostipes*, *Coprococcus*, and *Streptococcus* was increased. The data suggest that fungal and bacterial communities in the gut are co-dependent and that disruption of one community affects the other.

Oral Supplementation of Mice with Fungi Found to Expand during Antifungal Treatment Exacerbates Development of Allergic Airway Disease

The exacerbated immune responses to challenge (colitis or asthma) that we observed after fungal community dysbiosis induced by antifungal drug treatment could have been a consequence of the general reduction in fungal burden, reduction in specific fungi, or the relative expansion of specific fungi. Having unexpectedly observed that several fungi (*A. amstelodami*, *E. nigrum*, and *W. sebi*) appeared to expand during treatment with either drug, we hypothesized that this expansion might be sufficient to influence systemic immunity. Species-specific quantitative PCR of fecal DNA revealed that fluconazole treatment resulted in an increase in *A. amstelodami*, *E. nigrum*, and *W. sebi* rDNA (Figure 4A), indicating that levels of these species were truly increased and did not simply become more common in the context of a total reduction in fungal burden. We confirmed that all three fungi were resistant to fluconazole in vitro and showed little (*A. amstelodami*, *E. nigrum*) or no (*W. sebi*) susceptibility to amphotericin-B compared to *Candida albicans* (Figure S3A). It is unlikely that the effect of antifungal treatment is due to alterations of fungal populations within the lung, as we consistently recovered less fungal DNA from an entire lobe of lung tissue compared to a single fecal pellet and were unable to detect the presence of the fungi of interest in the mouse lung by quantitative PCR (Figures S3B and S3C).

To test whether expansion of these fungi would be sufficient to influence allergic airway disease, we fed mice by repeated oral

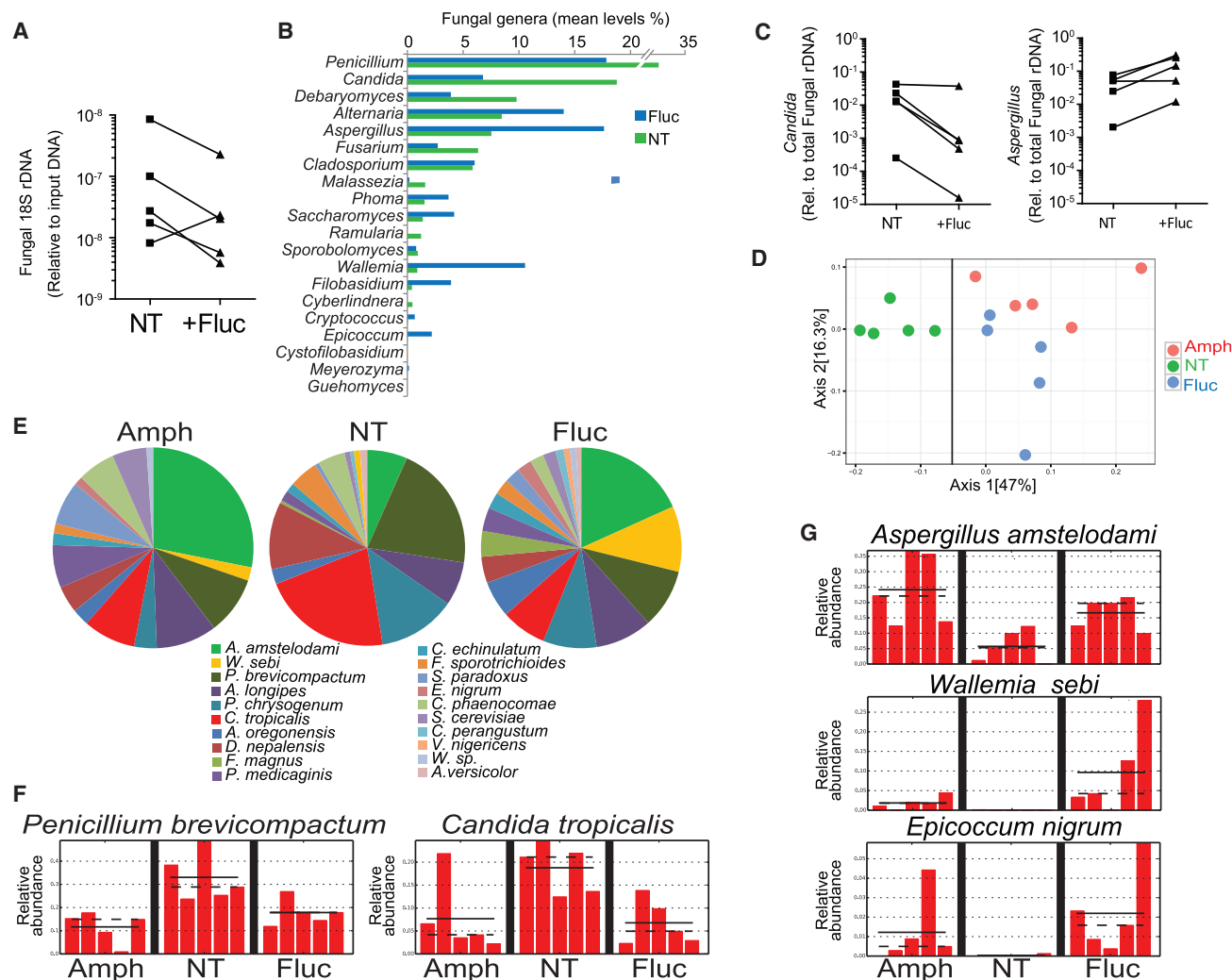


Figure 3. Antifungal Treatment Alters the Composition of Commensal Fungal Populations of the Gut

(A) Quantitative PCR for fungal 18S rDNA in feces of mice before and after 3 weeks of treatment with fluconazole. (B) Bar graphs show relative abundance of specific fungal genera sequences before and after treatment with fluconazole assessed by ITS1 amplicon sequencing. (C) PCR quantification of *Candida* and *Aspergillus* DNA, relative to total 18S fungal rDNA, in the feces of mice before and after fluconazole treatment. (D) PCoA analysis of fungal communities in control mice and mice treated with fluconazole or amphotericin. (E) Taxonomic distribution of most abundant fungal species in control mice and mice treated with fluconazole or amphotericin. (F and G) LEfSe analysis on samples from control mice and mice treated with fluconazole or amphotericin showed significant decrease (F) of some or expansion (G) of other fungal species. The horizontal straight lines in the panels (F and G) indicate the group means, and the dotted lines indicate the group medians. Each bar represents an individual mouse. Data are representative of three independent sequencing experiments with five mice/group for each condition. See also Figure S2.

gavage with a mixture of *A. amstelodami*, *E. nigrum*, and *W. sebi* and immunized with HDM as described (Figure 4B). Similar to treatment of mice with antifungal drugs, supplementation of mice with *A. amstelodami*, *E. nigrum*, and *W. sebi* was sufficient to cause exaggerated HDM-mediated allergic airway disease as measured by an increase in the number of BAL eosinophils and lymphocytes, as well as elevated serum IgE and HDM-specific IgG1 levels (Figures 4C and 4D). Oral supplementation with similar amounts of *Penicillium brevicompactum*, which does not expand with antifungal treatment (Figure 3F), did not exacerbate airway disease (Figures 4E and 4F). We did not observe exacerbated DSS colitis in mice supplemented with these fungi,

indicating that antifungal drug treatment modulates intestinal disease through a mechanism that is independent of expansion of these specific fungi (Figures S4A–S4F). Together, these results support the hypothesis that manipulation of the intestinal fungal community significantly impacts peripheral immune responses, and suggests that expansion of certain fungal populations by antifungal treatment might be a contributing factor.

DISCUSSION

In this study we utilized oral antifungal treatment to determine if disruption of the “healthy mycobiota” in the intestines alters

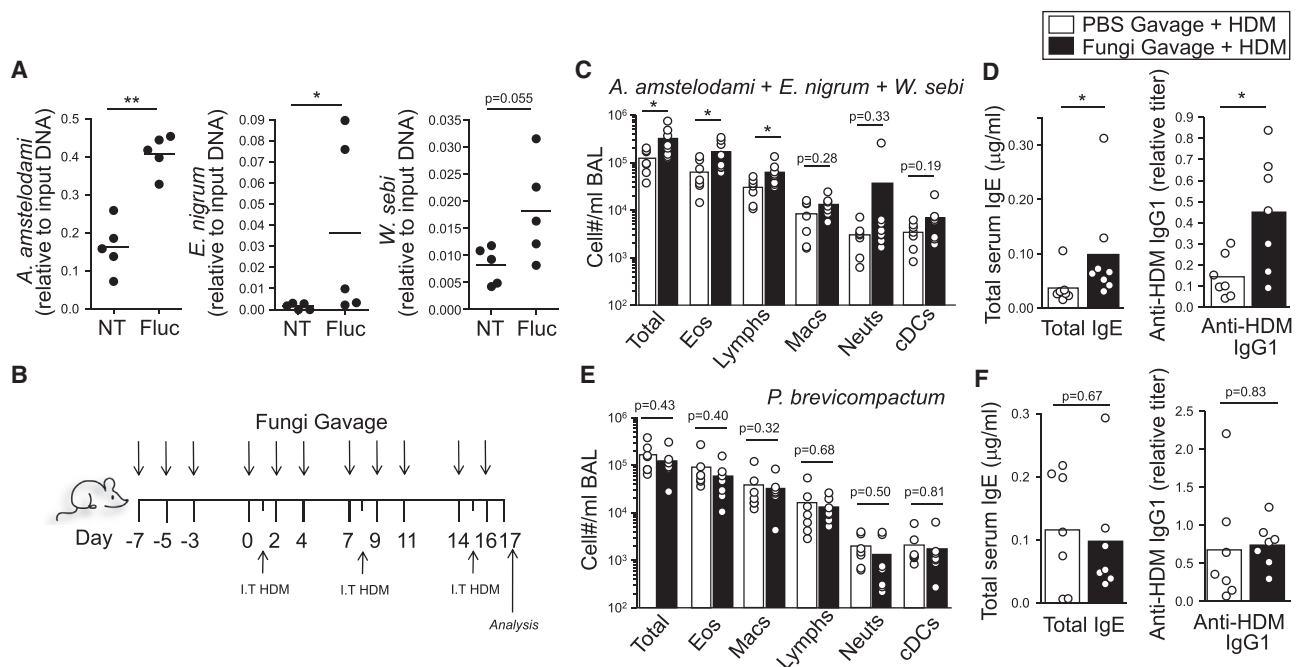


Figure 4. Allergic Airway Disease in Mice Supplemented with Fungi that Expand during Antifungal Treatment

(A) Species-specific PCR quantification (relative to the amount of input DNA in PCR reaction) of indicated fungi in feces of mice treated with or without fluconazole for 3 weeks.

(B) Experimental setup and time course of oral fungal inoculation and HDM immunization.

(C and E) Quantification of cell counts in the BAL of HDM-immunized mice treated (filled black bars) or not (open bars) with the fungal cocktail (C) or *P. brevicompactum* (E).

(D and F) Serum levels of IgE and HDM-specific IgG1 in HDM-immunized mice treated (filled black bars) or not (open bars) with the fungal cocktail (D) or *P. brevicompactum* (F). Each dot represents an individual mouse. Data are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, Student's *t* test. See also Figures S3 and S4.

local and peripheral immune homeostasis. Treatment of mice with antifungal drugs increased disease severity in chemically induced and T cell transfer-mediated models of experimental colitis and exacerbated development of HDM-induced allergic airway disease. High-throughput ITS1 and 16S sequencing of fungal and bacterial rDNA showed that antifungal treatment restructured intestinal fungal and bacterial communities. We specifically noted expansion of three commensal fungi (*A. amstelodami*, *E. nigrum*, and *W. sebi*) that, when orally delivered to mice, recapitulated the effects of antifungal treatment on allergic airway disease. These findings provide compelling evidence to support a functional role of the fungal “mycobiota” in modulating immune function and development of inflammatory disease.

The finding that antifungal treatment exacerbated disease during both intestinal and allergic lung inflammation was surprising. We have previously reported that acute treatment with fluconazole is protective during DSS colitis in situations where opportunistic fungi have expanded and invaded the intestinal mucosa (Iliev et al., 2012); however, it was not known if commensal fungi play a protective or detrimental role under steady-state conditions. The observation that extended pretreatment with antifungals exacerbated disease in both chemically induced and T cell transfer-mediated models of experimental colitis suggests that steady-state fungal populations of the gut directly or indirectly help to maintain healthy intestinal homeostasis. There is a sub-

stantial body of literature showing that intestinal bacterial populations can both positively and negatively influence the development and severity of inflammatory bowel disease (Clemente et al., 2012). Whether or not the effects we observed following antifungal drug treatment were due to primary alterations in the fungal community or to secondary effects on bacterial populations is unclear. It is interesting to note, however, that antifungal treatment resulted in substantial reduction in *Lactobacillus* populations (Figure S2), which are thought to be protective in the context of both intestinal and allergic airway inflammation (Karimi et al., 2009; Lyons et al., 2010; Tang et al., 2015a).

The observation that antifungal treatment also exacerbates the development of allergic airway disease was surprising. Disruption of commensal bacteria with antibiotics has been shown to promote the development of allergic disease in mice, and this has been attributed to a role for commensal bacteria-derived signals in suppressing systemic Th2 polarization of the immune system (Marsland and Salami, 2015). Interestingly, antibiotic-mediated depletion of gut microbiota in both mice and humans can also lead to intestinal overgrowth of commensal *Candida* spp. in the intestines (Giuliano et al., 1987; Samonis et al., 1990), and this has been shown to exacerbate allergic airway disease, potentially through the ability of *Candida* spp. to generate high levels of PGE₂ (Kim et al., 2014; Noverr et al., 2004, 2005). Furthermore, individuals with SAFS (severe asthma with fungal sensitivity) lack detectable fungal colonization of the

lung but surprisingly benefit from antifungal therapy (Denning et al., 2009; Pasqualotto et al., 2009). Given these observations, we predicted that antifungal treatment would be protective in the context of allergic airway disease. High-throughput sequencing of intestinal fungal populations demonstrated that antifungal treatment did not simply deplete commensal fungi but rather led to dysbiosis of the fungal community characterized by a commensurate increase in several fungal species, some of which are consistently found to be present in house dust samples from homes of asthma patients (Dannemiller et al., 2016; Lignell et al., 2008). These fungi are also consistently found at low levels in human stool samples by mycobiota sequencing (our unpublished data) and have been found in oral mycobiome studies (Dupuy et al., 2014; Ghannoum et al., 2010). Importantly, one of these species, *A. amstelodami*, was previously shown to induce increases in blood eosinophil levels in mice following repeated oral gavage (Bukelskiene et al., 2006). This is in line with our observation that oral inoculation of mice with *A. amstelodami*, along with *W. sebi* and *E. nigrum*, promotes exaggerated Th2 responses during HDM-induced allergic airway disease. Antifungal drug treatment appears to modulate intestinal disease through a mechanism that is independent of expansion of these specific fungi, since we did not observe exacerbated DSS colitis in mice supplemented with these fungi.

Studies suggest that overuse of antibiotics, particularly during early life, can contribute to inflammatory disease such as asthma and IBD (Marra et al., 2009; Murk et al., 2011; Ungaro et al., 2014). The data presented here indicate that antifungal treatment could similarly have significant impacts on the immune system and warrant further investigations into whether frequent antifungal treatment in humans could contribute to the development or severity of inflammatory disease. We anticipate that there are many factors contributing to the exacerbated inflammatory responses that we observe with antifungal drugs and that fungal dysbiosis is one initial driving component. It will be important to determine how commensal fungi of the “healthy” gut interact with the immune system, and furthermore how fungal and bacterial populations interact to modulate local and peripheral immune homeostasis.

EXPERIMENTAL PROCEDURES

Mice

Mice 8–10 weeks of age were used. *Rag1*^{−/−} mice were bred and housed under specific pathogen-free conditions in the Cedars-Sinai Medical Center animal facility. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Antifungal Treatments and Fungal Inoculations

Mice were provided with autoclaved water supplemented with 0.5 mg/ml fluconazole (Sigma-Aldrich PHR1160), 0.1 mg/ml amphotericin-B solubilized in DMSO (Sigma-Aldrich), or 1 mg/ml 5-fluorocytosine (Sigma-Aldrich) for 1–3 weeks prior to initiation of lung or intestinal disease. Antifungal treatment was continued throughout the course of experiments. *A. amstelodami* (ATCC 46362), *E. nigrum* (ATCC 42773), *W. sebi* (ATCC 42694), and *P. brevicompactum* (ATCC 9056) were cultivated on Sabouraud dextrose agar for 5–7 days and further propagated in liquid Sabouraud dextrose broth (both at 25°C) prior to oral gavage into mice. Fungi were mixed at equal ratios (by dry weight) and dispersed vigorously with a 20-gauge needle. Mice were inoculated by oral gavage with 20-gauge feeding needles with 100 μ l of the

fungi cocktail (approximately 0.5 g/kg in PBS) every other day, beginning 1 week prior to initiation of allergic airway disease or DSS colitis, and continued throughout the course of the experiment.

DSS and T Cell Transfer-Induced Experimental Colitis

Mice were given autoclaved water supplemented or not with 0.5 mg/ml fluconazole 3 weeks prior the induction of colitis. For the induction of acute colitis, mice were given drinking water supplemented with 3% (w/v) dextran sulfate sodium (DSS, MP Biomedicals, LLC, Aurora, OH) for 7 days and sacrificed at day 12. Body weight, gross blood, and stool consistency were analyzed on a daily basis. For chronic colitis, recipient *Rag1*^{−/−} mice were adoptively transferred with 5×10^5 CD4⁺CD45RB⁺ T cells from donor C57BL/6 mice. Mice were sacrificed at 6–7 weeks after the T cell transfer, and colons and MLNs were collected for analysis. For both models, paraffin-embedded colons were sectioned and stained with H&E for pathology assessment. Assessment of the severity of colitis was measured by the disease activity index (DAI) as described (Iliev et al., 2012).

House Dust Mite-Induced Allergic Airway Disease

Mice were given the indicated treatment (antifungal drug or fungal gavage) for 1 week prior to initiation of airway disease. Mice were given three intratracheal immunizations with 100 μ g HDM extract (*Dermatophagoides pteronyssinus*, Greer Laboratories) at 1 week intervals, and animals were sacrificed 2 days after the last immunization for analysis of airway inflammation. BAL was harvested for quantification of infiltrating inflammatory cells. One lobe of lung was fixed in 10% formalin for H&E histology and another was frozen for RNA extraction. Serum was harvested for measurement of antibody titers, and mediastinal lymph nodes were isolated for HDM-restimulation and T cell cytokine analysis.

ELISA Measurement of Serum Antibodies

Total serum IgE was measured by ELISA (BD Biosciences) according to manufacturer's instructions. For HDM-specific IgG1, 96-well plates (Corning) were coated overnight at 4°C with 25 μ g/ml HDM in PBS to capture serum HDM-specific antibodies followed by detection with biotinylated anti-mouse IgG1 (Biolegend), streptavidin-HRP (Biolegend) and development with BD OptEIA TMB substrate set (BD Biosciences). OD (450–570 nm) measurements were recorded.

Ethics Statement

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the institutional animal use and care committee of the Cedars-Sinai Medical Center (IACUC 5160).

ACCESSION NUMBERS

Sequences were deposited in the NCBI Sequence Read Archive under the accession number SRP073269.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article at <http://dx.doi.org/10.1016/j.chom.2016.05.003>.

AUTHOR CONTRIBUTIONS

M.L.W., D.M.U., and I.D.I. designed the study and wrote the paper. M.L.W., J.J.L., A.S.B., and I.D.I. performed experiments and analyzed data. C.A.L. and M.G. provided technical support. J.T., J.B., and V.F. performed ITS and 16s sequencing and provided bioinformatics support. H.L.W. provided pathology expertise. T.C. and M.A. provided advice and technical support. D.M.U. and I.D.I. supervised the project.

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REFERENCES

- Bukelskiene, V., Baltruikiene, D., and Repeckiene, J. (2006). Study of health risks associated with *Aspergillus amstelodami* and its mycotoxic effects. *Ekologija* 3, 42–47.
- Clemente, J.C., Ursell, L.K., Parfrey, L.W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–1270.
- Collins, S.M., Surette, M., and Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nat. Rev. Microbiol.* 10, 735–742.
- Dannemiller, K.C., Gent, J.F., Leaderer, B.P., and Peccia, J. (2016). Influence of housing characteristics on bacterial and fungal communities in homes of asthmatic children. *Indoor Air* 26, 179–192.
- Denning, D.W., O'Driscoll, B.R., Hogaboam, C.M., Bowyer, P., and Niven, R.M. (2006). The link between fungi and severe asthma: a summary of the evidence. *Eur. Respir. J.* 27, 615–626.
- Denning, D.W., O'Driscoll, B.R., Powell, G., Chew, F., Atherton, G.T., Vyas, A., Miles, J., Morris, J., and Niven, R.M. (2009). Randomized controlled trial of oral antifungal treatment for severe asthma with fungal sensitization: The Fungal Asthma Sensitization Trial (FAST) study. *Am. J. Respir. Crit. Care Med.* 179, 11–18.
- Dupuy, A.K., David, M.S., Li, L., Heider, T.N., Peterson, J.D., Montano, E.A., Dongari-Bagtzoglou, A., Diaz, P.I., and Strausbaugh, L.D. (2014). Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of *Malassezia* as a prominent commensal. *PLoS ONE* 9, e90899.
- Erb Downward, J.R., Falkowski, N.R., Mason, K.L., Muraglia, R., and Huffnagle, G.B. (2013). Modulation of post-antibiotic bacterial community re-assembly and host response by *Candida albicans*. *Sci. Rep.* 3, 2191.
- Fan, D., Coughlin, L.A., Neubauer, M.M., Kim, J., Kim, M.S., Zhan, X., Simms-Waldrup, T.R., Xie, Y., Hooper, L.V., and Koh, A.Y. (2015). Activation of HIF-1 α and LL-37 by commensal bacteria inhibits *Candida albicans* colonization. *Nat. Med.* 21, 808–814.
- Gallis, H.A., Drew, R.H., and Pickard, W.W. (1990). Amphotericin B: 30 years of clinical experience. *Rev. Infect. Dis.* 12, 308–329.
- Ghannoum, M.A., Jurevic, R.J., Mukherjee, P.K., Cui, F., Sikaroodi, M., Naqvi, A., and Gillevet, P.M. (2010). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog.* 6, e1000713.
- Giuliano, M., Barza, M., Jacobus, N.V., and Gorbach, S.L. (1987). Effect of broad-spectrum parenteral antibiotics on composition of intestinal microflora of humans. *Antimicrob. Agents Chemother.* 31, 202–206.
- Huffnagle, G.B. (2010). The microbiota and allergies/asthma. *PLoS Pathog.* 6, e1000549.
- Huffnagle, G.B., and Noverr, M.C. (2013). The emerging world of the fungal microbiome. *Trends Microbiol.* 21, 334–341.
- Iliev, I.D., Funari, V.A., Taylor, K.D., Nguyen, Q., Reyes, C.N., Strom, S.P., Brown, J., Becker, C.A., Fleshner, P.R., Dubinsky, M., et al. (2012). Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* 336, 1314–1317.
- Karimi, K., Inman, M.D., Bienenstock, J., and Forsythe, P. (2009). *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. *Am. J. Respir. Crit. Care Med.* 179, 186–193.
- Kim, Y.G., Udayanga, K.G., Totsuka, N., Weinberg, J.B., Núñez, G., and Shibuya, A. (2014). Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE₂. *Cell Host Microbe* 15, 95–102.
- Lignell, U., Meklin, T., Rintala, H., Hyvärinen, A., Vepsäläinen, A., Pekkanen, J., and Nevalainen, A. (2008). Evaluation of quantitative PCR and culture methods for detection of house dust fungi and streptomyces in relation to moisture damage of the house. *Lett. Appl. Microbiol.* 47, 303–308.
- Lyons, A., O'Mahony, D., O'Brien, F., MacSharry, J., Sheil, B., Ceddia, M., Russell, W.M., Forsythe, P., Bienenstock, J., Kiely, B., et al. (2010). Bacterial strain-specific induction of Foxp3⁺ T regulatory cells is protective in murine allergy models. *Clin. Exp. Allergy* 40, 811–819.
- Marra, F., Marra, C.A., Richardson, K., Lynd, L.D., Kozyrskyj, A., Patrick, D.M., Bowie, W.R., and Fitzgerald, J.M. (2009). Antibiotic use in children is associated with increased risk of asthma. *Pediatrics* 123, 1003–1010.
- Marsland, B.J., and Salami, O. (2015). Microbiome influences on allergy in mice and humans. *Curr. Opin. Immunol.* 36, 94–100.
- Mason, K.L., Erb Downward, J.R., Mason, K.D., Falkowski, N.R., Eaton, K.A., Kao, J.Y., Young, V.B., and Huffnagle, G.B. (2012). *Candida albicans* and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. *Infect. Immun.* 80, 3371–3380.
- Molloy, M.J., Bouladoux, N., and Belkaid, Y. (2012). Intestinal microbiota: shaping local and systemic immune responses. *Semin. Immunol.* 24, 58–66.
- Mukherjee, P.K., Sendid, B., Hoarau, G., Colombel, J.F., Poulain, D., and Ghannoum, M.A. (2015). Mycobiota in gastrointestinal diseases. *Nat. Rev. Gastroenterol. Hepatol.* 12, 77–87.
- Murk, W., Risnes, K.R., and Bracken, M.B. (2011). Prenatal or early-life exposure to antibiotics and risk of childhood asthma: a systematic review. *Pediatrics* 127, 1125–1138.
- Noverr, M.C., Noggle, R.M., Toews, G.B., and Huffnagle, G.B. (2004). Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect. Immun.* 72, 4996–5003.
- Noverr, M.C., Falkowski, N.R., McDonald, R.A., McKenzie, A.N., and Huffnagle, G.B. (2005). Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. *Infect. Immun.* 73, 30–38.
- Ott, S.J., Kühbacher, T., Musfeldt, M., Rosenstiel, P., Hellmig, S., Rehman, A., Drews, O., Weichert, W., Timmis, K.N., and Schreiber, S. (2008). Fungi and inflammatory bowel diseases: Alterations of composition and diversity. *Scand. J. Gastroenterol.* 43, 831–841.
- Pasqualotto, A.C., Powell, G., Niven, R., and Denning, D.W. (2009). The effects of antifungal therapy on severe asthma with fungal sensitization and allergic bronchopulmonary aspergillosis. *Respirology* 14, 1121–1127.
- Qiu, X., Zhang, F., Yang, X., Wu, N., Jiang, W., Li, X., Li, X., and Liu, Y. (2015). Changes in the composition of intestinal fungi and their role in mice with dextran sulfate sodium-induced colitis. *Sci. Rep.* 5, 10416.
- Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229–241.
- Samonis, G., Anaissie, E.J., and Bodey, G.P. (1990). Effects of broad-spectrum antimicrobial agents on yeast colonization of the gastrointestinal tracts of mice. *Antimicrob. Agents Chemother.* 34, 2420–2422.
- Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., and Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60.
- Semic-Jusufagic, A., Belgrave, D., Pickles, A., Telcian, A.G., Bakhsoliani, E., Sykes, A., Simpson, A., Johnston, S.L., and Custovic, A. (2014). Assessing the association of early life antibiotic prescription with asthma exacerbations, impaired antiviral immunity, and genetic variants in 17q21: a population-based birth cohort study. *Lancet Respir. Med.* 2, 621–630.
- Tang, C., Kamiya, T., Liu, Y., Kadoki, M., Kakuta, S., Oshima, K., Hattori, M., Takeshita, K., Kanai, T., Saijo, S., et al. (2015a). Inhibition of Dectin-1 Signaling Ameliorates Colitis by Inducing *Lactobacillus*-Mediated Regulatory T Cell Expansion in the Intestine. *Cell Host Microbe* 18, 183–197.

Tang, J., Iliev, I.D., Brown, J., Underhill, D.M., and Funari, V.A. (2015b). Mycobiome: approaches to analysis of intestinal fungi. *J. Immunol. Methods* **421**, 112–121.

Underhill, D.M., and Iliev, I.D. (2014). The mycobiota: interactions between commensal fungi and the host immune system. *Nat. Rev. Immunol.* **14**, 405–416.

Ungaro, R., Bernstein, C.N., Gearry, R., Hviid, A., Kolho, K.L., Kronman, M.P., Shaw, S., Van Kruiningen, H., Colombel, J.F., and Atreja, A. (2014). Antibiotics associated with increased risk of new-onset Crohn's disease but not ulcerative colitis: a meta-analysis. *Am. J. Gastroenterol.* **109**, 1728–1738.

Virgin, H.W. (2014). The virome in mammalian physiology and disease. *Cell* **157**, 142–150.