



HHS Public Access

Author manuscript

Cell Host Microbe. Author manuscript; available in PMC 2020 March 13.

Published in final edited form as:

Cell Host Microbe. 2019 March 13; 25(3): 377–388.e6. doi:10.1016/j.chom.2019.01.007.

***Malassezia* is Associated with Crohn's Disease and Exacerbates Colitis in Mouse Models**

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SUMMARY

Inflammatory bowel disease (IBD) is characterized by alterations in the intestinal microbiota and altered immune responses to gut microbiota. Evidence is accumulating that IBD is influenced by not only commensal bacteria but also commensal fungi. We characterized fungi directly associated with the intestinal mucosa in healthy people and Crohn's disease patients and identified fungi specifically abundant in patients. One of these, the common skin resident fungus *Malassezia restricta*, is also linked to the presence of an IBD-associated polymorphism in the gene for CARD9, a signaling adaptor important for anti-fungal defense. *M. restricta* elicits innate

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AUTHOR CONTRIBUTIONS

Conceptualization, D.M.U. and J.J.L.; Investigation, J.J.L., K.S.M., I.D.I., V.I.M., C.N., P.S. M.G., A.J.W., and J.H.S.; Resources, D.P.B.M., J. Braun, S.R.T., C.L. and J. Borneman; Formal Analysis, J.T., D.L., V.F., J. Brown, D.L. and D.P.B.M.; Writing – Original Draft, D.M.U. and J.J.L.; Writing – Review & Editing, D.M.U. and J.J.L.; Funding Acquisition, D.M.U.;

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables.

DECLARATION OF INTERESTS

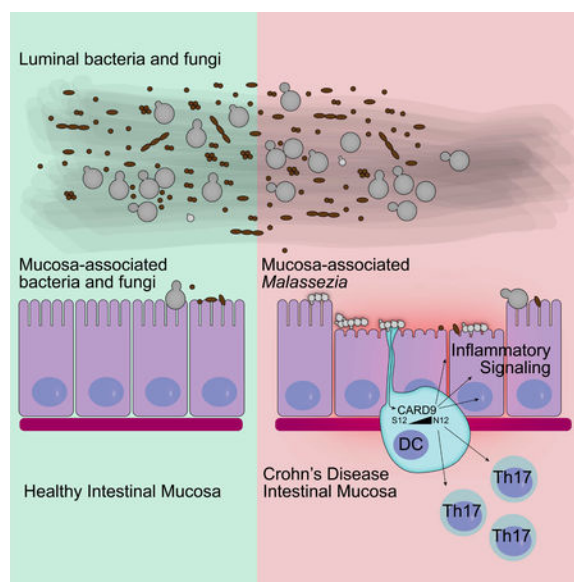
The authors declare no competing interest.

inflammatory responses largely through CARD9 and is recognized by Crohn's disease patient anti-fungal antibodies. This yeast elicits strong inflammatory cytokine production from innate cells harboring the IBD-linked polymorphism in CARD9 and exacerbates colitis via CARD9 in mouse models of disease. Collectively, these results suggest that targeting specific commensal fungi may be a therapeutic strategy for IBD.

eTOC BLURB

Limon et. al. surveyed intestinal wall-associated fungi in patients with Crohn's Disease (CD), and healthy controls and found a common commensal skin yeast called *Malassezia* preferentially in CD patients. *Malassezia* exacerbates colitis in mouse models through mechanisms requiring CARD9, a signaling protein involved in antifungal immunity.

Graphical Abstract



INTRODUCTION

Inflammatory bowel disease (IBD) susceptibility and severity are understood to be influenced by a combination of genetics, microbiota, and environment. The intestinal microbiota includes fungi (the mycobiota), and changes in the mycobiota have been reported in patients with Crohn's disease (CD) (Chehoud et al., 2015; Hoarau et al., 2016; Lewis et al., 2015; Liguori et al., 2016a, b; Sokol et al., 2016), especially increased prevalence of *Candida* spp., although how this relates to disease is not yet clear. Circumstantial evidence of a role for commensal fungi in inflammatory diseases of the gut has been accumulating for years. Serological evidence suggests that IBD is associated with changes in how the immune system interacts with commensal fungi. Development of anti-*Saccharomyces cerevisiae* antibodies (ASCA) that recognize yeast cell wall mannans found in many, but not all, fungi is a clinical biomarker identifying a large portion of Crohn's disease patients (Joossens et al., 2002; Reese et al., 2006). Serological markers including ASCA have proven useful in

defining IBD subtypes and predicting responses to therapies. Anti-tumor necrosis factor- α biologics (e.g. Infliximab) are useful in treating IBD, however, the presence or absence of ASCA together with other markers is linked to failure of Infliximab therapy in CD and ulcerative colitis (Esters et al., 2002; Ferrante et al., 2007; Taylor et al., 2001).

Genome-wide association studies have identified a common polymorphism in the gene for CARD9, a signaling adapter protein that is essential for anti-fungal innate immunity in mice and humans, as among the strongest genetic risk factors linked to Crohn's disease and ulcerative colitis (Jostins et al., 2012; Rivas et al., 2011). CARD9 is required for inflammatory signaling by C-type lectin receptors involved in innate sensing of fungi including Dectin-1, Dectin-2 and Mincle (Perez de Diego et al., 2015; Roth and Ruland, 2013). The primary phenotype in people with rare loss-of-function mutations in *CARD9* is susceptibility to fungal infection (Glocker et al., 2009). The common disease-associated risk allele confers an amino acid change in CARD9 (S12N) that has recently been shown in a mouse model to alter rather than ablate signaling (Xu et al., 2018). A polymorphic haplotype of the gene for Dectin-1, an innate receptor for fungal β -glucan that signals via CARD9, has been linked to severe disease in patients with ulcerative colitis, and we have observed that mice lacking the gene for Dectin-1 are more susceptible to experimental colitis (Iliev et al., 2012).

Recent studies suggest that changes in the mycobiota may be observed in IBD (Lewis et al., 2015; Ott et al., 2008). Sequencing of highly variable regions in bacterial rDNA (16S sequencing) has proven to be a powerful and widely-adopted approach for in-depth characterization of complex bacterial communities. The analogous approach for fungi is based on sequencing the "Internal Transcribed Spacer (ITS)" regions of fungal rDNA. A recent study of fecal samples from pediatric IBD patients and controls using this approach suggested that disease may be associated with a decrease in overall fungal diversity and an increase in the presence of *Candida* spp. (Chehoud et al., 2015), an idea that has some history and may suggest that *Candida* could be a factor in a subset of CD patients (Pineton de Chambrun et al., 2008). Subsequent studies with adult patients investigating fecal or biopsy samples have described similar associations with *Candida* and, to a lesser and more variable degree, other fungi (Chehoud et al., 2015; Hoarau et al., 2016; Liguori et al., 2016b; Sokol et al., 2016). However, accurately identifying fungi in ITS sequencing projects is still a challenge, and how detection of fungal DNA relates to host-microbe interactions at mucosal surfaces is still unclear (Limon et al., 2018).

Together, the data suggest that immune responses to intestinal fungi may influence intestinal inflammation in a subset of patients with IBD. Using a fungal ITS sequencing approach and a custom-curated database of fungal ITS sequences (Tang et al., 2015), we have examined in depth the mucosa-associated intestinal mycobiota of healthy people and of patients with CD. We found several mucosa-associated fungi that were significantly more abundant in CD patients, and we found that one of these, *Malassezia restricta*, was especially present in patients carrying the IBD *CARD9* risk allele and that ASCA antibodies can recognize this yeast. Indeed, we found that the *CARD9*^{S12N} variant caused human immune cells to more potently produce inflammatory cytokines in response to *M. restricta*. *M. restricta* is known as a common member of the skin microbiome, but a role for *M. restricta* in the gut has not

been previously established. We found that *M. restricta* exacerbates colitis in mice, even in gnotobiotic mice in which *M. restricta* is the only fungus present, and that it elicits its inflammatory responses via CARD9.

RESULTS

To focus our mycobiota analysis on fungi that are associated with intestinal mucosal surfaces (as opposed to those found in fecal material), we obtained water-lavage samples from Crohn's disease patients and healthy controls undergoing screening colonic endoscopy (McHardy et al., 2013). The patient preparation associated with endoscopy clears out fecal material, leaving behind selectively microbes capable of sticking firmly to the gut mucosa or otherwise evading the general flushing of the gut. We isolated and sequenced fungal ITS1 regions from 166 samples representing the sigmoid colon and cecum (Tables S1, S2). Three samples were excluded from further analysis due to obtaining too few sequences for sufficiently deep analysis (Figure S1A). For the remaining samples, an average of $\approx 100,000$ sequences per sample were assessed. Of these sequences, 77%–83% were identified by comparison to a custom-curated fungal ITS database, an approach designed to mitigate current challenges associated with inaccurate and non-specific data in public repositories (Figure S1B) (Tang et al., 2015). For 28 patients, we had paired sigmoid and cecum samples collected at the same time. Analysis demonstrated that these samples are more closely related to each other than they were to the sites from which they were collected (Figure S1C), suggesting that heterogeneity between samples is a reliable measure of the source of the samples and not due to technical variations in sample handling.

Overall, of the two major fungal phyla Ascomycota were substantially more common than Basidiomycota in all groups, consistent with previous studies surveying the human intestinal mycobiota (Figure S1D) (Chehoud et al., 2015; Hoarau et al., 2016; Lewis et al., 2015; Liguori et al., 2016a, b; Sokol et al., 2016). 70 genera of fungi were identified, with the most prevalent 8 genera accounting for more than 60% of the sequences (Figure 1A, 1B, Table S3). *Candida* and *Pichia* were the most highly detected, although there was considerable heterogeneity, even among healthy control samples. While many samples were dominated by *Candida*, other groups of samples were dominated by *Pichia* or *Fusarium*. In healthy control samples (sigmoid/cecum), almost half were dominated by a single genus (48%/48%), while in the Crohn's disease samples, this number fell to less than a quarter (15%/23%).

To determine whether there are disease-associated alterations in fungi we applied the MaAsLin (Multivariate Association with Linear Models) method developed by Huttenhower and coworkers (Morgan et al., 2012). This is a multivariate statistical framework specifically designed to be used to discover associations between clinical metadata (here being gender, age, diagnosis, and site) and microbial community abundance. Like a previous study, we observed that CD samples are associated with a significant loss of Ascomycota and an associated gain in Basidiomycota (Figure 1C) (Sokol et al., 2016). Specifically, we found that several fungal genera (*Malassezia*, *Cladosporium*, *Aureobasidium*, and *Fusarium*) stand out as associated with CD (Figure 1D, Table S4A). The first three are more common in samples taken from patients with Crohn's disease and are rarely found in samples from

healthy controls. *Malassezia* (identified as 65% *M. restricta* and 34% *M. globosa*) is responsible for the increase in basidiomycetes in CD patients. *Fusarium*, an ascomycete, is significantly reduced in CD patients. Other ascomycete fungi commonly of interest such as *Candida* and *Pichia* were not distributed unequally between samples from healthy controls and patients with Crohn's disease (Figure S1E). CD is characterized by diverse clinical characteristics including the primary location of the disease. We observed that the CD-associated increases in detection of *Malassezia* and *Cladosporium* were mainly in patients with ileocolonic disease, while the decrease in *Fusarium* was mainly in patients with colonic disease (Figure 1E, and Table S4B).

The Crohn's disease risk variant of CARD9 is a non-synonymous single nucleotide polymorphism (SNP) leading to a serine to asparagine change at codon 12 (S12N) that may alter the function of the protein (Xu et al., 2018; Zhernakova et al., 2008). We examined whether the *CARD9* genotype was specifically linked to the presence of any fungi. Of the fungi we identified, *Malassezia* spp. was the most strongly linked to *CARD9*^{S12N} (Figure 2A). This association is strikingly illustrated in samples from the sigmoid colon of all CD patients or selectively in CD patients with ileocolonic disease in which *Malassezia* spp. was increasingly present as the number of *CARD9*^{S12N} alleles increased (AA, Figure 2B). In contrast, *Pichia* was nearly absent in patients homozygous for the *CARD9*^{S12N} risk allele.

As noted above, ASCA are common in CD patients, so we investigated whether patient sera with high ASCA reactivity also recognize *Malassezia restricta*, the primary species of *Malassezia* found associated with CD. We observed that ASCA-high patient sera are substantially more reactive against *M. restricta* than ASCA-low sera (Figure 2C). Together, the data support a link between CD, CARD9, and *Malassezia*.

Malassezia restricta is a common fungus that is a natural commensal colonizer of the skin of many animals including humans (Findley et al., 2013). It can grow aerobically as well as anaerobically and requires long chain fatty acids for growth (and is thus commonly associated with oilier regions of the skin). Diseases associated with *Malassezia* spp. range from benign (dandruff) to life-threatening sepsis (typically associated with indwelling catheters). To experimentally determine whether *M. restricta* might exacerbate colitis, we treated specific pathogen-free (SPF) mice by oral gavage with *M. restricta* and examined its effect on DSS-induced colitis (Figure S2A). Under these conditions, *M. restricta* levels were modestly elevated in feces compared to control mice (Figure S2B). Oral gavage with *M. restricta* by itself had no apparent adverse effects, but it exacerbated DSS-induced colitis as measured by a shortening of the colon (Figure 3A, 3B), a worsening of disease activity (Figure 3C), increased lipocalin-2 levels in the feces (Figure 3D), and more severe intestinal inflammation characterized by increased mucosal erosion, crypt destruction and inflammatory cell infiltration in the colon (Figure 3E, F). Consistent with the histology, restimulation of colonic lamina propria T cells revealed stronger production of IL-17A- and IFN- γ -producing CD4⁺ cells which correlated with higher numbers of inflammatory Th1 and Th17 cells (Figure 3G, 3H and Figure S2C). Further, we detected increased activated CD4⁺ T cells in the lamina propria and mesenteric lymph nodes (Figure S2D). The effects of *M. restricta* were consistently more pronounced than *C. albicans*, which had little effect in these experiments. *S. cerevisiae* has also been reported to exacerbate disease in mouse

models of colitis (Chiaro et al., 2017), so we also compared the effects of *M. restricta* to *S. cerevisiae*. *M. restricta* promoted more severe disease compared to *S. cerevisiae* (Figure S3).

Mechanistically, *M. restricta* might exacerbate disease directly, or its presence might alter other bacterial or fungal members of the microbiota to exacerbate disease. To better understand whether increased levels of *M. restricta* alone are sufficient to exacerbate colitis, we made use of germ-free mice colonized with altered Schaedler flora (ASF). Being colonized with a defined set of 8 bacteria, ASF mice are healthier than germ-free mice, have more mature immune systems, and are fungal-free (Figure 4A) (Wymore Brand et al., 2015). Oral gavage with *M. restricta* makes *M. restricta* the only fungus present, and we found that this does not alter relative levels of any of the ASF bacteria (Figure 4B). As in SPF mice, *M. restricta* exacerbated DSS-induced colitis in ASF mice as measured by a shortening of the colon (Figure 4C, 4D), a worsening of disease activity (Figure 4E), increased lipocalin-2 levels in the feces (Figure 4F), and stronger production of IL-17A- and IFN- γ -producing lamina propria CD4⁺ cells (Figure 4G, 4H). To further investigate this, we evaluated the response to DSS-induced colitis in germ-free animals exposed to *M. restricta*. Like the ASF mice, disease was more severe when *M. restricta* was present (Figure S4). These data suggest that *M. restricta* is sufficient to directly exacerbate disease.

Given the ability of *M. restricta* to exacerbate colitis in mice, we sought a better understanding of how it activates inflammatory immune responses compared to other common budding yeasts. Although *M. restricta* is substantially smaller than *C. albicans* or *S. cerevisiae* yeasts (Figure 5A), it evokes a stronger pro-inflammatory response per organism from human monocyte-derived dendritic cells (Figure 5B) and mouse bone marrow-derived dendritic cells and macrophages (Figure 5C, 5D). *M. restricta* is also particularly potent at inducing expression of co-stimulatory molecules on mouse dendritic cells (Figure 5E) and, when co-cultured with naïve T cells and anti-CD3 ϵ antibodies, these cells induce proliferation and Th1/Th17 polarization more potently than *C. albicans* yeast (Figure 5F, 5G).

Consistent with prior reports that *Malassezia pachydermatis* and *Malassezia furfur* are recognized by Dectin-2 and Mincle (Ishikawa et al., 2013; Yamasaki et al., 2009), receptors that signal through CARD9 (Plato et al., 2015), the inflammatory response of mouse bone marrow-derived dendritic cells and neutrophils to *M. restricta* was highly dependent on CARD9 (Figure 6A, 6B). We further found that Dectin-2 was especially important for responses to *M. restricta*, while Dectin-1 and Mincle did not contribute significantly (Figure 6C).

To investigate the consequences of the IBD-associated *CARD9*^{S12N} polymorphism on host responses to *M. restricta*, we generated human peripheral blood monocyte-derived dendritic cells from healthy donors homozygous for the S (GG) or N (AA) alleles and stimulated them with fungi. AA dendritic cells produced significantly more TNF- α and IL-8 in response to *M. restricta* than GG dendritic cells (Figure 6D). Other cytokines including IL-1 β , IL-10, and IL-6 were unaffected, consistent with a recent report suggesting that making the analogous *Card9*^{S12N} polymorphism in mice alters some, but not all CARD9-dependent signaling (Xu et al., 2018). Cytokine production was not noticeably affected when cells were

stimulated with *C. albicans* or *S. cerevisiae*, consistent with the observation that, relative to other yeasts, *M. restricta* is a strong activator of innate inflammatory signaling via the C-type lectin/CARD9 pathway.

Together, the data suggest that *M. restricta* exacerbates colitis directly by stimulating inflammatory responses via CARD9. To test this, we evaluated the capacity of *M. restricta* to exacerbate disease in mice lacking CARD9. We observed that loss of CARD9-mediated signaling prevented *M. restricta* from exacerbating disease as measured by reduced shortening of the colon (Figure 7A, 7B), amelioration of disease activity (Figure 7C, 7D), and decreased lipocalin-2 levels in the feces (Figure 7E).

DISCUSSION

Together, the data suggest that changes in intestinal fungi and host responses to intestinal fungi may contribute to disease in a subset of patients with Crohn's disease. Our data suggest that colonization of the colonic mucosa with *M. restricta*, a common commensal fungus found typically on the skin, may increase disease severity, especially in patients carrying the *CARD9*^{S12N} risk allele. We find that the N12 *CARD9* variant in humans promotes a stronger inflammatory response to *M. restricta* than the S12 version. In the United States, over 1.6 million people are diagnosed with IBD, and the *CARD9* allele has a 25%–50% frequency depending on the subgroup. Thus, the observation might be expected to affect a substantial proportion of patients with Crohn's disease. We find that, compared to other common yeasts, *M. restricta* elicits a particularly strong inflammatory response from myeloid phagocytes (macrophages and dendritic cells) and that this response is largely dependent on CARD9 signaling. In mice, *M. restricta* exacerbates DSS-induced colitis, and experiments using gnotobiotic mice in which *M. restricta* is the only fungus present and no fungus-induced changes in bacterial microbiota are observed we find that the presence of *M. restricta* is sufficient to exacerbate disease. Finally, we find that the ability of *M. restricta* to exacerbate disease in mice is dependent on the presence of functional CARD9.

Previous studies have reported an association of *Candida* spp. in the stool with CD, although the functional consequences of this association are not yet clear (Chehoud et al., 2015; Hoarau et al., 2016; Lewis et al., 2015; Liguori et al., 2016a, b; Sokol et al., 2016). These studies have variously specified *C. albicans* or *C. tropicalis*. Curiously, we do not observe a *Candida* association in our data set, perhaps because we have sampled specifically mucosa-associated fungi, which will report different microbial contents than stool samples (Zmora et al., 2018). That we have not observed a *Candida* association does not indicate that *Candida* plays no role in the dysbiosis associated with IBD, only that we did not observe it. Due to the sampling methodology, it was not possible to obtain stool samples paired with the mucosal washings that we have analysed. It will be valuable in future studies to attempt to pair such samples to determine if the *Candida* association is specific to luminal contents. In healthy mice, we find that gavage with *Candida* has negligible effect on DSS colitis, although we have previously noted that *Candida* exacerbates disease in animals with an underlying deficiency in anti-fungal immunity (Dectin-1 knockout animals) (Iliev et al., 2012). Others have observed in gastric infection models (resulting in enormous *Candida* burdens) that *Candida* variously exacerbates or mitigates disease in wild type animals (Jiang

et al., 2017; Leonardi et al., 2018; Sovran et al., 2018). It will be important going forward to develop a better understanding of when, where, and how *Candida* is relevant in IBD.

The data indicate that *Malassezia* is more prevalent in the colonic mucosa of CD patients carrying the *CARD9*^{S12N} variant. The observation that *Malassezia* stimulates a stronger/different innate immune response when *CARD9*^{S12N} is present is consistent with its association with an inflammatory disease, but how this increased or altered inflammatory response might promote colonization or survival of *Malassezia* is unknown. The data suggest that *Malassezia* is uncommon in the colonic mucosa of healthy individuals regardless of *CARD9* genotype, suggesting that factors other than *CARD9* dictate the potential to be colonized. It is possible that the nature of the disease in *CARD9*^{S12N} patients is such that it favors *Malassezia* colonization that, in turn, evokes a strong inflammatory response. Future studies will have to address the inflammatory, dietary, physiological, and/or microbiome factors that lead to *Malassezia* association with the intestinal mucosa.

The combined roles of a patient's genetic susceptibility and environmental factors provides a basis for subtyping Crohn's Disease (Jostins et al., 2012). Abiotic environmental factors such as smoking have recently been shown to lead to Paneth cell dysfunction in mice harbouring the *ATG16L1*^{T300A} risk allele, and CD patients with the *ATG16L1*^{T300A} genotype who smoked had a poorer clinical prognosis (Liu et al., 2018). The bacterial microbiota as an environmental factor contributing to CD has also been shown to play a probable role in pathogenesis. Mice deficient of Atg1611 and Nod2 in the dendritic cell compartment are not able to process protective immunomodulatory signals in the form of outer membrane vesicles (OMVs) containing polysaccharide A from *Bacteroides fragilis* leading to a defect in the induction of protective T regulatory cell differentiation (Chu et al., 2016). In DC-T cell co-culture experiments DCs harbouring the *ATG16L1*^{T300A} allele failed to induce T regulatory cell differentiation, thus negating a health-promoting signal from the microbiome. Here we report a fungal organism whose presence is strongly linked to the *CARD9*^{S12N} risk allele. The pathogenesis of CD in this context, however, is likely due to a strong inflammatory reaction to the fungal organism that may be principally sensed through Dectin-2.

Malassezia spp. are the dominant fungal members of the skin microbiota and, because they depend on processing external lipids for growth, they grow mainly in sebaceous glands that produce oily secretions (Wheeler et al., 2017). To make use of extracellular fatty acids *Malassezia* secrete lipases and other enzymes necessary for fatty acid metabolism. These enzymes and their products have been reported to irritate skin (DeAngelis et al., 2005), and it is interesting to speculate that their production in the gut mucosa could contribute to disease. Some of these enzymes drive generation of short-chain fatty acids that have antimicrobial activity in vitro against bacteria and fungi found on the skin. Certain short-chain fatty acids in the gastrointestinal tract, either produced by commensal microbes as a by-product of carbohydrate fermentation or introduced by dietary supplementation, promote production of colonic regulatory T cells (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). It is interesting to hypothesize that such regulatory mechanisms could also be altered due to *Malassezia* fatty acid metabolism in the gut.

Malassezia have been reported to produce AhR ligands including indirubin, malassezin, formylindolo[3,2-b]carbazole, indolo[3,2-b]carbazole, pityriazepin, pityriacitrin, and tryptanthrin (Wheeler et al., 2017). AhR is an orphan nuclear receptor that promotes wound healing and contributes to overall skin homeostasis (Barouti et al., 2015; Di Meglio et al., 2014). AhR expression in CD4⁺ T cells has been shown to increase the production of the proinflammatory cytokines IL-17 and IL-22 (Veldhoen et al., 2008). AhR ligands have also been implicated in inhibition of TLR-induced responses in phagocytes (Vlachos et al., 2012), and AhR functions have been implicated in the homeostasis of cutaneous invariant $\gamma\delta$ T cells (Kadow et al., 2011). Thus, it is also interesting to hypothesize that *Malassezia*-induced AhR stimulation in the gut may directly modulate immune activation in health and disease.

Together, our data suggest that changes in intestinal fungi and host responses to intestinal fungi may contribute to disease in a subset of patients with Crohn's disease. The data suggest that intestinal colonization with *M. restricta*, a common commensal fungus found typically on the skin, may increase disease severity and that genetic factors, especially *CARD9* polymorphisms are important in defining the inflammatory response to colonization. The findings suggest that a precision approach therapeutically targeting specific members of the fungal microbiota in certain individuals should be explored.

STAR METHODS TEXT

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by Dr. David Underhill (David.Underhill@csmc.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human studies—A previously-assembled and newly-expanded cohort of healthy individuals undergoing screening colonoscopy and Crohn's disease patients undergoing colonoscopy for clinical reasons (Li et al., 2011) was examined in accord with human subject protocols approved by the institutional review board of Cedars-Sinai Medical Center including written informed consent of each subject to participate in the study. For IBD subjects, colonoscopy procedures were performed during periods of inactive disease, and clinical data recorded included site of the diagnosed disease. All CD patients were undergoing standard care for their disease, although differences in care were not considered further in this study. All enrolled subjects were prepared for colonoscopy by taking Golytely® the day before the procedure. Mucosal lavage samples representing the mucosal luminal interface were collected from different intestinal regions as described previously (Li et al., 2011; McHardy et al., 2013). All samples were collected from non-inflamed intestinal regions, which excluded the potential influence of active inflammation on the mucosal microbiota as much as possible. For CD subjects, colonoscopy procedures were performed during periods of inactive disease, and clinical data recorded included site of the diagnosed disease. All CD patients were undergoing standard care for their disease, although differences in care were not considered further in this study. Subjects' metadata, including gender, age, and colon regions sampled, were recorded (Table S1, S2).

For human monocyte derived dendritic cells, human blood was collected from male and female healthy donors, in similar proportions, after informed consent by the Cedars-Sinai Medical Center MIRIAD Biobank in accordance with Cedars-Sinai Medical Center Institutional Review Board procedures.

Animal studies—C57BL/6 mice 8–10 weeks of age were used for most experiments and purchased from Jackson Laboratories. All mice were healthy before initiation of studies, were not subjected to previous procedures, and were naive to drugs. All standard mice were maintained in an SPF environment. Mice were provided with water and a standard laboratory diet ad libitum (PicoLab rodent diet 20) except if noted otherwise. They were supplied with hardwood chips as bedding and housed in a temperature-controlled, air-conditioned room on a 12-hr light-dark cycle. Bone marrow-derived dendritic cells and macrophages were prepared from C57BL/6 mice from Jackson Laboratories or from Card9, Dectin-1, Dectin-2, or Mincle knockout mice maintained under specific pathogen-free conditions. Mincle knockout bones were kindly provided by Dr. Andrew Limper (Mayo Clinic, Rochester, MN). A colony of Altered Schaedler Flora (ASF)-colonized mice was generated by colonizing germ-free mice with ASF stool (Taconic). Colonization was verified by PCR analysis of the ASF component bacteria, and absence of fungi was assessed by PCR as previously described (Wheeler et al., 2016). ASF mice were kept under sterile conditions in CBC (Class Biologically Clean) flexible film isolators, exposed to a 14/10-hr light/dark cycle and provided standard, autoclaved water and mouse chow (Purina Gamma Irradiated Diet 5066) ad libitum. For in vitro experiments, male and female mice were used, and no differences were noted. For in vivo experiments, female mice were used unless otherwise stated. Mice were housed in specific pathogen-free conditions in the Cedars-Sinai animal facility, and all animal experiments were conducted according to Cedars-Sinai Medical Center Institutional Animal Care and Use Committee guidelines.

Fungi—*Candida albicans* (ATCC 90028) and *Saccharomyces cerevisiae* (ATCC 201388) yeasts were grown with shaking overnight at 37°C in Sabouraud dextran broth (SDB). *Malassezia restricta* (clinical isolate MYA-4611) was grown statically for 3 days at 30°C in modified Dixon broth (mDixon) supplemented with glycerol monostearate. For in vitro experiments yeasts were fixed in 2% paraformaldehyde at room temperature for 1 hour then washed 3 times with cell culture media and counted. For in vivo experiments fungi were grown as described above washed 3 times with 0.2 M sodium bicarbonate and resuspended at a concentration of 1×10^9 yeasts/mL of 0.2 M sodium bicarbonate. Mice were dosed with 1×10^8 yeast cells at the times indicated.

METHOD DETAILS

High-throughput ITS1 sequencing.—During DNA isolation and sequencing, investigators were blinded to sample identities. DNA was extracted from 166 samples using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories) including a 30-second bead-beating in a Mini-Beadbeater-16 (BioSpec). Fungal ITS1 amplicons were generated in 20 μ L PCR reactions using 3 μ L of each sample with 35 cycles using Phusion DNA Polymerase (New England BioLabs) at an annealing temperature of 56.1°C using the primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC)

incorporating an extra 6 bases at the 5' end as sample-specific bar codes. Resultant ITS amplicons were purified using Agencourt AmPure Magnetic Beads (Beckman Coulter), resuspended in 20 μ L of nuclease-free water, and quantified using a Qubit fluorometer. Amplicons were further qualified using the DNA 1000 assay on the Agilent Bioanalyzer (Agilent Technologies). To generate Illumina TruSeq libraries, 8 barcoded libraries were pooled per TruSeq index to be used, and TruSeq adapters were ligated to the amplicons via the DNA Nano LT kit (Illumina). Final libraries were quantified with Qubit's high sensitivity dsDNA assay, and qualified with the Agilent Bioanalyzer DNA 1000 assay.

Sequencing was performed on the Illumina MiSeq platform (Illumina) with paired end 250bp sequencing chemistry, giving the possibility to obtain relatively long overlapping reads with less than 1% error rates. Fungal ITS1 libraries were clonally amplified directly onto paired end flowcells and sequenced with standard Illumina sequencing primers according to manufacturer's instructions. Raw data processing and run de-multiplexing was performed using on-instrument analytics as per manufacture recommendations.

Antisera analyses—Human serum was collected from CD patients (n=18–22 per group, equally distributed between male and female) after informed consent by the Cedars-Sinai Medical Center MIRIAD Biobank in accordance with Cedars-Sinai Medical Center Institutional Review Board procedures. ASCA-IgA and ASCA-IgG level determination was made by the Cedars-Sinai Medical Center MIRIAD Biobank using the method described previously (Landers et al., 2002). *M. restricta* was grown as described above and washed two times with sterile PBS. Five million live yeasts were plated per well onto 96 well U-bottom plates and stained with human serum diluted in FACS buffer at 1:100 in 100 μ L. Primary staining was done for 30 minutes at room temperature followed by washing with 150 μ L of FACS buffer. Samples were then stained with Alexa Fluor-488 conjugated goat anti-human IgA (Southern Biotech) and Alexa Fluor-647 conjugated goat anti-human IgG (Southern Biotech) in FACS buffer at a 1:300 dilution for 30 minutes at room temperature followed by washing. Stained cells were fixed in 2% paraformaldehyde, washed and analyzed on a BD LSR2 flow cytometer (BD Biosciences). Flow cytometry analysis was performed using FlowJo software (TreeStar). Flow cytometry data was reported as mean fluorescence intensity.

Macrophage, dendritic cell, and neutrophil preparation and stimulation—

Mouse bone marrow-derived macrophages and dendritic cells were grown as previously described (Goodridge et al., 2009). For in vitro assays dendritic cells or macrophages were plated at 400,000 cells per well in 24 well plates and stimulated with indicated doses of fungal cells for 24 hours. Supernatants were collected and used for cytokine measurements by ELISA. Mouse neutrophils were negatively purified from bone marrow using MojoSort Mouse Neutrophil Isolation Kit (Biolegend, San Diego, CA) and stimulated as indicated above.

For human monocyte derived dendritic cells, peripheral blood mononuclear cells were isolated by Ficoll-Paque Premium gradient (GE Healthcare) and CD14⁺ monocytes were negatively selected using EasySep™ Human Monocyte Enrichment Kit (Stem Cell Technologies). Purified CD14⁺ monocytes were cultured in the presence of human GM-CSF

and human IL-4 (PeproTech) for 7 days. Differentiated cells were plated at 200,000 cells per well in 48 flat bottom tissue culture treated plates (Corning) and stimulated for 24 hours with indicated doses of fungal cells. Supernatants were collected for measuring cytokine production by Meso Scale Diagnostics V-Plex Plus Human Proinflammatory Panel 1 or ELISA (BioLegend).

DSS colitis—Where indicated mice were supplemented with 3% DSS (MP Biomedicals LLC) in their drinking water for 7 days, for the induction of colitis, followed by 4–5 days of recovery on regular water. For mice administered fungi, the dosing is as described above. Mice were gavaged 3 times before the introduction of DSS water every other day and 3 times every other day following DSS water. Disease activity index was assessed by measuring weight loss, stool consistency and presence of blood in stool. Histological analyses were performed by a trained pathologist blinded to the experimental conditions as previously described (Iliev et al., 2012; Wheeler et al., 2016).

Fecal lipocalin-2—As a non-invasive biomarker for intestinal inflammation, we measured fecal lipocalin-2 levels (Chassaing et al., 2012). Mouse fecal pellets were collected in sterile 1.7 ml microcentrifuge tubes and resuspended at 100 mg/mL in sterile PBS with protease inhibitor cocktail (Cell Signaling Technology). Samples were shaken using a bead beater at medium speed for 90 seconds followed by centrifugation. Supernatants were assayed for lipocalin-2 using LEGEND MAX mouse NGAL (Lipocalin-2) ELISA Kit (BioLegend).

Colonic lymphocyte lamina propria isolation and ex-vivo stimulation—Lymphocytes from the colonic lamina propria were isolated by using the mouse Lamina Propria Dissociation Kit and AutoMacs per the manufacturer's instructions (Miltenyi Biotec). After tissue dissociation the samples were re-suspended in a 42% Percoll gradient solution and layered on top of a 72% Percoll gradient for centrifugation and isolation of single cells. Isolated cells were re-suspended in PBS containing 2% fetal bovine serum counted and stained with fixable viability dye, Zombie UV followed by Fc blocking and staining with mouse CD4 antibody (BioLegend). After staining, cells were washed and re-suspended in complete media and stimulated for 6 hours using cell stimulation cocktail (eBioscience) in the presence of brefeldin A (BioLegend) and GolgiStop (BD Biosciences). After stimulation, cells were fixed in 2% paraformaldehyde washed and stained for intracellular cytokine production using Intracellular Staining Permeabilization Wash Buffer (BioLegend) per the manufacturer's instructions.

Antibody staining and flow cytometry—Single cells preparations were first treated with fixable cell viability dye, Zombie UV followed by TruStain fcX (BioLegend) to block Fc receptors. Fluorophore conjugated antibodies were used as follows: anti-CD4 (clone GK1.5), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-CD86 (clone GL-1) (BioLegend), anti-IFN γ (clone XMG1.2), anti-IL17A (clone eBio17B7), anti-CD45 (clone 30-F11) (eBioscience). Stained cells were fixed in 2% paraformaldehyde and analyzed on a BD LSR2 flow cytometer (BD Biosciences). Flow cytometry analysis was performed using FlowJo software (TreeStar).

Mouse dendritic cell and CD4⁺ T cell co-culture—Mouse dendritic cells were generated and plated as described above. Naïve CD4⁺ T cells were purified from mouse spleen and peripheral lymph nodes using a CD4 Naïve Enrichment Kit (Life Technologies). Prior to adding naïve T cells, dendritic cells were stimulated with indicated fungal cell doses for 3 hours to allow for complete fungal cell internalization. Naïve CD4⁺ T cells were then added at a ratio of 2:1 with or without anti-CD3e antibodies (BioLegend) for poly-clonal T cell stimulation. Cells were co-cultured for 5 days and processed for intracellular cytokine production as described above.

Colonic RNA purification, cDNA synthesis and qPCR analysis—Colonic sections were collected and homogenized in TRIzol reagent per the manufacturer's instruction. The aqueous fractions were processed with RNeasy Mini Kit (Qiagen) for RNA purification. cDNA synthesis was carried out using iScript cDNA Synthesis Kit (BioRad) per the manufacturer's instructions. Gene expression of colonic samples was performed using iTaq Universal SYBR Green Supermix (BioRad), on an Eppendorf Mastercycler ep realplex2 and realplex 2.2 software. Mouse β -actin was used as the housekeeping gene. Primers used are as follows: tnfa forward primer 5'-TCTCATGCACCACCATCAAGGAC-3' and reverse primer 5'-TGACCACTCTCCCTTTGCAGAAC-3', il-6 forward primer 5'-ATCCAGTTGCCTTCTTGGGACTG-3' and reverse primer 5'-TAAGCCTCCGACTTGTGAAGTGG-3', β -actin forward primer 5'-GGCTGTATTCCCCTCCATCG-3' and reverse primer 5'-CCAGTTGGTAACAATGCCATGT-3'.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data Processing—For analysis of ITS1 sequence data, raw FASTQ data were filtered to enrich for high quality reads including removing the adapter sequence by cutadapt v1.4.1 (Martin, 2011), demultiplexing and truncating reads not having an average quality score of 20 (Q20) over a 3 base pair sliding window, removing any reads that do not contain the proximal primer sequence or any reads containing a single N (unknown base) by a custom script. Filtered pair-end reads were then merged with overlap into single reads using SeqPrep v1.0 wrapped by QIIME v1.6 (Caporaso et al., 2010) with default settings.

The processed high-quality reads were firstly aligned to previously observed host sequences (including rRNA, olfactory receptor and uncharacterized genes in human and mouse) to deplete potential contamination, then operational taxonomic unit (OTU) were picked by aligning filtered reads to the Targeted Host Fungi (THF) custom fungal ITS database (version 1.6) (Tang et al., 2015), using BLAST v2.2.22 in the QIIME v1.6 wrapper with an identity percentage 97%. Only less than 0.36% reference sequences in THF database could not be annotated down to genus level (and assigned as "Unidentified" for genus name). OTUs with average relative abundance >0.00001 were considered to be present as well as compiled into genera for downstream analysis.

Bioinformatic analysis—While intestinal disease can be exacerbated or ameliorated by organisms that do not change in overall abundance, we focused our first line of analysis on evaluating whether specific mucosa-associated fungi are enriched or depleted in the context

of Crohn's Disease. The multivariate association analysis was conducted by using MaAsLin v0.0.3 with minimum OTU prevalence of 0.05, minimum abundance of 0.001 and FDR-corrected p-value cutoff of 0.10, on the variables of interest: gender, age, body site and diagnosis (Morgan et al., 2012).

CARD9^{S12N} allele data were acquired using ImmunoChip. Genetic association between the *CARD9*^{S12N} allele and fungi was performed in the generalized linear model (GLM) framework, with adjustment for Principle Components (PCs) from population stratification analysis. Permutation test was performed to control for false-positives that can be caused by the skewness of the fungal data. The corrected significance threshold for this analysis is $0.05/75=6.7\times 10^{-4}$.

Statistical analysis—All experiments were conducted with at least triplicate measurements a minimum of two times unless otherwise stated in the text or figure legends. Statistics were measured by Student's t-test or One-Way ANOVA with Tukey's multiple comparison test for significance using GraphPad Prism software or R version 3.4.0. Mann-Whitney U Test performed where data failed tests for normality.

DATA AND SOFTWARE AVAILABILITY

Sequence reads from this study are available from the Sequence Read Archive under the project ID "PRJNA306760".

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study was supported by grants to D.M.U. from the NIH (R01 DK093426, P01 DK046763) and the Janis and William Wetsman Chair in Inflammatory Bowel Disease. The MIRIAD IBD Biobank is supported by the Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute & the NIH (P01 DK046763). This research was further supported by NIH National Center for Advancing Translational Science (NCATS) UCLA CTSI Grant Number UL1TR001881.

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HIGHLIGHTS

- *M. restricta* is associated with the colonic mucosa in Crohn's disease (CD) patients.
- *M. restricta* exacerbates colitis in wild type and gnotobiotic mice.
- *M. restricta* is found in CD patients with a disease-linked polymorphism in *CARD9*.
- *Malassezia*-exacerbated colitis in mice requires signaling via *CARD9*.

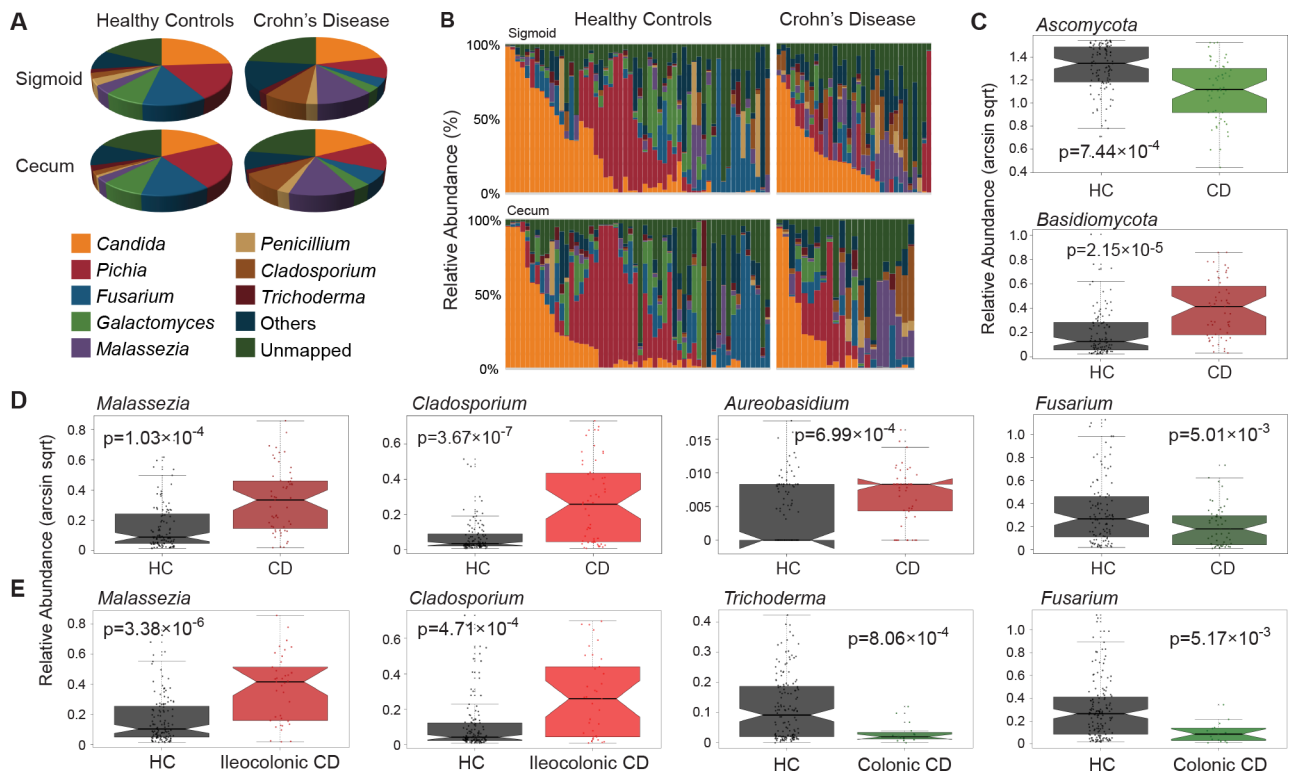


Figure 1. Crohn's disease mucosae-associated mycobiome characterization

(A) Sequences from healthy control and Crohn's disease sigmoid colon and cecum were identified to the genus level, and the relative representation of each genus in the datasets is illustrated. "Others" include 67 relatively rare genera, and "Unmapped" indicates the fraction of the sequences that could not be convincingly identified as belonging to specific genera (defined as $< 97\%$ identity to a known reference sequence).

(B) The data are shown broken down by individual patient (x-axis), showing the relative abundance of sequences detected in each sample.

(C) MaAsLin analysis reveals that Crohn's disease (CD) is associated with a decrease in ascomycetes and an increase in basidiomycetes compared to healthy controls (HC). Notched box plots show individual arcsine square-root transformed relative abundance's median and confidence intervals as well as the first and third quartiles. The strength of the difference in the CD samples is noted by the brightness (lower in CD – green, higher in CD – red).

(D) Specific genera and their associations with CD (all patients) are illustrated.

(E) Analysis as in (D) but illustrating associations specific to the indicated types of Crohn's disease (ileocolonic or colonic).

See also Figure S1 and Tables S1–S4.

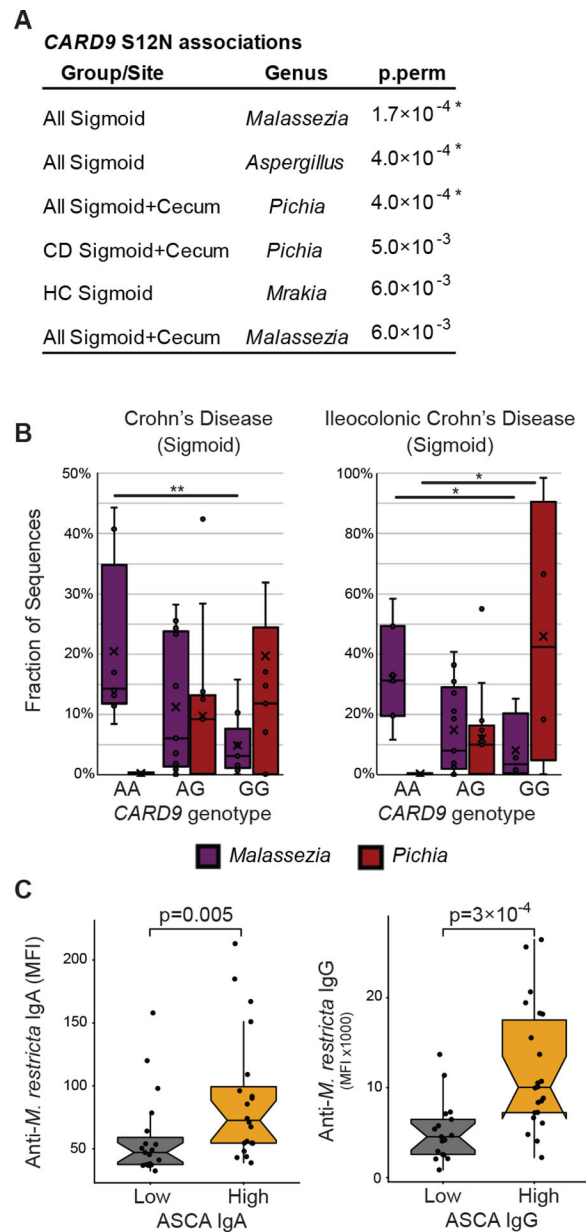


Figure 2. The presence of *Malassezia* is linked to the Crohn's disease *CARD9* risk allele

(A) Presence or absence of the CD-associated variant of *CARD9* was assessed for association with fungal genera. Fungal genera approaching or exceeding statistical significance (Bonferroni-corrected 6.7×10^{-4}) are listed.

(B) Box-and-whisker plots showing relative abundance of *Malassezia* and *Pichia* in sigmoid colon CD samples (all patients, left, and patients with ileocolonic CD, right) according to the *CARD9*^{S12N} SNP genotype. (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA with Tukey's multiple comparison test).

(C) Serum samples were selected from a biobank of CD patient sera previously characterized as "High" or "Low" for ASCA IgG or IgA ($n = 18$ – 22 per group) and screened by flow cytometry for IgA and IgG reactivity against *Malassezia restricta*. (Mann-Whitney

U Test) Notches indicate 95% confidence interval and whiskers extend no further than 1.5 times IQR from the hinge.

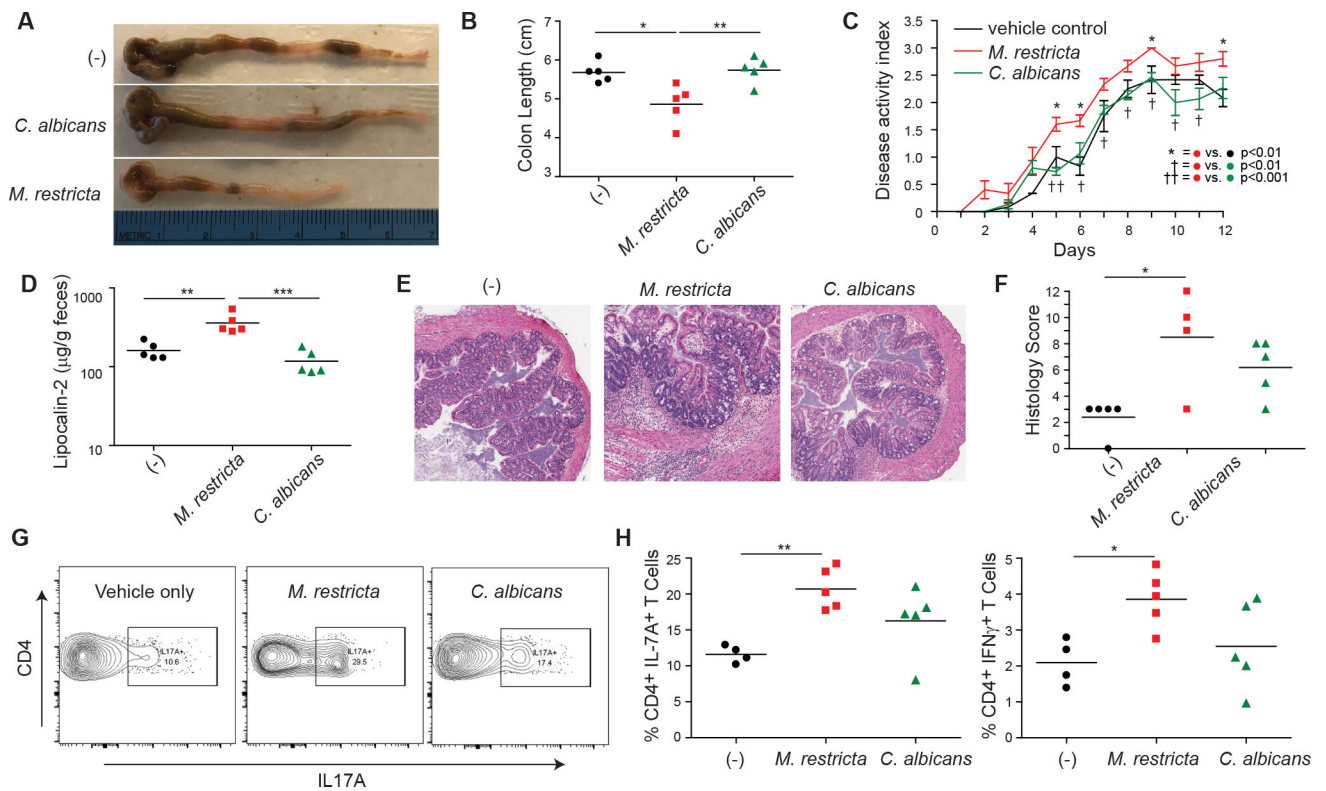


Figure 3. *M. restricta* exacerbates colitis in mice

(A, B) Colon length upon termination of experiment in which mice were gavaged with the indicated live yeast and exposed to DSS in their drinking water for 7 days and then without DSS for 5 days (n=5/group).

(C) Disease activity over duration of experiment.

(D) Fecal lipocalin-2 levels were measured by ELISA on day 6.

(E, F) Representative H&E-stained colon sections of an experiment terminated on day 7 (E) and histological assessment of disease severity (F).

(G, H) Percentage of IL-17A- and IFN- γ -producing colonic lamina propria CD4⁺ T cells was determined upon sacrifice (day 12).

(* , p<0.05; ** , p<0.01; *** , p<0.001; one-way ANOVA with Tukey's multiple comparison test). Each dot represents an individual mouse. Data are representative of three independent experiments.

See also Figures S2 and S3.

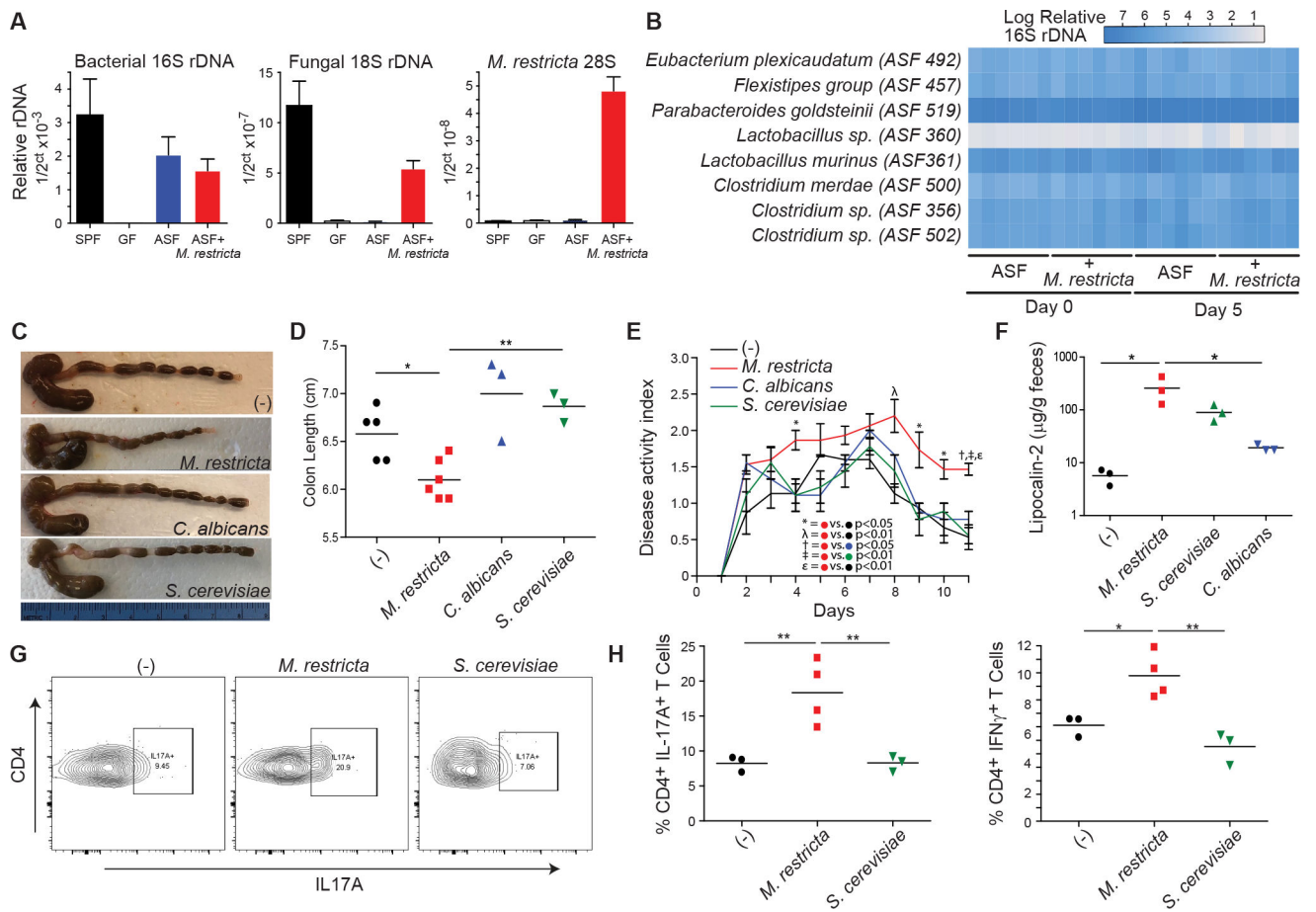


Figure 4. *M. restricta* is sufficient to exacerbate colitis in gnotobiotic mice

(A) Detection of bacterial and fungal rDNA by quantitative PCR in feces of specific pathogen-free (SPF), germ-free (GF), altered Schaedler flora-colonized (ASF) mice, and ASF mice gavaged with *M. restricta* (n=5/group).

(B) Levels of the 8 ASF bacteria were assessed by quantitative PCR of 16s rDNA before and after exposure to *M. restricta*. Each column is a different mouse as labeled

(C, D) Colon length upon termination of experiment in which fungal-free altered Schaedler flora (ASF) mice were gavaged with live yeast and exposed to DSS in their drinking water.

(E) Disease activity over duration of experiment.

(F) Fecal lipocalin-2 levels were measured by ELISA on day 5.

(G, H) Percentage of IL-17A- and IFN- γ -producing colonic lamina propria CD4⁺ T cells was determined upon sacrifice.

(*, p<0.05; **, p<0.01; ***, p<0.001; one-way ANOVA with Tukey's multiple comparison test). Each dot represents an individual mouse.

See also Figure S4.

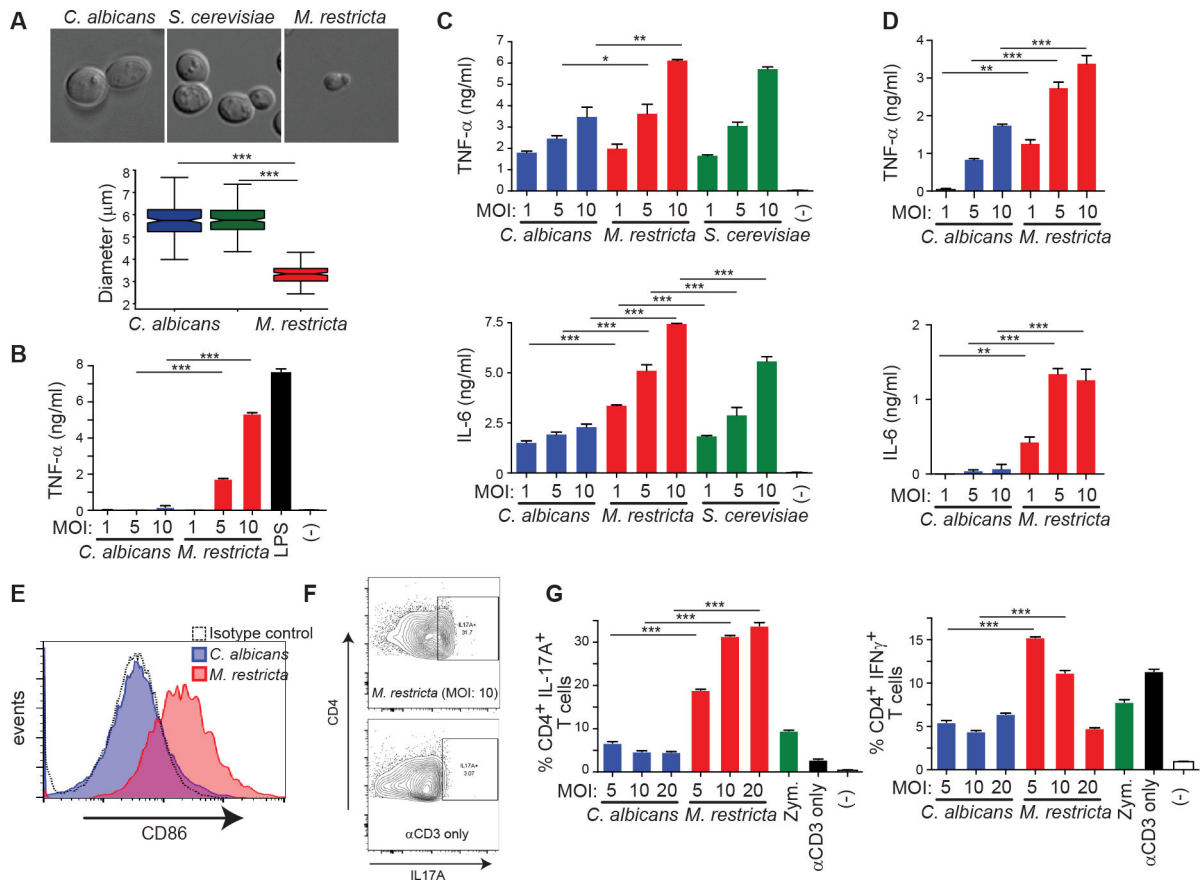


Figure 5. *M. restricta* elicits a strong inflammatory response from myeloid phagocytes

(A) *C. albicans*, *S. cerevisiae*, and *M. restricta* were grown in liquid culture and imaged by differential interference contrast microscopy. Diameters ($n > 150$ each) were measured using ImageJ.

(B) *M. restricta*, *C. albicans* or *S. cerevisiae* were fixed (killed) in paraformaldehyde, and human dendritic cells were exposed to killed yeasts at the indicated multiplicities of infection (MOI), or to *E. coli* lipopolysaccharide (LPS, 100 ng/ml) for 24 hours. TNF- α in culture supernatants was measured by ELISA.

(C) Mouse bone marrow-derived dendritic cells were stimulated as in (B) and TNF- α and IL-6 levels were measured in culture supernatants.

(D) Mouse bone marrow-derived macrophages were stimulated as in (B) and TNF- α and IL-6 levels were measured in culture supernatants.

(E) Mouse bone marrow-derived dendritic cells were stimulated with fungi as in (B) and expression of CD86 was assessed by flow (MOI 10).

(F, G) Mouse bone marrow-derived dendritic cells were stimulated with the indicated fungi in the presence of anti-CD3 ϵ anti-bodies and co-cultured with naïve CD4⁺ T cells.

Production of IL-17A and IFN- γ by CD4⁺ cells was assessed by flow cytometry.

(* , $p < 0.05$; ** , $p < 0.01$; *** , $p < 0.001$; one-way ANOVA with Tukey's multiple comparison test). Data are representative of three independent experiments.

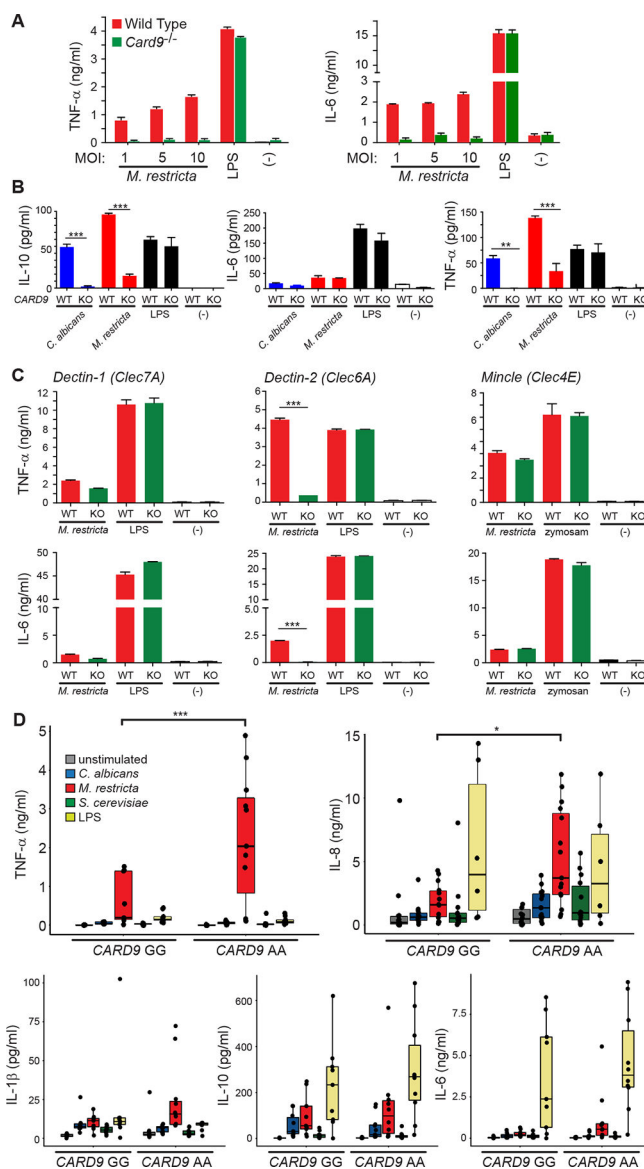


Figure 6. Innate inflammatory responses to *M. restricta* are *CARD9*/C-type lectin dependent and are enhanced by the Crohn's disease-associated *CARD9* polymorphism

(A) Mouse bone marrow-derived dendritic cells from wild-type (WT) or *Card9*^{-/-} mice were stimulated with fixed *M. restricta* at the indicated multiplicities of infection (MOI), or to *E. coli* lipopolysaccharide (LPS, 100 ng/ml) for 24 hours. TNF- α and IL-6 levels were measured in culture supernatants.

(B) Neutrophils from wild type (WT) or *Card9*^{-/-} (KO) mice were purified from bone marrow and stimulated with *C. albicans* or *M. restricta* yeast (MOI 5, fixed in paraformaldehyde) or *E. coli* lipopolysaccharide (LPS, 100 ng/ml) for 24 hours. Cytokines in culture supernatants were measured by ELISA.

(C) Mouse bone marrow-derived dendritic cells from wild-type (WT) or the indicated knockout (KO) mice were stimulated with *M. restricta* yeast (MOI 5, fixed in paraformaldehyde), *E. coli* lipopolysaccharide (LPS, 100 ng/ml), or zymosan (30 μ g/ml) as indicated for 24 hours. TNF- α and IL-6 levels were measured in culture supernatants.

(D) Human peripheral blood-derived dendritic cells were prepared from healthy donors determined to be homozygous for the *CARD9*^{S12N} A (risk) or G (protective) alleles. Cells were stimulated (MOI 10) with the indicated yeasts or LPS (100 ng/ml) for 24 hours and production of TNF- α , IL-8, IL-1 β , IL-10, and IL-6 (n=6–15) was measured. Each dot is an individual patient (measured in duplicate), and the boxes indicate means and standard deviations.

(*, p<0.05; **, p<0.01; ***, p<0.001; one-way ANOVA with Tukey's multiple comparison test). Data in A and B are representative of three independent experiments.

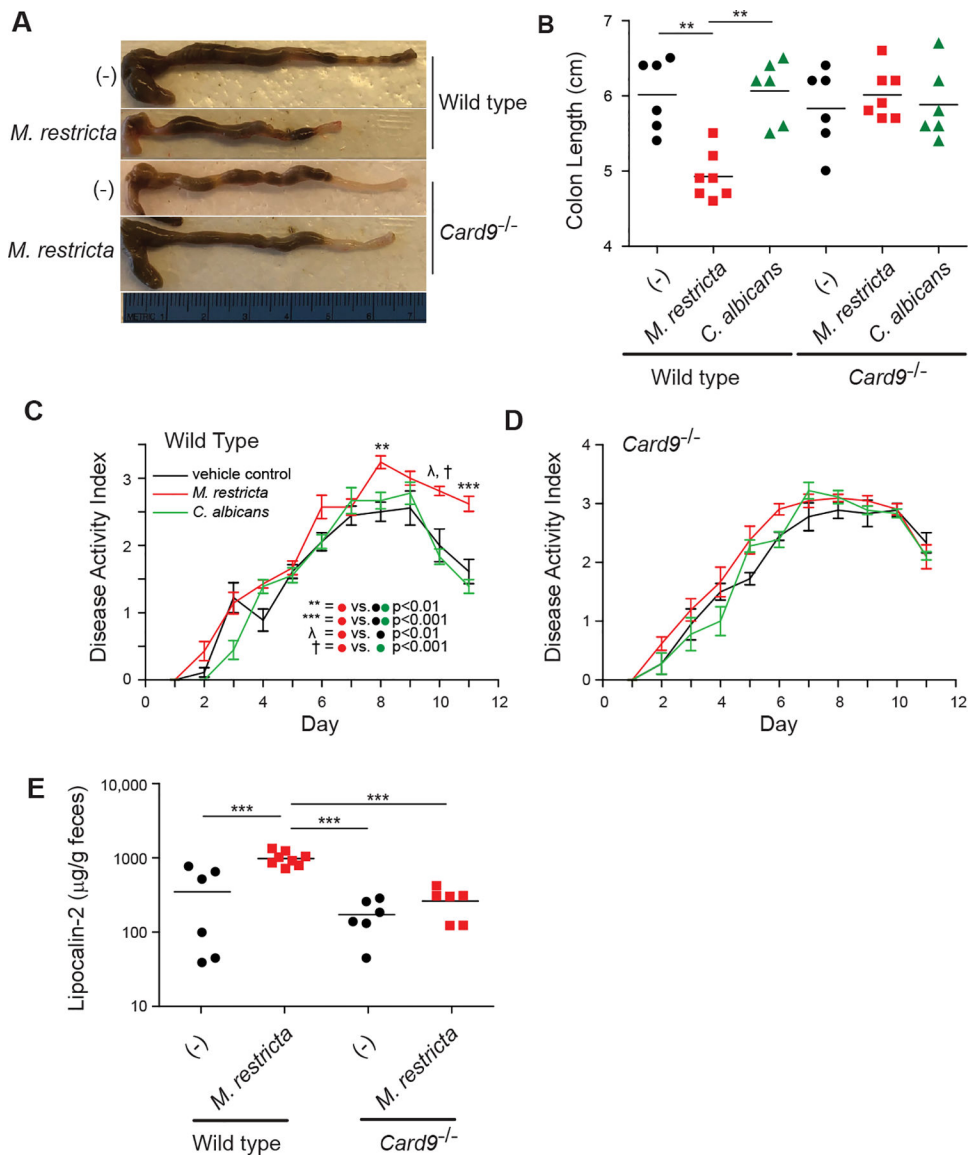


Figure 7. In vivo effects of *M. restricta* require CARD9

(A, B) Colon length upon termination of experiment in which *Card9*^{-/-} mice or wild type littermates were gavaged with the indicated live yeast and exposed to DSS in their drinking water for 7 days and then without DSS for 5 days (n=6–7/group).

(C, D) Disease activity over duration of experiment.

(E) Fecal lipocalin-2 levels were measured by ELISA on day 6.

(*, p<0.05; **, p<0.01; ***, p<0.001; one-way ANOVA with Tukey's multiple comparison test). Each dot represents an individual mouse. Data are representative of at least two independent experiments.