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Commensal fungi recapitulate the protective benefits of intestinal bacteria

Tony T. Jiang[1], **Tzu-Yu Shao**[1,2], **W.X. Gladys Ang**[1], **Jeremy M. Kinder**[1], **Lucien H. Turner**[1], **Giang Pham**[1], **Jordan Whitt**[2], **Theresa Alenghat**[2], and **Sing Sing Way**[1,3,*]

[1]Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, 45229, USA

[2]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, 45229, USA

SUMMARY

Commensal intestinal microbes are collectively beneficial in preventing local tissue injury and augmenting systemic antimicrobial immunity. However, given the near-exclusive focus on bacterial species in establishing these protective benefits, the contributions of other types of commensal microbes remain poorly defined. Here we show that commensal fungi can functionally replace intestinal bacteria, by conferring protection against injury to mucosal tissues and positively calibrating the responsiveness of circulating immune cells. Susceptibility to colitis and influenza A virus infection that occur upon commensal bacteria eradication are efficiently overturned by monocolonization with either *Candida albicans* or *Saccharomyces cerevisiae*. The protective benefits of commensal fungi are mediated by mannans, a highly conserved component of fungal cell walls, since intestinal stimulation with this moiety alone overrides disease susceptibility in mice depleted of commensal bacteria. Thus, commensal enteric fungi safeguard local and systemic immunity by providing tonic microbial stimulation that can functionally replace intestinal bacteria.

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[*]Correspondence: singsing.way@cchmc.org (SSW).
[3]Lead Contact and corresponding author

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AUTHOR CONTRIBUTIONS

All authors participated in the design or execution of the experiments. T.T.J and S.S.W. wrote the manuscript with editorial input from all the authors.

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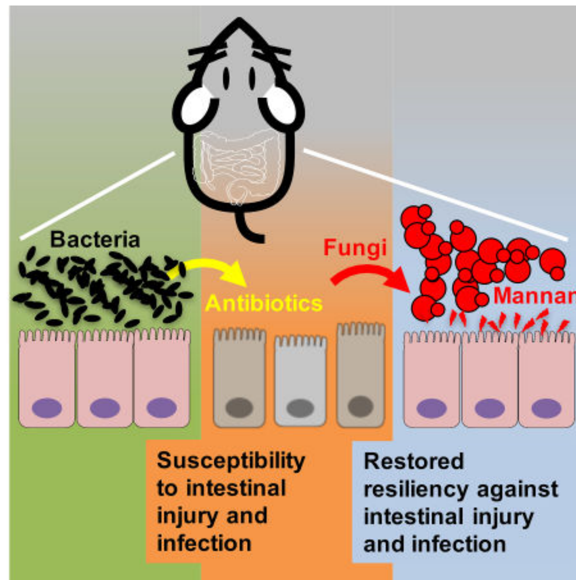

Intestinal colonization is not restricted to bacteria alone, but includes fungi whose symbiotic interactions with the mammalian host remain incompletely defined. Jiang *et al.* demonstrate that diverse species of fungi can functionally replace enteric bacteria by conferring protection against infectious and inflammatory disorders.

INTRODUCTION

Commensal intestinal microbes collectively play beneficial roles in calibrating immunological responsiveness to improve the outcomes of inflammatory disorders and infections (Chung and Kasper, 2010; Hooper et al., 2012; Round and Mazmanian, 2009). This symbiotic relationship between enteric commensal microbes and the mammalian host has primarily been probed by analyzing intestinal bacteria. For example, oral administration of broad-spectrum antibiotics that deplete enteric bacteria impairs resiliency and survival against dextran sodium sulfate (DSS)-induced colitis (Rakoff-Nahoum et al., 2004). Interestingly, these protective benefits are not confined to local intestinal tissues, but extend distally to enhance responsiveness of immune cells that protect against pathogens that disseminate or replicate in extra-intestinal tissues (Abt et al., 2012; Ichinohe et al., 2011). These collective benefits of commensal bacteria are reproduced by intestinal stimulation using conserved bacterial structural components such as peptidoglycan or LPS (Abt et al., 2012; Clarke et al., 2010; Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004). Thus, commensal bacteria, through their principal molecular components, play important immune-modulatory roles in protecting against disease locally in the intestine and systemically in extra-intestinal tissues.

Importantly however, microbial commensalism is not restricted only to bacteria, but includes viral and fungal species each capable of colonizing mammalian hosts. In this regard, mono-colonization with individual strains of murine norovirus has been recently shown to replace commensal bacteria in promoting resistance against DSS induced colitis and maintaining intestinal homeostasis (Kernbauer et al., 2014). This functional overlap between commensal

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bacteria and viruses gives rise to fundamental questions regarding whether other classes of intestinal microbes play similar beneficial roles in positively calibrating local and systemic immunity.

Fungi are a ubiquitous component of the mammalian microbiome. Despite being estimated to comprise <1% of commensal microbial species by genomic equivalence (Arumugam et al., 2011; Qin et al., 2010), individual fungi are also of >100-fold increased size compared with bacteria (Underhill and Lliev, 2014). Host sensing of intestinal fungi through the microbial pattern recognition receptor, dectin-1, in mice harboring commensal enteric bacteria has been associated with protective immune responses during DSS induced tissue injury (Iliev et al., 2012). Nonetheless, since intestinal bacteria can independently provide immune modulatory signals, it remains unclear if these beneficial properties attributed to intestinal fungi reflect direct interactions with the mammalian host, or indirect consequences from shifts in the composition of bacterial communities. For example, dectin-1 deficient mice have increased proportions of lactobacilli that can independently ameliorate DSS induced colitis by enhancing colonic accumulation of protective regulatory T cells (Tang et al., 2015). Thus, whether fungal-host interactions are autonomously beneficial for influencing disease outcomes remains undefined.

RESULTS

C. albicans intestinal mono-colonization overrides the protective necessity of commensal bacteria

Fungi are naturally impervious to antibiotics that eradicate commensal bacteria, and therefore are poised to bloom during antibiotic treatment (Erb Downward et al., 2013; Fan et al., 2015; Mason et al., 2012). This ability of fungi to rapidly accumulate after antibiotic induced eradication of intestinal bacteria was exploited to investigate whether commensal fungi – in isolation – can functionally recapitulate the protective benefits of enteric bacteria. Supplementing the drinking water with a previously described cocktail of broad-spectrum antibiotics efficiently eliminates recoverable anerobic and aerobic bacteria from the feces of mice housed in our specific pathogen free facility (Figure 1A) (Abt et al., 2012; Jiang et al., 2015). 16S rDNA copies were also sharply reduced (>400-fold) in the feces of antibiotic treated compared with control mice (Figure 1B), in agreement with prior studies demonstrating commercial rodent chow subsequently becomes the major contributor and source of residual bacterial 16S rDNA in mice receiving this antibiotic cocktail (Hill et al., 2010). In turn, *Candida albicans* efficiently establishes intestinal colonization among mice sustained on this broad-spectrum antibiotic cocktail (Figure 1C). Interestingly, despite high-density intestinal *C. albicans* colonization, mice gained weight at a comparable tempo compared with antibiotic treated controls not administered *C. albicans* (Figure 1D). Fungal intestinal colonization also occurs without disrupting the architecture and morphology of local tissues. Crypt height, goblet cell density and paneth cell granularity were each histologically unchanged throughout the small and large intestine among *C. albicans* colonized mice (Figure S1). Together, these results show *C. albicans* can replace intestinal bacteria in a commensal fashion without overt harmful consequences.

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Mice housed in our specific pathogen free facility are devoid of endogenous commensal fungi and noroviruses since the levels of these microbes measured in the feces by nucleic acid qPCR were comparable to the background observed for germ-free mice (Figure 1E and data not shown). We therefore reasoned that any *in vivo* shifts in disease susceptibility will reflect the biological properties of mono-colonization with this fungal species. Remarkably, *C. albicans* mono-colonization efficiently overturned mortality induced by DSS among antibiotic treated mice, with overall survival and average time-to-death rebounding to levels comparable to commensal bacteria replete conventional mice (Figure 1F). Commensal *C. albicans* also protects against DSS induced colonic shortening, intestinal permeability and inflammation compared with antibiotic treated control mice (Figure S2). Importantly, this protection cannot be explained by potential bacteria that persist despite antibiotic treatment since *C. albicans* mono-association also significantly improved survival of germ-free mice, which are highly susceptible to DSS induced intestinal injury (Kitajima et al., 2001) (Figure S3). Thus, enteric mono-colonization with a single fungal species can override the protective necessity of commensal bacteria in averting local tissue injury.

We next investigated the immune modulatory properties of commensal fungi in extra-intestinal tissues, given the active calibration of systemic immune cell responsiveness previously shown for intestinal bacteria (Abt et al., 2012; Clarke et al., 2010; Ichinohe et al., 2011). For these studies, influenza A virus with restricted tropism to respiratory tissues (Chaturvedi et al., 2015), and natural resistance to the antibiotics used to facilitate *C. albicans* colonization (Abt et al., 2012; Ichinohe et al., 2011), makes it an ideal pathogen to probe the extra-intestinal impacts of commensal fungi. We found *C. albicans* mono-colonization efficiently overturned the fatal susceptibility to influenza A virus infection amongst commensal bacteria depleted mice (Figure 1G). Commensal fungi also reversed the blunted accumulation and IFN-γ production of viral-specific CD8 T cells for mice treated with antibiotics, to levels comparable to commensal bacteria replete conventional mice housed under specific pathogen free conditions (Figure 1H). Collectively, these findings highlight *C. albicans* mono-colonization can replace the systemic benefits of commensal enteric bacteria.

S. cerevisiae intestinal mono-colonization overturns disease susceptibility induced by commensal bacteria depletion

Saccharomyces cerevisiae, another yeast naturally found in the mammalian gut (Hoffmann et al., 2013; Sokol et al., 2017), was used to evaluate whether these protective benefits are shared by other species of commensal fungi. Similar to *C. albicans*, mice treated with the same cocktail of broad-spectrum antibiotics and then inoculated with *S. cerevisiae* consistently have high-density of this yeast in their feces (Figure 2A). In turn, the uniform mortality and colonic shortening induced by DSS among commensal bacteria depleted mice was dramatically improved in mice with *S. cerevisiae* mono-colonization (Figure 2B and 2C). Commensal *S. cerevisiae* also reduced susceptibility to intranasal influenza A virus infection among antibiotic treated mice (Figure 2D), which coincided with rebounded accumulation and IFN-γ production by protective viral-specific CD8 T cells (Figure 2E). These near identical benefits conferred by *S. cerevisiae* and *C. albicans* suggest universal protective properties shared across fungal species capable of mammalian host colonization.

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Protective benefits of enteric commensal fungi require persistent colonization

To investigate the durability of protection conferred by commensal fungi, the impacts of fungal eradication with the antimycotic agent, fluconazole, on protection against DSS colitis and respiratory influenza A virus infection were evaluated. We found recoverable fungi in the feces of *C. albicans* mono-colonized mice rapidly declined to undetectable levels 10 days after adding fluconazole to antibiotic-supplemented drinking water that is consistent with recent studies (Figure 3A) (Underhill and Lliev, 2014; Wheeler et al., 2016). Interestingly, fungal eradication efficiently abolished the improved survival and delayed time-to-death after DSS challenge conferred by *C. albicans* mono-colonization (Figure 3B). Protection against influenza A was similarly overturned following depletion of commensal fungi (Figure 3C). This necessity for persistent fungal colonization in protection against DSS colitis and influenza A virus infection is consistent with susceptibility to chemical colitis and allergic airway disease unleashed after fluconazole treatment of mice with a diverse repertoire of commensal microbe (Wheeler et al., 2016). However, this requirement for tonic presence of commensal fungi in systemic immune modulation contrast with the concept of “trained immunity” recently shown to be primed by invasive fungi (Cheng et al., 2014), which may reflect contextual differences in sensing fungi as intestinal microbes as opposed to pathogens in sterile tissues after parental injection.

Mannans mediate the protective benefits conferred by commensal intestinal fungi

This requirement for persistent intestinal colonization by commensal fungi, combined with the functional overlap between *C. albicans* and *S. cerevisiae*, led us to investigate the molecular basis for these fungal-driven immunological shifts. Since mannans are a major cell wall component shared by nearly all fungi (Klis et al., 2006; Ruiz-Herrera et al., 2006), we evaluated if this fungal moiety could functionally replace the protective benefits of intact fungi in the absence of commensal bacteria. Intrarectal administration was employed to mimic the high-density commensal fungi colonization observed in the lower intestinal tract. Remarkably, susceptibility to DSS and influenza A virus infection among antibiotic treated mice were each mitigated by mannan reconstitution, with survival and accumulation of protective immune cells restored to levels comparable to *C. albicans* colonized controls (Figure 4A and S4). By contrast, intrarectal inoculation with other fungal cell wall components such as curdlan or zymosan failed to improve mortality among antibiotic treated mice (Figure 4A). Thus, the protective benefits of commensal fungi are recapitulated by mannans, a highly conserved fungal cell wall constituent. Fungal derived mannans can stimulate host cells through a variety of microbial pattern recognition receptors. For example, mannan induced TNF-α production is sharply reduced among TLR2 or TLR4-deficient macrophage cells (Jouault et al., 2003; Netea et al., 2006; Tada et al., 2002), and the ability of mannan-rich fungal moieties such as zymosan to activate host cells is blunted in the absence of dectin-1 (Brown et al., 2002; Taylor et al., 2007). The stimulation of other proinflammatory cytokines such as IL-17 by mannan is dependent on the presence of the mannose receptor CD206, and can be further amplified through co-activation of TLR2 (van de Veerdonk et al., 2009). More recently, an essential role has been demonstrated for dectin-2 in mediating responsiveness of bone marrow derived dendritic cells to mannans (Saijo et al., 2010). A potential explanation for these apparently discordant results may be heterogeneity in the mannan preparation or cell activation markers evaluated. Therefore, to

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investigate the necessity of each host receptor in recognizing our purified, biologically active mannan preparation, we evaluated how neutralizing each molecule impacts mannan stimulation *in vitro*. For these studies, we used the RAW-Blue 264.7 macrophage NF-κB reporter cell line that are responsive to stimulation through a wide variety classical microbial pattern recognition receptors including toll-like receptors and c-type lectins (i.e., dectin-1, dectin-2, mannose receptor) (Bi et al., 2010; Ying et al., 2015). Interestingly, neutralization of dectin-1 and TLR4 each significantly reduced mannan induced NF-κB. By contrast, mannan stimulated cell activation was not significantly impacted by antibody blockade against TLR2, dectin-2 or the mannose receptor (Figure 4B).

We next addressed the *in vivo* necessity of dectin-1 and TLR4 in mediating the protective benefits of *C. albicans* by evaluating the impact of fungal colonization on susceptibility to DSS induced intestinal injury among mice with targeted defects in these molecules. Similar to our findings for isogeneic WT mice on the C57BL/6 background, susceptibility to DSS was sharply increased after antibiotic induced eradication of commensal bacteria in both dectin-1-deficient and TLR4-deficient mice (Figure 4C, 4D). Interestingly, *C. albicans* mono-colonization efficiently overturned DSS induced mortality among dectin-1 deficient mice (Figure 4C). By contrast, the protective benefits of fungal colonization against DSS were sharply reduced among TLR4 deficient mice, with only marginally improved survival amongst *C. albicans* colonized mice compared with antibiotic treated controls ($P = 0.90$) (Figure 4D). Thus, TLR4 plays essential non-redundant roles for conferring the protective benefits of commensal *C. albicans* that bypass the protective necessity of enteric bacteria through fungal specific mannans.

DISCUSSION

Tonic stimulation by commensal bacteria is increasingly recognized to improve many aspects of host health (Abt et al., 2012; Chung and Kasper, 2010; Hooper et al., 2012; Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004; Round and Mazmanian, 2009). Here we show that susceptibility to intestinal injury and extra-intestinal infection caused by absent bacteria is overturned with fungal colonization. Antifungal administration eliminates the beneficial impacts of commensal fungi, in agreement with the necessity for endogenous enteric fungi to protect against intestinal injury or airway inflammation in the presence of commensal bacteria (Wheeler et al., 2016). These benefits of commensal fungi, when evaluated in the absence of intestinal bacteria, are in sharp contrast to their deleterious roles in exacerbating intestinal injury in dectin-1 deficient mice (Iliev et al., 2012). This discrepancy likely reflects additional stimulation by commensal bacteria or differences in commensal bacteria composition among dectin-1 deficient mice (Tang et al., 2015), or discordant features of the fungal “mycobiome” across institutions (Iliev et al., 2012). By exploiting the absence of detectable endogenous fungi in our facility, we further demonstrate that individual fungal species, in isolation, can take the place of commensal bacteria in positively calibrating local and systemic immunity.

The protective benefits of commensal fungi are mediated by mannan, a highly conserved structural component of fungal cell walls. These results parallel the biological properties of commensal bacteria conferred by their principal molecular constituents. For example,

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lipotechoic acid or LPS administration, in lieu of live commensal bacteria, can each avert DSS-induced mortality (Rakoff-Nahoum et al., 2004). Likewise, peptidoglycan, LPS, CpG or poly(I:C) reconstitution augments systemic antimicrobial immunity among antibiotic treated mice (Abt et al., 2012; Ichinohe et al., 2011). These benefits of commensal bacteria conferred by their individual structural components, require host microbial pattern recognition receptors as LPS-mediated protection against DSS colitis is abolished in absence of TLR4 (Rakoff-Nahoum et al., 2004). Interestingly however, host recognition of mannans has been demonstrated to occur with considerably more functional redundancy spanning multiple pattern recognition receptors including TLR2, TLR4, dectin-1, dectin-2, DC-SIGN, mincle and the mannose receptor (Hardison and Brown, 2012; Jouault et al., 2003; Netea et al., 2008; Nigou et al., 2008; Saijo et al., 2010). While these conclusions have been shown using complementary models of *in vitro* stimulation with purified mannans or parenteral infection with invasive fungal pathogens, our data show an essential role for TLR4 in sensing commensal fungi *in vivo*.

In the broader biological context, we show the protective benefits of commensal microbes are not limited to bacteria and viruses, but also shared by enteric fungi. Host sensing of type I interferons is essential for the immune modulatory properties of commensal murine norovirus (Kernbauer et al., 2014), and macrophage cells show diminished interferon responsiveness in the absence of commensal bacteria (Abt et al., 2012). Considering invasive fungi also induce type I interferons (Biondo et al., 2011; Bourgeois et al., 2011; del Fresno et al., 2013), it is tempting to speculate that diverse commensal microbial types – spanning fungi, bacteria and viruses – all converge to stimulate type I interferon production that potentiates inflammasome-dependent IL-1β and IL-18 activation to positively calibrate beneficial host responses (Fang et al., 2014; Fernandes-Alnemri et al., 2010; Henry et al., 2007; Ichinohe et al., 2011). Interestingly, IFN-β supplementation can blunt *C. albicans* induced pro-IL-1β and IL-1β release by LPS primed macrophage cells, highlighting that type I interferons can also suppress inflammasome activation causing increased susceptibility to invasive fungal infection (Guarda et al., 2011). Therefore, further investigating how host receptors that sense intestinal microbes can facilitate their protective benefits yet simultaneously suppress their potential for invasive infection may unveil the fundamental dichotomy between commensal and pathogenic microbes.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sing Sing Way (singsing.way@cchmc.org)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—Age- and sex-matched wild-type, Dectin-1$^{-/-}$ and TLR4$^{-/-}$ mice on the C57BL/6 background were purchased from Charles River and housed under specific pathogen-free conditions. Germ-free C57BL/6 mice were maintained in gnotobiotic isolator units, exclusively fed autoclaved chow and water, and routinely monitored to ensure the absence of

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microbial contamination. All experiments were conducted under Cincinnati Children's Hospital Animal Care and Use Committee approved protocols.

Fungi—The commonly used *C. albicans* wild-type laboratory strain SC5314 was a kind gift from Dr. Daniel H. Kaplan (University of Minnesota) (Igyarto et al., 2011). *S. cerevisiae* strain MYA797 was purchased from the ATCC.

Viruses—Influenza A virus expressing $OVA_{257-264}$ (SIINFEKL) peptide (OVA-Influenza A) derived from the PR8 H1N1 strain was a kind gift from Drs. Thomas Moran (Ichan School of Medicine at Mount Sinai) and Paul Thomas (Saint Jude Children's Research Hospital).

METHOD DETAILS

Experimental replication, randomization and blinding—For each experiment, age and sex-matched groups of mice were randomly allocated to experimental groups. Each experiment was independently performed at least twice to ensure reproducibility. Histological scoring was performed by a board-certified veterinarian pathologist (T.A.) in a double blinded fashion. Sample sizes per group in each experiment reflect 80% power to detect a Cohen's effect size of 1.5 with an α error probability of 0.05 (G*Power 3.1).

Antibiotic, antimycotic and DSS treatment—To eradicate commensal bacteria, filter-sterilized drinking water was supplemented with ampicillin (0.5 mg/mL, Sigma), gentamicin (0.5 mg/mL, Sigma), metronidazole (0.5 mg/mL Sigma), neomycin (0.5 mg/mL, Sigma), vancomycin (0.25 mg/mL, MP Biomedicals) and sucralose (4 mg/mL, Sigma) (Abt et al., 2012; Elahi et al., 2013). For depletion of intestinal *C. albicans*, the antibiotic cocktail was supplemented with fluconazole (0.5 mg/mL, Sigma) (Iliev et al., 2012). To induce intestinal injury, the drinking water of mice was supplemented with DSS (40,000 kDa, Alfa Aesar) for 6 days with or without the antibiotic cocktail, and then received untreated or antibiotic supplemented drinking water for the remainder of the experiment. Wild-type mice were administered 3% DSS. To account for the increased DSS susceptibility that occurs in the absence of dectin-1 or TLR4, or among gnotobiotic germ-free mice (Iliev et al., 2012; Kitajima et al., 2001; Rakoff-Nahoum et al., 2004), 2% DSS was used for comparing the impacts of fungal colonization and/or commensal bacteria eradication in these animals.

Fungal colonization—Fungi were cultured the day prior in yeast extract-peptone-adenine-dextrose media at 30°C (200 rpm). The following day, the culture was washed and suspended in sterile saline. Antibiotic treated mice were administered an oral lavage of 10^6 fungal CFUs (in 30 μL phosphate-buffered saline) via P200 micropipette (Xin et al., 2014).

Recoverable bacterial or fungal burden—Tissues were sterilely collected, homogenized, and serial dilutions of each homogenate (into PBS) were spread onto brain heart infusion media (Sigma) agar plates. For isolating fungi, brain heart infusion media used for agar plates were supplemented with ampicillin (2.5 μg/mL, Sigma), gentamicin (2.5 μg /mL, Sigma), metronidazole (2.5 μg /mL Sigma), neomycin (2.5 μg/mL, Sigma),

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vancomycin (1.25 μg /mL, MP Biomedicals). Colony forming units were enumerated after incubation for 24 hours at 37°C.

DNA/RNA isolation and qPCR—Bacterial DNA was isolated using the QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's instructions. For isolating fungal DNA, individual fecal pellets were suspended in 50 mM Tris buffer (pH 7.5) supplemented with 1 mM EDTA, 0.2% β-mercaptoethanol and 1000 units/ml of lyticase (Sigma), incubated at 37°C for 30 minutes to disrupt fungal cells as described (Iliev et al., 2012) prior to processing through the QIAamp DNA stool mini kit (Qiagen). Viral RNA was extracted with the QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. qPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the following primers: Bacterial 16S, Forward 5'-ACTCCTACGGGAGGCAGCAGT-3', Reverse 5'-ATTACCGCGGCTGCTGGC-3'; Fungal ITS1-2, Forward 5'-CTTGGTCATTTAGAGGAAGTAA-3, Reverse 5'-GCTGCGTTCTTCATCGATGC-3'; Eukaryotic 18S, Forward 5' ATTGGAGGGCAAGTCTGGTG-3', Reverse 5'-CCGATCCCTAGTCGGCATAG-3'; MNV RdRp, Forward 5'-CCAAAGTGGGAT AGAAATGGTAGTC-3', Reverse 5'-TCACTCATCCTCATTCACAAAGACT-3'.

Intestinal permeability—Fasting was initiated and maintained throughout the experiment starting 2 hours prior to intragastric gavage with 0.6 mg/g body weight of FITC-labeled dextran solution (from 100 mg/mL, FD4, Sigma). After 4 hours, blood from the retro-orbital sinus was collected in heparinized tubes and serum analysis for FITC concentration was performed with a fluorescence spectrophotometer (Synergy HTX, Biotek) at an excitation wavelength of 485 nm and emission wavelength of 528 nm. Standard curves were obtained by diluting FITC–dextran in saline.

Influenza A infection—OVA-Influenza A virus was grown and titered in Madin-Darby canine kidney epithelial cell monolayers, and stored at −70°C. For infection, individual virus aliquots were thawed, diluted in PBS, and administered intranasally (6×10^4 PFUs in 30 μL) to mice anesthetized with xylazine and ketamine (Ichinohe et al., 2011).

Microscopy—Hematoxylin and eosin (H&E) or alcian blue/periodic acid-Schiff (AB/PAS) staining of intestinal tissue was performed by the Pathology Core at Cincinnati Children's Hospital. Sections were imaged on a Nikon Eclipse 80i microscope. Slides were analyzed using ImageJ software. Villi width was measured where the base of the villi meets the crypt; at least 50 villi per mouse were measured for villi width. Paneth cell granules were counted in at least 30 crypts per mouse (Kernbauer et al., 2014). Mean values were calculated for each mouse and used as individual data points. The histological scoring for DSS treated mice was graded on a severity scale of 1–5 for epithelial ulceration, inflammatory cell infiltration, and edema.

Isolation of lymphocytes from lung parenchyma—Lungs were minced into small pieces with a razor and digested with collagenase D (1 mg/mL, Sigma), DNAse (0.1 mg/mL, Sigma) in DMEM (Gibco) supplemented with 10% (vol/vol) FBS, 1% (vol/vol) L-glutamine (Cellgro), 1% (vol/vol) penicillin-streptomycin (Cellgro) and 10 mM HEPES (Cellgro) for 60 minutes (37°C, 200 rpm), and then mashed through a 70 μm filter. Residual red blood

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cells were lysed with hypertonic solution (10 mM $HKCO_3$, 16mM NH_4Cl, pH 7.3) prior to tetramer staining or cytokine stimulation.

Tetramer staining and flow cytometry—Single cell suspensions from the lung or mediastinal lymph node were incubated with brilliant violet 421-conjugated $OVA_{257–264}$:H2-K^b tetramer (60 minutes, 25°C) prior to staining with the following fluorophore-conjugated antibodies purchased from eBioscience: FITC anti-mouse CD4 (clone GK1.5), APC anti-mouse CD8α (clone 53-6.7), PE-Cy5 anti-mouse CD11b (clone M1/70), PE-Cy5 anti-mouse CD11c (clone N418), PE-Cy5 anti-mouse F4/80 (clone BM8), PE-Cy5 anti-mouse B220 (clone RA3-6B2), eFluor 450 anti-mouse IFN-γ (clone XMG1.2). Samples were acquired on a BD FACSCanto and analyzed with FlowJo software (Treestar). $OVA_{257–264}$:H-2K^b specific CD8 T cells were gated on lymphocytes, single cells, B cell and myeloid (B220, CD11b, CD11c, F4/80) negative, $CD8^+CD4^-$ tetramer positive cells.

Cytokine production—Single cell suspensions from the lung were stimulated with 50 μM $OVA_{257–264}$ peptide in media supplemented with BD GolgiPlug (BD Biosciences) according to manufacturer's instructions for 4–5 hours at 37°C.

Rectal inoculation with fungal cell wall moieties—Mice were intrarectally administered the indicated dosage of mannan (Sigma), curdlan (Wako) or zymosan (Sigma) suspended in 50 μl saline after anesthetization with xylazine and ketamine beginning one day prior to DSS or influenza A challenge, and re-administered every other day thereafter.

RAW-Blue stimulation and quantification of NF-κB activity—RAW-Blue cells (InvivoGen) cells are derived from the murine RAW 264.7 macrophages with chromosomal integration of a secreted embryonic alkaline phosphatase reporter construct induced by NF-κB. 5×10^4 RAW-Blue cells were seeded 24 hours prior to addition of the indicated blocking antibody. One hour thereafter, mannan (Sigma) was added at a final concentration of 500 μg/mL. Supernatants were collected after 24 hours to quantify NF-κB activity by colorimetric assay using QUANTI-Blue reagent (InvivoGen).

QUANTIFICATION AND STATISTICAL ANALYSIS

The number (n) of individual animals used per group are described in each individual figure panel, or shown by individual data points that represents the results from an individual animal (Figures 1, 2, S1, S2, S3 and S4) or individual well for cell stimulation assays (Figure 4). The number of replicate experiments are described in each figure legend. All statistics and data distribution analysis were performed with Prism (GraphPad). The unpaired two-tailed Student's *t*-test with Welch's correction was used to compare differences between two groups (Ruxton, 2006). The non-parametric Kruskal-Wallis test with Dunn's correction, one-way ANOVA with Holm-Sidak's correction or Mann-Whitney U test was used to evaluate experiments containing more than two groups depending on the distribution pattern of the data. Survival curves were analyzed by the Log-rank (Mantel-Cox) test. The upper threshold for statistical significance for all experiments was set at $P < 0.05$.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC anti-mouse CD4 (clone GK1.5)	eBioscience	Cat#11-0041-85
APC anti-mouse CD8α (clone 53-6.7)	eBioscience	Cat#17-0081-83
PE-Cy5 anti-mouse CD11b (clone M1/70)	eBioscience	Cat#15-0112-83
PE-Cy5 anti-mouse CD11c (clone N418)	eBioscience	Cat#15-0114-82
PE-Cy5 anti-mouse F4/80 (clone BM8)	eBioscience	Cat#15-4801-82
PE-Cy5 anti-mouse B220 (clone RA3-6B2)	eBioscience	Cat#15-0452-83
eFluor 450 anti-mouse IFN-γ (clone XMG1.2)	eBioscience	Cat#48-7311-82
Anti-mouse Dectin-2 (clone D2.11E4)	Thermo Fisher Scientific	Cat#MA1-82675
Anti-mouse TLR4/MD2 (clone MTS510)	Hycult Biotech	Cat#HM1029-FS
Anti-mouse Dectin-1 (clone R1-8g7)	InvivoGen	Cat#mabg-mdect
Anti-mouse TLR2 (clone C9A12)	InvivoGen	Cat#mabg-mtlr2
Anti-mouse Mannose Receptor (clone 15-2)	Abcam	Cat#ab8918
Fungal and Virus Strains		
Candida albicans SC5314	(Igyarto et al., 2011)	N/A
Saccharomyces cerevisiae Sb49	ATCC	ATCC MYA797
Influenza A H1N1 strain PR8	(Moltedo et al., 2009)	N/A
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Ampicillin	Sigma-Aldrich	Cat#A0166-100G
Gentamicin	Sigma-Aldrich	Cat#G3632-25G
Metronidazole	Sigma-Aldrich	Cat#M3761-100G
Neomycin	Sigma-Aldrich	Cat#N6386-100G
Vancomycin	MP Biomedical	Cat#0219554005-5g
Sucralose	Sigma-Aldrich	Cat#69293-100G
Fluconazole	Sigma-Aldrich	Cat#PHR1160-1G
FITC-dextran	Sigma-Aldrich	Cat#FD4-1G
Mannan	Sigma-Aldrich	Cat#M7504-5G
Curdlan	Wako Chemicals	Cat#034-09901
Zymosan	Sigma-Aldrich	Cat#Z4250-5G
Dehydrated Culture Media: Brain Heart Infusion	Thermo Fisher Scientific	Cat#B11060
QUANTI-Blue	InvivoGen	Cat#raw-sp
$OVA_{257-264}$ peptide	United Biochemical Research, Inc.	N/A
BD GolgiPlug (Brefeldin A solution)	BD Biosciences	Cat#555029
Lyticase	Sigma-Aldrich	Cat#L4025-100KU
Critical Commercial Assays		

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
QIAamp DNA Stool Mini Kit	QIAGEN	Cat#51504
QIAamp Viral RNA Mini Kit	QIAGEN	Cat#52904
Deposited Data		
Experimental Models: Cell Lines		
Raw-Blue Macrophage Cells	InvivoGen	Cat#raw-sp
Madin-Darby canine kidney Cells (line MDCK.2)	American Type Culture Collection	CRL-2936
Experimental Models: Organisms/Strains		
Oligonucleotides		
Bacterial 16S, F: 5'-ACTCCTACGGGAGGCAGCAGT-3'; R: 5'-ATTACCGCGGCTGCTGGC-3'	Thermo Fisher Scientific	N/A
Fungal ITS-1, F: 5'-CTTGGTCATTTAGAGGAAGTAA-3'; R: 5'-GCTGCGTTCTTCATCGATGC-3'	Thermo Fisher Scientific	N/A
Eukaryotic 18S, F: 5'-ATTGGAGGGCAAGTCTGGTG-3'; R: 5'-CCGATCCCTAGTCGGCATAG-3'	Thermo Fisher Scientific	N/A
MNV RdRp, F: 5'-CCAAAGTGGGATAGAAATGGTAGTC-3', R: 5'-TCACTCATCCTCATTCACAAAGACT-3'	Thermo Fisher Scientific	N/A
Recombinant DNA		
Software and Algorithms		
Prism 6.07	GraphPad	https://www.graphpad.com/
FlowJo 9.9.6	Treestar	https://www.flowjo.com/solutions/flowjo
G*Power 3.1	G*Power	http://gpower.software.informer.com/3.1/
Other		
$OVA_{257-264}$:H2-K^b Tetramer	NIH Tetramer Core	N/A

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Commensal fungi functionally replace intestinal bacteria in mitigating tissue injury
- Commensal fungi positively calibrate the activation of protective CD8 T cells
- Protective benefits of commensal fungi require persistent intestinal colonization
- Fungal cell-wall mannans recapitulate the protective benefits of commensal fung

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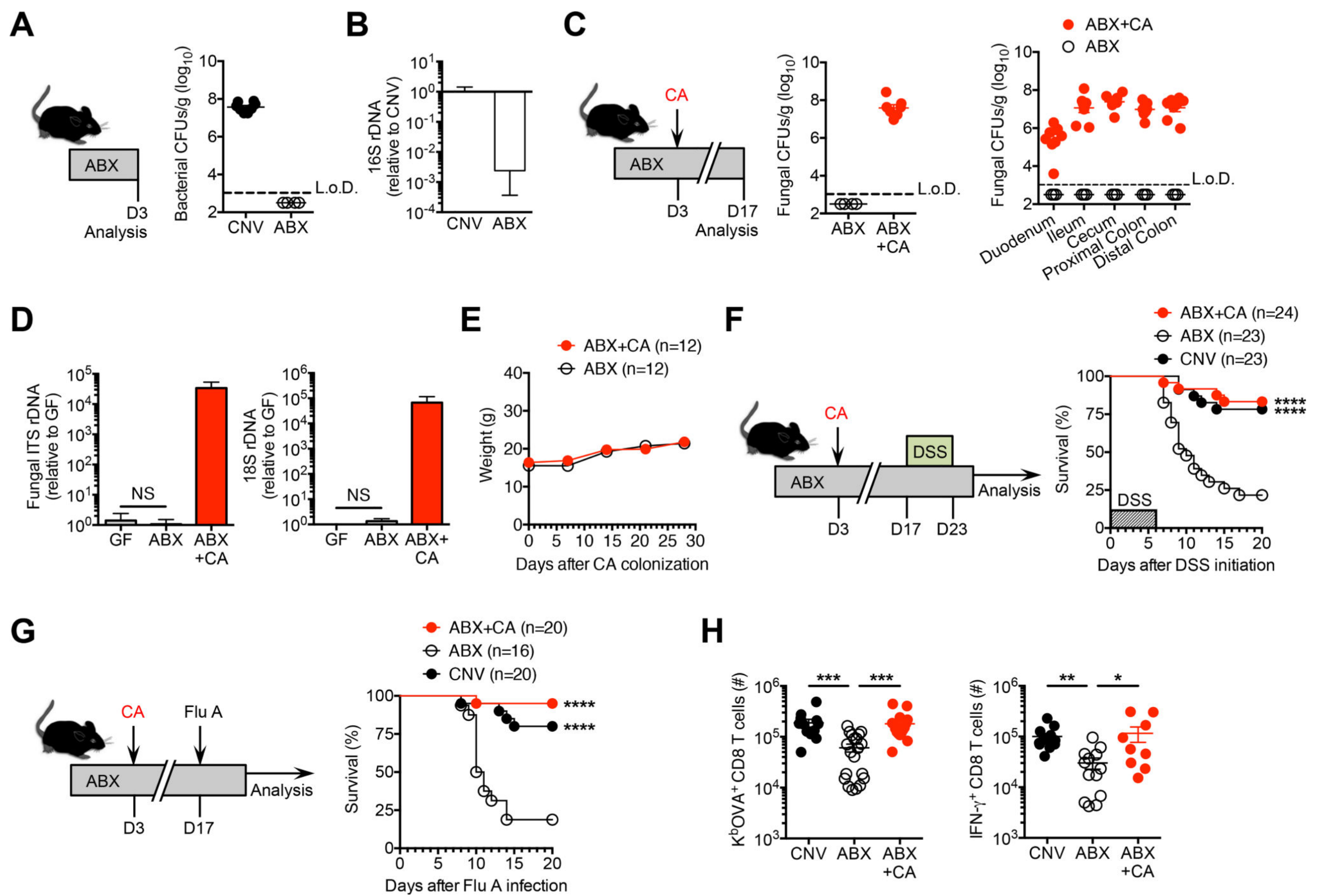

Figure 1. ***Candida albicans*** **intestinal mono-colonization bypasses the protective necessity of commensal enteric bacteria**

(A) Recoverable bacterial colony forming units (CFUs) from feces of mice after supplementing the drinking water with an antibiotic cocktail (ABX) containing ampicillin, gentamicin, metronidazole, neomycin, vancomycin, compared with no-antibiotic treated conventional (CNV) controls housed under specific-pathogen free conditions.

(B) Bacterial-specific 16S rDNA qPCR of feces for mice described in panel (A) normalized to conventional (CNV) controls housed under specific-pathogen free conditions.

(C) Recoverable fungal CFUs in the feces (left) and each intestinal segment (right) for mice inoculated with *C. albicans* (CA) and maintained on ABX treatment for 14 days (ABX+CA), compared to ABX treated controls without CA administration (ABX).

(D) Weight gain after *C. albicans* inoculation among antibiotic treated mice (ABX+CA) compared with ABX only controls.

(E) Fungal-specific internal transcribed spacer (ITS-1) rDNA (left) and eukaryotic 18S rDNA (right) qPCR of feces for mice described in panel (C) normalized to germ-free (GF) controls. (E) Percent survival after DSS supplementation in the drinking water (for six days) for mice described in panels (A,C).

(F) Percent survival after influenza A PR8-OVA (Flu A) intranasal infection (6×10^4 PFUs) for mice described in panels (A,C).

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(G) Total number of Flu A-specific K^bOVA tetramer$^+$ CD8 T cells (left), and IFN-γ^+ CD8 T cells after *in vitro* $OVA_{257–264}$ peptide stimulation (right), from lungs nine days after Flu A infection for mice described in panels (A,C).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, by Log-rank (Mantel-Cox) test (F,G) or nonparametric Kruskal-Wallis test with Dunn's correction (H). Data are representative of at least two independent experiments each with similar results. Bars, mean ± s.e.m. L.o.D., limit of detection.

See also Figures S1–3.

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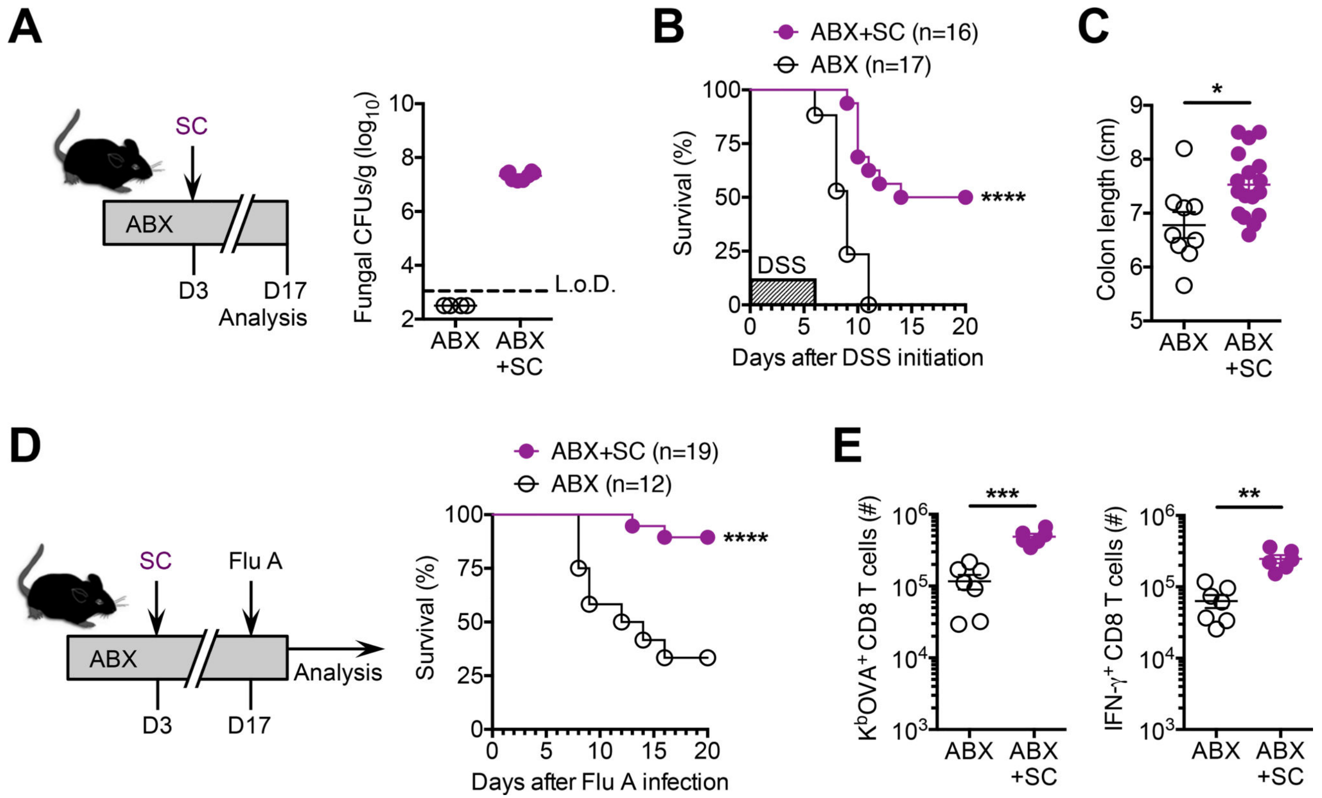

Figure 2. *Saccharomyces cerevisiae* intestinal mono-colonization overturns disease susceptibility induced by commensal bacteria depletion

(A) Recoverable fungal colony forming units (CFUs) in the feces of specific-pathogen free mice administered *S. cerevisiae* (SC) and maintained on drinking water supplemented with an antibiotic cocktail containing ampicillin, gentamicin, metronidazole, neomycin, vancomycin for 14 days (ABX+CA), compared with antibiotic treated controls without SC inoculation (ABX).

(B) Percent survival after DSS supplementation in the drinking water (for six days) for mice described in panel (A).

(C) Colon length after DSS treatment (for six days) for mice described in panel (A).

(D) Percent survival after influenza A PR8-OVA (Flu A) intranasal infection (6×10^4 PFUs) for mice described in panel (A).

(E) Total number of Flu A-specific K^bOVA tetramer$^+$ CD8 T cells (left), and IFN-γ^+ CD8 T cells after *in vitro* $OVA_{257-264}$ peptide stimulation (right), from lungs nine days after Flu A infection for mice described in panel (A).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by ****$P < 0.0001$, Log-rank (Mantel-Cox) test (B,D) or unpaired t-test with Welch's correction (C,E). Data are representative of at least two independent experiments each with similar results. Bars, mean ± s.e.m. L.o.D., limit of detection.

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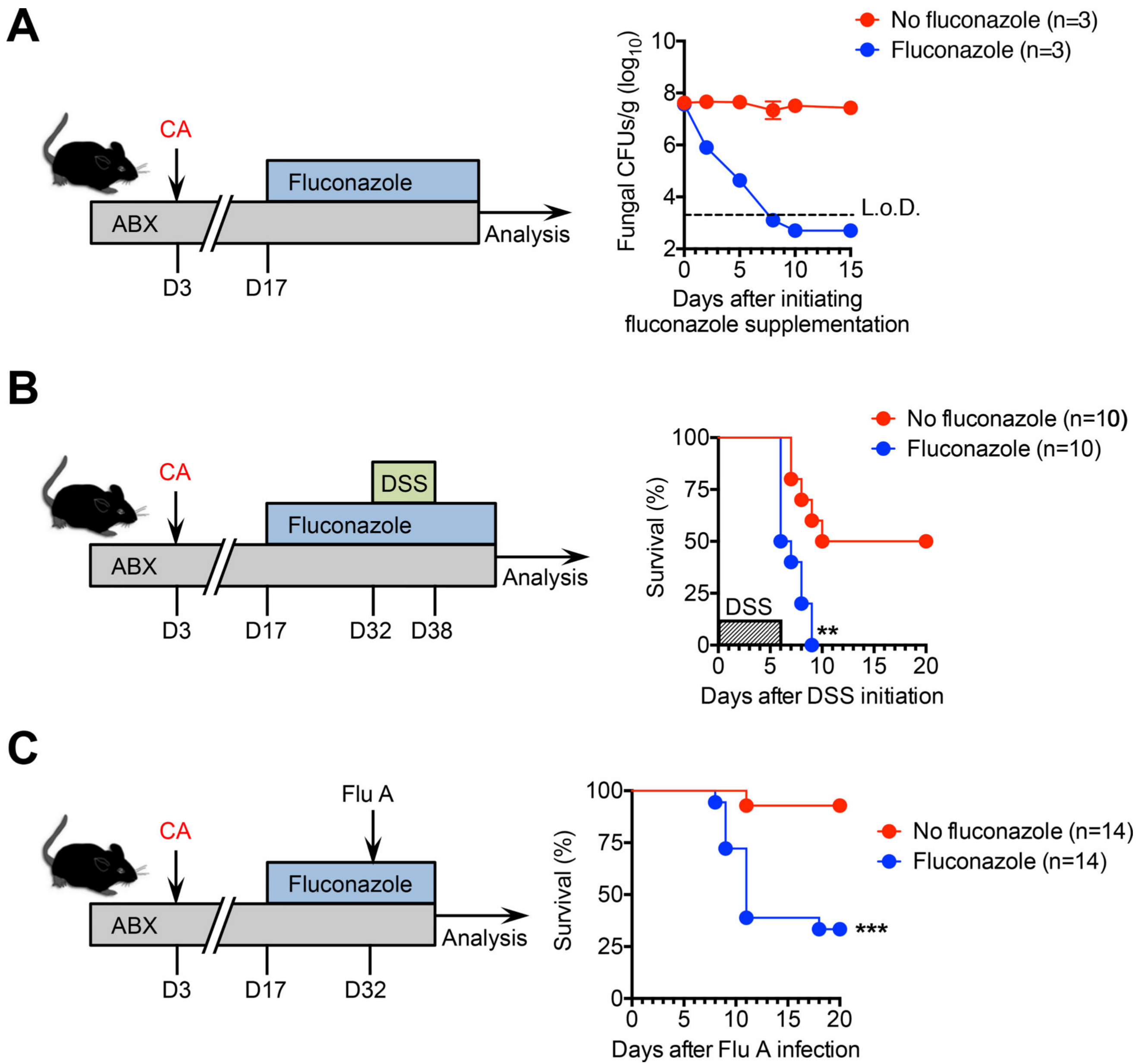

Figure 3. Persistent fungal intestinal colonization is required for maintaining their protective benefits

(A) Fungal colony forming units (CFUs) in the feces with or without supplementing fluconazole (FLUC) to specific-pathogen free mice previously administered *C. albicans* (CA) and maintained on drinking water supplemented with an antibiotic cocktail (ABX) containing ampicillin, gentamicin, metronidazole, neomycin, and vancomycin.

(B) Percent survival after DSS supplementation in the drinking water (for six days) for mice described in panel (A).

(C) Percent survival after influenza A PR8-OVA (Flu A) intranasal infection (6×10^4 PFUs) for mice described in panel (A).

*$P < 0.05$, **$P < 0.001$, by Log-rank (Mantel-Cox) test. Data are representative of at least two independent experiments each with similar results. Bars, mean ± s.e.m. L.o.D., limit of detection.

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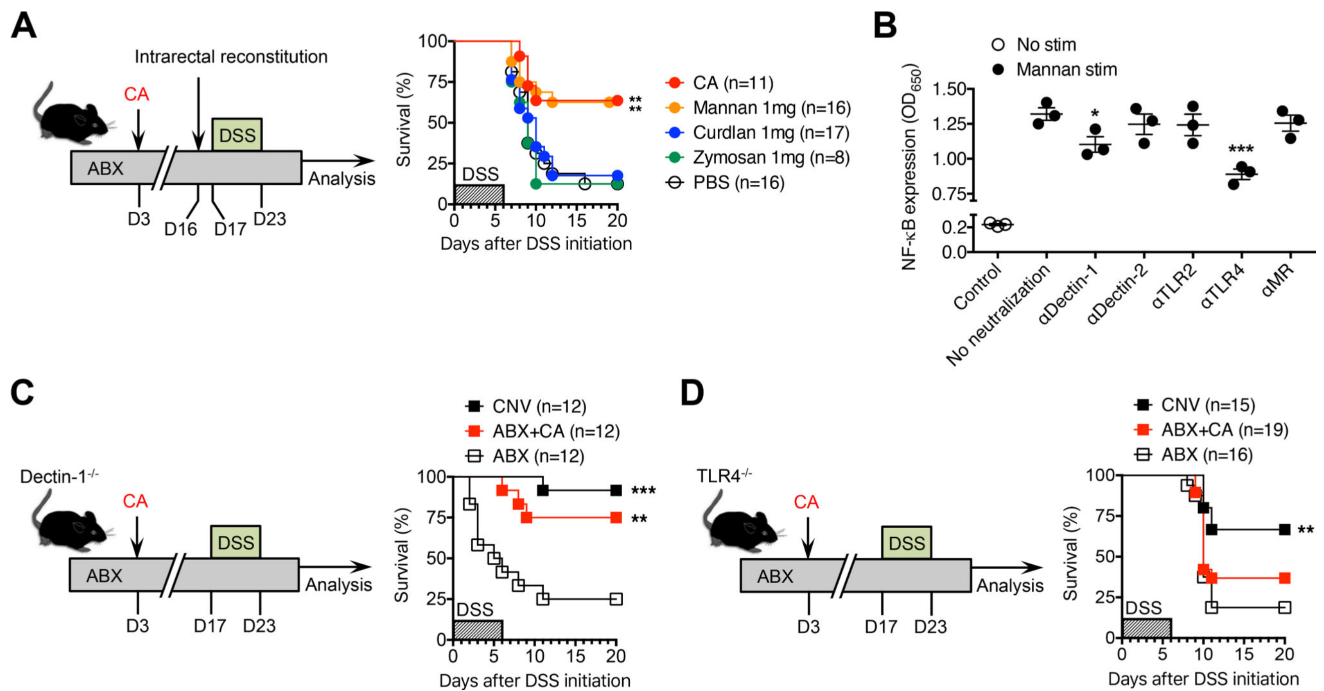

Figure 4. Protective benefits of commensal fungi are mediated by mannans and through TLR4 dependent pathways

(A) Percent survival after DSS supplementation (for six days) among specific-pathogen free mice maintained on drinking water supplemented with an antibiotic cocktail (ABX) containing ampicillin, gentamicin, metronidazole, neomycin, vancomycin, and administered *C. albicans* (CA) three days after initiating antibiotic treatment, or intrarectally administered mannan, curdlan, zymosan or saline (PBS) every other day starting one day prior to DSS challenge.

(B) NF-kB expression induced among RAW-blue macrophages after incubation with or without mannan in the presence or absence of the indicated neutralizing antibodies.

(C) Percent survival after DSS supplementation (for six days) among conventional (CNV) dectin-1 deficient mice housed in specific-pathogen free conditions, administered antibiotics (ABX), or *C. albicans* (CA) inoculated three days after initiating antibiotic treatment (ABX +CA).

(D) Percent survival after DSS supplementation (for six days) among conventional (CNV) TLR4 deficient mice housed in specific-pathogen free conditions, administered antibiotics (ABX), or *C. albicans* (CA) inoculated three days after initiating antibiotic treatment (ABX +CA).

$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, by one-way ANOVA with Holm-Sidak's correction (B) or Log-rank (Mantel-Cox) test (C,D). Data are representative of at least two independent experiments each with similar results. Bars, mean ± s.e.m.

See also Figure S4.