The role of SFB in the mouse model of VVC.

Abstract

Candida albicans is a dimorphic, biofilm forming, opportunistic fungal pathogen found in association with human mucosal surfaces. *C. albicans* can induce several diseases of the mucosal epithelium, such as oropharyngeal candidiasis (OPC), and vulvovaginal candidiasis (VVC). Once *C. albicans* transitions to its pathogenic hyphal form, and infiltrates into the epithelium, the host mounts an immune response to control the infection. In OPC the immune response is protective, however, in VVC the immune response exacerbates the disease. Understanding the complex fungal-pathogen interactions at the vaginal mucosa is an ongoing area of study. Utilizing a mouse model, we can look at the interactions of the immune system during VVC, in particular the Th17 response. We show here that IL-17RA KOs are susceptible to VVC without the use of estrogen, which is known to play a role in VVC, and that IL-17RA KOs clear infection slower than the WT mice. As VVC is a disease of a mucosal surface, we also looked at the role of other commensals in the model, specifically SFB, because of its role in Th17 proliferation. Co-housing mice together ensured equal SFB exposure across treatment groups, and we show here that results were similar irrespective of co-housing.

Introduction

Several species of microorganisms colonize the human body, collectively making up the human microbiome (1-3). These symbiotic relationships can be mutualistic, commensalisitc, amensalistic, and parasitic (1). The microbiome usually exists in a state of homeostasis with the host's immune system, however, the host becoming immunocompromised, or undergoing some sort of damage, such as trauma, can disrupt the host-pathogen relationship, switching it from a commensal to a parasitic role (1-2, 4). Fungal pathogens are one such example of microorganisms that can switch roles from commensalistic to parasitic. Many fungal pathogens exist in two forms: the yeast form, and the parasitic hyphal form, which damages the host. One of the most well characterized and studied host-fungal relationships is with the fungal commensal *Candida albicans* (1).

Candida albicans primarily inhabits mucosal surfaces of the gastrointestinal tract, skin, and genital tract (1, 5, 6). Under normal circumstances *C. albicans* remains confined in mucus in its yeast form, where it remains dormant to the host immune system, however, when it adheres to host epithelial cells, it undergoes a switch from yeast to hyphal form (4,7-8). There is evidence that the epithelial cells recognize invading Candida through TLR4, SIGNR1, and most recently TLR2 (9-10). Once *C. albicans* attaches to epithelial cells, the hyphae release a toxic peptide called candidalysin, which the epithelial cells recognize and react against by releasing DAMPs, S100 alarmins, and pro-inflammatory cytokines (IL-1β). These activate signaling cascades, including the NLRP3 inflammasome, culminating in production and release of pro-inflammatory cytokines (1-2, 7–12). Cytokine release by epithelial cells leads to immune cell recruitment. *C. albicans* infiltration into the vaginal epithelium, and the subsequent immune response, creates and exacerbates vulvovaginal candidiasis (VVC) in otherwise healthy women (13).

Vulvovaginal candidiasis (VVC) infects many women worldwide, and causes discomfort, characterized by burning, itching, and redness of the vaginal tissues, and in some cases is a recurrent infection (1, 8, 12, 14). Several exogenous factors increase a womans' risk of developing VVC, such as, the use of estrogen contraceptives, and prolonged use of antibiotics (1,15-16)**.** Under normal immune homeostatic conditions, the vaginal mucosa is protected against VVC, however, once the immune system mounts a response against infiltrating *C. albicans*, specifically through the induction of polymorphonuclear neutrophils (PMNs), a woman develops VVC (8, 10, 17-18). In VVC, PMNs fail to clear the fungus, while also mounting a strong immune response that aggressively targets the epithelial cells and exacerbates the infection (8). The mechanisms behind VVC and the host-pathogen interactions that occur at the vaginal mucosa remain undefined.

The factors associated with how *C. albicans* breaches the vaginal epithelium, and why the immune system cannot clear the fungal infection, is an ongoing topic of study. The role of the immune system, in particular the Th17 pathway in *C. albicans* pathogenesis, has been widely studied in *oropharyngeal candidiasis*, and it plays a protective role (7, 19, 20-23), however, this protective role does not extend to all the mucosal surfaces that *C. albicans* can infect (2, 6). Of note, the role of Th17 cells in the vaginal mucosa is still controversial (17). Although, evidence exists that IL-17a and IL-22 play no role in PMN recruitment or protection of the vaginal mucosa (17) (and) the historical importance of Th17 in fungal pathogenesis (2) ensures that without further study it cannot effectively be ruled out as a mechanism in VVC.

Using a mouse model, we examine the role of the immune system and the Th17 pathway in VVC**.** The VVC mouse model is an essential tool for studying the *in vivo* responses of *C. albicans* at the vaginal mucosa. Of note, the mouse model of VVC commonly utilizes estrogen to sustain the infection because mice are not natural hosts for *C.albicans* (18, 24-25). We must take into account the use of estrogen in the mouse model of VVC, when looking at immune response, because of the discovery that estrogen diminishes pathogen induced immune responses, including Th17 response, and mice treated with estrogen and challenged with *C. albicans* can not clear the infection (24, 26). Utilizing IL-17RA KOs not treated with estrogen, we can test our hypothesis that Th17 plays a role in VVC. The *in vivo* mouse model also lets us study the role that other members of the microbiome play in the VVC model, in particular segmented filamentous bacteria (SFB).

SFB is a gram-positive, spore-forming, filamentous commensal microbe that is found associating with epithelial cells of the gut (3). SFB is primarily studied in mice and rat models, but it has been found in many mammals (27–29). In the gut, SFB interacts with the immune system, ensuring full development of immune signals that create the basis for the formation of the gut immune barrier (28). SFB also helps establish the balance of CD4+ T cell subsets in the gut (3, 28, 30). In particular, it is shown that SFB strongly induces Th17 cell differentiation in the gut (3, 27-28, 30).

The influence of SFB on immune cells begs the question if the immune response that we observe in our mouse models can be fully attributed to an actual change in immune cell populations, or is it due to basal levels induced by the presence of SFB, with our major interest being in the Th17 cell population. Interestingly, we can test this question in our VVC model by comparing Jackson mice, which are bred without SFB (27), with mice that have SFB, and also by co-housing mice. (Co-housing mice) If mice are co-housed prior to the VVC experiment we can ensure that SFB colonizes every mouse, and that the responses we see with the VVC model are not due to SFB presence.

Results

Results from our first VVC experiment showed equal fungal burdens between IL-17RA KO and WT sesame oil treatment groups on days 3 and 7 (Fig. 1a-b). We also saw that IL-17RA KO mice had higher fungal burden than WT sesame oil treatment groups at day 7 (Fig. 1c-d), and therefore we concluded that in our study IL-17 KOs cleared the infection slower. When we compared WT sesame oil treated and IL-17RA KO sesame oil treated mice to WT estrogen treated mice, we saw higher fungal burdens in the estrogen treated groups, which was expected.

The results from our first experiment pointed toward a role for Th17 in the VVC model, however, prior to our second VVC experiment we wanted to look at the presence of SFB. Our results showed that WT C57Bl/6 mice from Jackson labs have no SFB, and all of our other mice groups, including our IL-17RA KOs, have varying levels of SFB (Table 1). Of note, when we looked at older WT C57Bl/6 mice from Jackson labs, we did detect the presence of SFB, which we attributed to exposure of SFB over time by outside forces, such as during cage changes.

After we determined that SFB was present in all of our treatment groups we co-housed our mice to ensure that SFB was in all of our treatment groups prior to running our second

experiment. In our second experiment we also used Taconic WT mice rather than C57Bl/6 mice from Jackson lab, which as mentioned previously have no SFB (Table 1). The results from our second VVC experiment (Fig. 2a-d), were consistent to our first experiment (Fig 1a-d), with IL-17RA KOs treated with sesame oil being susceptible to VVC with equal fungal burdens to WT sesame treated groups.

Discussion

Vulvovaginal candidiasis is a common disease in women, and as of now, the role of the immune system in VVC is still unknown. We may, with a greater understanding of the immune response, discover new pathways for treatment options.

In this study we focused on the Th17 response, and found that knocking out IL-17RA, leaves mice susceptible to VVC without the use of estrogen. It is important to note, however, that we will need to add more mice to our treatment groups in order to see greater significance in our model.

This study also observed the role of other commensals that associate with mucosal surfaces to determine if they play any role in VVC. The connection between Th17 proliferation and SFB presence made these other commensals of particular interest to our studies. From our studies, we show that when comparing WT Jackson mice, without SFB, to WT Taconic mice the change in percentage of mice that have a fungal load is very similar. However to make a definitive conclusion on the role of SFB more trials will have to be run.

It is also important to note that the VVC model is highly variable, which could point to multiple mechanisms involved in VVC. Further areas of study include; looking at the role of other commensals, and running the VVC model on different strains of mice to determine the role of other immune mechanisms in this model: Cfos Flox:K13cre, IL22RA1 Flox:K13cre. Another area of study is running ELISAs on cytokines related to the Th17 pathway (IL-17A/F, IL-22), as well as other proinflammatory cytokines.

Experimental Procedures

Isolation of SFB from stool samples.

Stool samples were collected from mouse cages of interest. The stool samples were measured prior to DNA extraction (180-220mg). We used the QIAmp DNA stool mini kit (QIAGEN cat#51504) to elucidate DNA from the collected stool samples.

The presence of SFB was determined using QPCR. All DNA samples were standardized to a concentration of 20ng/uL. We used SYBR green (Quanta cat#95073) to determine the presence of SFB (F:5'-GACGCTGAGGCATGAGAGCAT-3', R:5' GACGGCACGGATTGTTATTCA-3'), and used eubacteria as a control (F:5'-ACTCCTACGGGAGGCAGCAGT-3', R:5'-ATTACCGCGGCTGCTGGC-3').

VVC Model

Our VVC model (based off of Brian Peters' (University of Tennessee Health Science Center) model Fig. 3) used WT C57BL/6 (Jackson), WT C57BL/6 (Taconic), and IL-17RA KO (Gaffen lab) female mice greater than 5 weeks old. Co-housing was done to determine the effects of SFB on the VVC model. We did not do co-housing in experiment one, which used Jackson mice as a WT control, however, two weeks prior to running our second experiment we co-housed WTs from Taconic with our IL-17RA KOs, and separated them on day-3 prior to beginning VVC.

On day -3, we injected mice with either sesame oil, or sesame oil + estrogen (1mg/mL). We cultured *C. albicans*, strain SC5314, overnight at 30 degrees celsius at 300 rpm on day -1. Mice were challenged with *C.albicans* on day 0 (experiment 1: 5*10^5 CFU, experiment 2: 5*10^6 CFU) by directly administering *C. albicans* in the vaginal canal using a pipette tip (10uL culture/mouse). Vaginal canals were lavaged on day 3 with PBS (100uL). The collected lavage was either plated at different dilutions (10,100, and 1000x) to determine fungal burden, or smeared on slides (1x) for future pap-smear staining. On day 4 the mice received a second

injection corresponding to their initial injection treatment from day -3. Mice were sacrificed on day 7, lavage was collected to repeat day 3 experiments, and vaginal tissues were harvested.

Figures

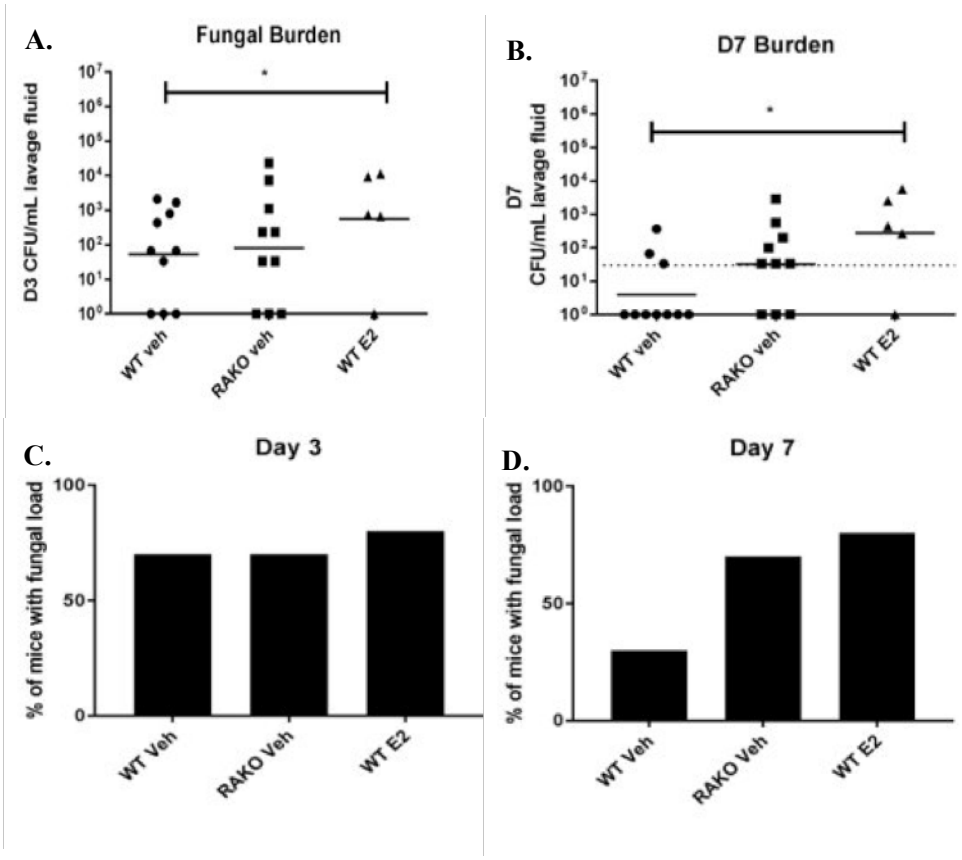

Figure 1. Experiment 1 fungal burdens and percentage of mice with a fungal load from vaginal lavage fluid days 3 and 7. Student T tests were performed on fungal burden (D3 p: 0.0387, D7 p: 0.0336) and chi-square correlation was performed for percentage of mice with a fungal load.

Table 1. Presence of SFB in mouse strains.

Date	Samples	Eubacteria	SFB	dct
10/31/17	WT Jax 1	13.566	40.00	26.434
	WT Jax 2	13.294	40.00	26.706
	WT Jax 5	13.424	40.00	26.576
	RAKO 3	13.3785	24.08	10.698
	RAKO 4	13.5665	27.43	13.862
11/28/17	WT JAX 3	13.4855	24.76	11.275
	WT JAX 4	13.3355	21.61	8.2695
	Taconic 1	13.356	23.17	9.8165
	Taconic 2	13.467	21.95	8.4805
	RAKO 1	13.233	21.23	8.0015
	RAKO 2	13.1165	20.07	6.957
	RAKO 3	12.949	20.96	8.007
	RAKO 4	13.583	20.95	7.363
	B6NT 1	13.419	31.39	17.9695
	B6NT 2	13.4275	33.67	20.2455

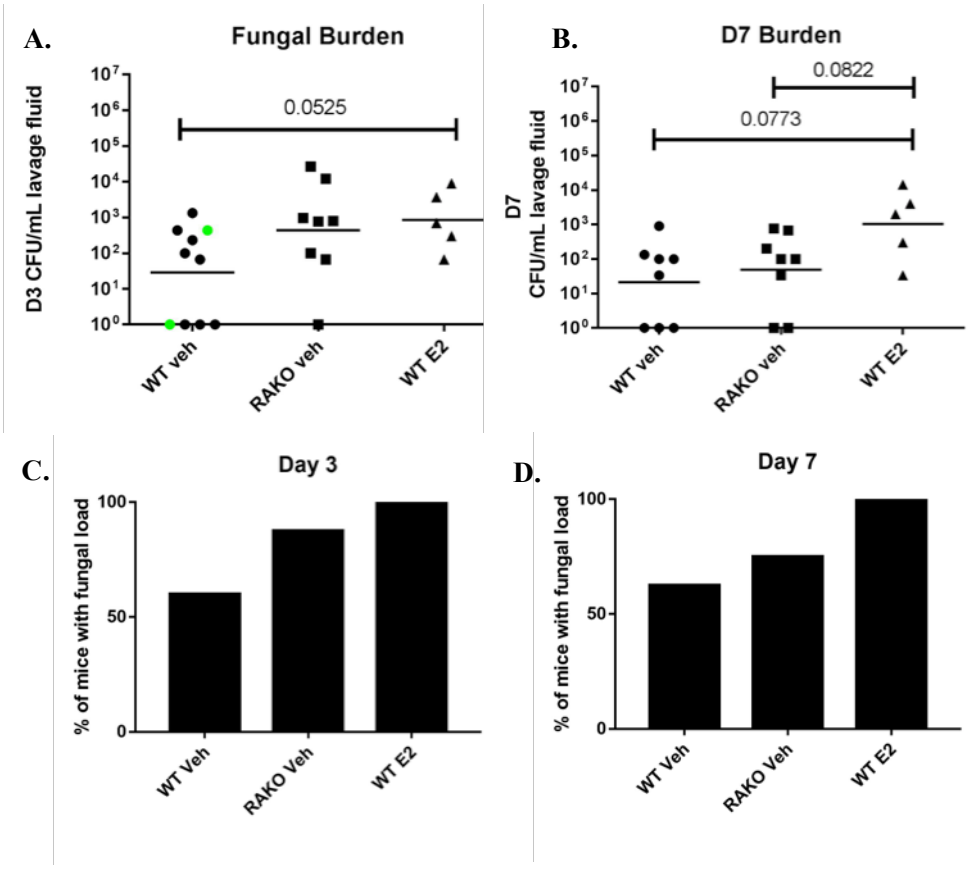

Figure 2. Experiment 2 fungal burdens and percentage of mice with a fungal load from vaginal lavage fluid days 3 and 7. Student T tests were performed on fungal burden, and chi-square correlation was performed for percentage of mice with a fungal load. Green dots in A. represent 2 mice where day 7 lavage could not be collected.

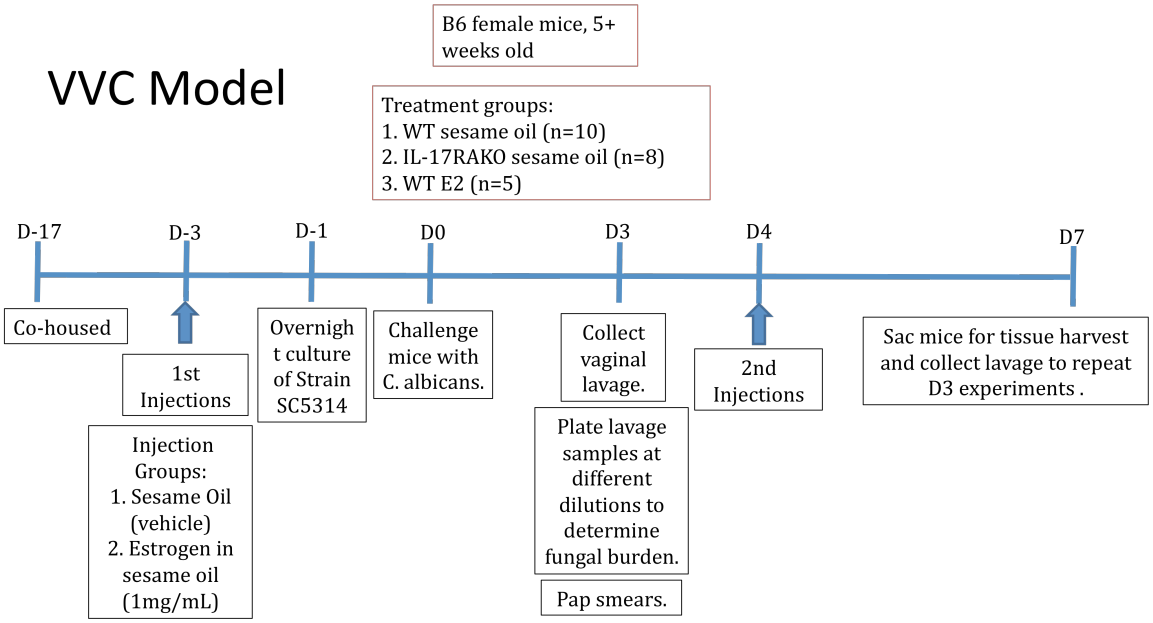

Figure 3. VVC model timeline.

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