

An organ-on-chip model of vulvovaginal candidiasis to study the interplay between fungal pathogenicity and inflammation

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Article

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Abstract

Vulvovaginal candidiasis (VVC) is a mucosal yeast infection that affects most women at least once in their lifetime, and exhibits severe recurrence (RVVC) in a subset of patients. The onset of (R)VVC is linked to a complex interplay between fungal pathogenicity and excessive innate immune responses driving neutrophilic inflammation that mediates disease progression. The current state of knowledge of VVC pathogenesis is established based on patient studies, animal models, and *in vitro* cell culture models. Conventional monolayer epithelial infection models cannot simulate recruitment of inflammatory neutrophils, a hallmark of VVC pathogenesis, whereas murine animal models exhibit some interspecies differences in the immune system. Recent advances in microfluidic organ-on-chip systems enabled us to establish a vulvovaginal-candidiasis (VVC)-on-chip model that simulates infection of vaginal mucosa by *Candida albicans* and can be used to study innate immune responses driving neutrophil-mediated inflammation.

C. albicans infection elicits proinflammatory responses that induce neutrophil recruitment to the vaginal epithelium in the chip. Neutrophils were found to swarm towards sites of *C. albicans*-infected epithelium, which was visualized and quantified *via* 3D object-based segmentation analysis. While recruited neutrophils fail to fully clear the infection, they exacerbate proinflammatory cytokine responses, consistent with clinical observations.

Overall, this VVC-on-chip model represents a novel tool to dissect mechanisms driving the immunopathology of VVC. Moreover, it also provides a platform for studying host-pathogen interactions at the vaginal epithelial interface as well as evaluation of both pathogen- and host-directed therapies.

Introduction

Vulvovaginal candidiasis (VVC), also called vaginal thrush, is a common infection of the vulvovaginal mucosa, affecting approximately 75% of immunocompetent women at least once in their lifetime [1–3]. Clinical symptoms of VVC include itching, burning, pain, swelling, and redness of the vulva and vagina, often accompanied by vaginal discharge, which strongly impacts the quality of life of affected women [4, 5]. With approximately 138 million cases being reported globally every year, VVC is regarded as the most prevalent *Candida*-associated infection and generates a heavy healthcare burden [6]. Globally, around 5–8% of women experience recurrent VVC (RVVC) [4, 5], a group of patients where deep insight into disease pathogenesis may be able to direct novel sustainable treatment strategies.

Unlike other forms of candidiasis, VVC is not associated with compromised immunity, but with predisposing factors, such as hormone replacement therapy, increased oestrogen levels, diabetes mellitus, and antibiotic usage that disrupts microbiota [2, 7–10]. The vaginal epithelium mounts initial responses against *C. albicans* [11]. Colonization of the vaginal mucosa by *Candida* species is tolerated by the epithelial cells without activating immune responses. However, inflammation is triggered when *C. albicans* forms hyphae, and the fungal burden increases beyond the tolerated epithelial threshold [1, 12–14], which is hypothesized to be lowered in RVVC patients. Hyphae that invade the vaginal epithelium

secrete the toxin candidalysin [15, 16], which is an important driver of tissue damage and subsequent proinflammatory immune responses [17–19]. With the release of proinflammatory cytokines and chemokines, as well as the fungal zincophore Pra1 [20], neutrophils are recruited to infiltrate the tissue and attack the pathogen [21, 22]. While being compromised in their ability to clear the infection [23], the excessive inflammation prompted by deployed neutrophil effector functions exacerbates tissue damage, release of alarmins and DAMPs, and inflammatory responses, leading to symptoms associated with VVC and a vicious cycle of persistent and recurring inflammation [1, 24, 25]. Consequently, VVC is considered an immunopathological condition [26]. Nonetheless, current therapeutic strategies rather aim at controlling fungal burden using antifungals [27, 28], than modulating immune responses.

The predominant models currently used to study VVC are murine *in vivo* models, and *in vitro* or *ex vivo* human epithelial infection models [14, 17, 29–31]. Rodents, due to their well-characterized physiology, are protagonists of hundreds of studies in elucidating complex host-*Candida* interactions that drive disease severity [30]. However, animal models are the subject of ethical concerns [32], and interspecies discrepancies between rodents and human immune responses limit translational capacity [33]. Aside from the differences in vaginal pH and microbiome [34–36], *C. albicans* does not naturally colonise mice [37]. In contrast, *in vitro/ex vivo* human epithelial infection models are well-established for studying host-pathogen interactions at the vaginal epithelial interface [17, 31, 38, 39]. Yet, these models typically lack tissue-resident immune cells, vascular flow, and circulating neutrophils that can be recruited, and therefore, require combinatorial experiments to dissect crosstalk between the epithelium and innate immune cells [40]. To obtain a more physiologically relevant model of early innate immune responses of VVC, neutrophils circulating through a bloodstream-like channel is necessary to model rolling, arrest, polarization, crawling, and migration through the endothelial layer [41, 42].

The past two decades have seen a revolution of developments in organ-on-chip (OoC) technology, which provides the opportunity to model microbial infections with increased or even near physiological complexity *in vitro* [43]. OoC models have already been established to study invasion of the intestinal epithelial barrier at the onset of invasive candidiasis [44, 45] and colitis [46]. Furthermore, organ-on-chip models can also mimic the human female reproductive system [47], vaginal microbiome-host interactions [48], and treatment of vaginal infections caused by *Neisseria gonorrhoeae* [49]. Other organ niches, such as the lung, blood-brain barrier, and intestine organ-on-chip models, have also been implemented to study fungal infections [44, 50–52].

In this study, we set out to develop a microfluidics-based vaginal *C. albicans* infection model that can specifically be employed to study the interplay between fungal pathogenicity and innate immune responses of VVC. This vulvovaginal-candidiasis (VVC)-on-chip model consists of a mucosal compartment lined with epithelial cells and tissue-resident macrophages, and a vascular compartment lined with endothelial cells perfused with neutrophils, providing a humanised platform for studying VVC.

Results

Establishment of *C. albicans* infection in the VVC-on-chip model

VVC involves complex crosstalk between epithelium and innate immune cells in response to *C. albicans*, which conventional *in vitro* models fail to fully capture. Therefore, we established a VVC-on-chip model to study the interplay between *C. albicans*, vulvovaginal epithelial cells, tissue-resident macrophages, endothelial cells, and circulating neutrophils (Fig. 1a). The model consists of an epithelial- and a vascular-compartment separated by a porous membrane. The epithelial compartment, mimicking the vulvovaginal epithelium, includes A-431 vulvovaginal epithelial cells (VECs) expressing cytosolic Nano-luciferase (Nluc) [53] and macrophages, an abundant resident immune cell type within the human vaginal niche [54, 55] (Fig. 1a). The vascular compartment is lined with immortalized human umbilical vascular endothelial cells (HUVEC/TERT 2) and is perfused with neutrophils (Fig. 1a).

To establish the dynamic VVC-on-chip model, we isolated primary human monocytes and differentiated them into macrophages (hMDMs) outside the chip for 7 days. During the final day of differentiation, endothelial cells (HUVEC/TERT 2) were seeded in the vascular compartment. Upon full hMDM differentiation, vulvovaginal epithelial cells (A-431) and hMDMs were seeded in the epithelial compartment. After establishing the different cell populations in their respective compartments at day – 3 and – 2, respectively, circular perfusion was applied to the vascular compartment for 24 hours (day – 1) to differentiate the endothelium [56]. The next day (day 0), the epithelial compartment was infected with *C. albicans* (Fig. 1b). Subsequently, a series of readouts of infection progression and inflammation, as well as fluorescence microscopy and image-based analysis were carried out 24 hours post-infection (hpi) (**Supplementary Fig. 1**).

Supplementary Fig. 1: Representative example of the VVC-on-chip 3D reconstructions. (left) Single-channel image layers from a three-dimensional confocal microscopy Z-stack recorded from a *C. albicans*-infected chip. From bottom to top: membrane pores (grey), DAPI-stained nuclei (cyan), Phalloidin-stained actin (green), anti-*Candida*-stained fungal hyphae (magenta), anti-CD15-stained neutrophils (orange), neutrophil-bounded fungal hyphae (overlay of orange and magenta). **(right)** The corresponding 3D surface reconstructions of the left panel provided by Imaris 11.0.1. From bottom to top: membrane pores (random colours), nuclei (cyan), actin (green), fungal hyphae (magenta), neutrophils (random colours), neutrophil-bounded fungal hyphae (overlay of magenta and random colours). See the Methods for segmentation details.

C. albicans infection of the VVC-on-chip model

After infecting the epithelial compartment of the VVC-on-chip with the *C. albicans* vaginal isolate CA3153 [57] (Fig. 2a), Nano-luciferase (Nluc)-expressing A-431 vulvovaginal epithelial cells (VECs), that release luciferase in the culture supernatant upon cell death were used to specifically assess the damage the epithelial tissue sustained during infection [53]. A moderate, yet significant, increase in epithelial cell

death was detected upon *C. albicans* infection, based on luciferase release (Fig. 2b). Similarly, consistently higher diffusion of Fluorescein Isothiocyanate-Dextran (FITC)-Dextran from the epithelial compartment into the vascular compartment in each experiment suggested a subtle loss of barrier integrity upon infection (Fig. 2c). Morphological changes in the epithelial actin-cytoskeleton organization caused by *C. albicans* infection could be observed, and in some cases, also formation of actin-cuffs around invading hyphae (Fig. 2d, **Supplementary Fig. 2**) [58, 59]. We found that the CD68⁺ macrophages in the epithelial compartment closely interacted with the infecting fungal hyphae, and CD68 staining was more intense in infected chips (Fig. 2d), which could reflect an activated macrophage state.

In addition to the *C. albicans* vaginal isolate, we wanted to understand how the *C. albicans* reference strain SC5314 (a highly virulent bloodstream isolate [60]) would behave in our VVC-on-chip model. Interestingly, based on colony forming unit (CFU) measurements, we found that the vaginal isolate CA3153 was more fit to establish infection in the epithelial compartment (**Supplementary Fig. 3a**) and importantly, invade the epithelial tissue (**Supplementary Fig. 3b**). Given the better ability of the vaginal isolate CA3153 to infect the vulvovaginal epithelium, we used this strain for our remaining study.

Supplementary Fig. 2: Magnified view of the representative images in Fig. 2d showing morphological changes in the epithelial actin-cytoskeleton organization upon CA3153 infection. (left) Uninfected. **(middle)** Epithelial actin-cytoskeleton disorganization in an infected chip. **(right)** Actin cuff formation in an infected chip (white arrowheads). Middle and right images are the magnified views of different regions in the same image. F-actin is stained with phalloidin (green) and *C. albicans* is stained with anti-*Candida* antibody (magenta). Magnification 20×. Scale bars represent 20 μm.

Supplementary Fig. 3: Comparison between reference strain SC5314 and vaginal isolate CA3153. (a) Epithelial fungal burden from SC5314 and CA3153 measured by quantifying colony-forming units (CFU; $n = 6$). **(b)** Epithelial fungal burden in epithelial lysate from SC5314 and CA3153 measured by quantifying CFU ($n = 4$). The symbols represent the data from each independent experiment, bars represent the mean + standard error of the mean (SEM), and the numbers represent the *P*-values obtained in each statistical test. Statistical significance was determined using paired t-test (a-b).

Circulating neutrophils infiltrate the *C. albicans* infected epithelial compartment

Neutrophil recruitment and activation are hallmarks of VVC [1, 6]. This was not only observed in experimental human vaginal *Candida* infection [26], but also in vaginal samples from women who developed symptomatic (R)VVC infection [61–63]. To study neutrophil-mediated inflammation during infection in our VVC-on-chip, primary human neutrophils were perfused through the vascular compartment during *Candida* infection (Fig. 3a). CA3153 infection led to significant release of myeloperoxidase (MPO) (Fig. 3b), a neutrophil enzyme converting reactive oxygen species into antimicrobial oxidants that is released upon degranulation [64, 65]. In contrast, MPO levels in the vascular compartment were similar under uninfected and infected conditions (Fig. 3c). Interestingly,

neutrophil influx did not result in fungal clearance as evidenced by the similar retrieval of CFU levels from the epithelial tissue (Fig. 3d), despite active engagement of the neutrophils with *C. albicans* (Fig. 3e). However, immunofluorescence microscopy suggests that the neutrophils did confine hyphal outgrowth (**Supplementary Fig. 4a**).

To validate the physiological relevance of our chip model, we compared our observations of neutrophil recruitment and activation with data from an *in vivo* vaginal *C. albicans* infection in mice. Histology of the vagina similarly showed neutrophil influx specifically to sites of the infected vaginal mucosa (Fig. 3f). Similar as in our chip model, increased release of neutrophil MPO was observed in *C. albicans*-infected mice (Fig. 3g). Moreover, detection of IL-1 β suggested an inflammatory state of the mouse vagina due to the infection (Fig. 3h).

Supplementary Fig. 4: Representative confocal microscopy of infected chips perfused without and with neutrophils. Nuclei are stained with DAPI (blue), F-actin is stained with phalloidin (green), human neutrophils are stained using anti-CD15 (orange), and *C. albicans* is stained with an anti-*Candida* antibody (magenta). Magnification 20 $\times$. Scale bars represent 100 μ m.

3D reconstructions of the VVC-on-chip confirms neutrophil influx and swarming behaviour

Optical sectioning of the VVC-on-chip model by Z-stack acquisition using confocal microscopy allowed us to perform 3D reconstruction to facilitate object-based analysis. For the reconstruction, we used phalloidin-based actin staining, DAPI-based nuclear staining, antibody-mediated *C. albicans* and neutrophil CD15 staining. In addition to CD15, neutrophils were characterised by their multi-lobed nuclei [66], which can be easily distinguished in the DAPI signal. The combination of the two stainings facilitated the segmentation and reconstruction of individual neutrophils. These 3D-reconstructed images allowed us to quantify characteristics of the behaviour of neutrophils during *C. albicans* infection. Determining the location of reconstructed neutrophils relative to the chip membrane confirmed an increased neutrophil migration to the infected vulvovaginal epithelial compartment while decreasing in the vascular compartment (Fig. 4a). Remarkably, the data suggests that some neutrophils tended to invade the tissue and wander randomly when there was no infection (Fig. 4b). Interestingly, some neutrophils tended to stay inside the pores of the chip membrane, which was slightly increased during *C. albicans* infection (Fig. 4b).

To attack large clusters of hyphae, neutrophils engage in swarming behaviour, where they collectively attack the fungi [67]. Immunofluorescence staining suggested that neutrophils both engaged *C. albicans* individually (Fig. 3e, **top row**), as well as in swarms (Fig. 3e, **lower row**). To quantify neutrophil swarming behaviour from the 3D-reconstructed images, we calculated distances to the three nearest neighbouring neutrophils. Aside from the high neutrophil counts being observed in *C. albicans*-infected chips, neutrophils were observed to have closer proximity to each other and showed adhesion to the *C. albicans* hyphae (Fig. 4c-e), characteristic for neutrophil swarms. Interestingly, we found that neutrophil volume increased upon *C. albicans* infection, while the cell surface area to volume ratio remained unchanged,

suggesting the migrated neutrophils are likely becoming flatter or more elongated in order to engage the *C. albicans* hyphae (Fig. 4f).

Neutrophil infiltration of the infected epithelium exacerbates inflammatory responses

Neutrophil infiltration into the vaginal mucosa can lead to immunopathology. Nevertheless, despite significant neutrophil recruitment in the infected VVC-on-chip model, we did not observe luciferase release into supernatants, suggesting no alterations in total tissue damage (Fig. 5a). However, IL-1 α , an alarmin released from cells upon cell death [68, 69], was significantly increased upon infection and further increased by the presence of neutrophils (Fig. 5b). Interestingly, the barrier permeability measured *via* a FITC-Dextran permeability assay was lower in the presence of neutrophils (Fig. 5c), which might be explained by reduced membrane disruption due to fungal growth restriction by neutrophils, or by neutrophils in the membrane pores hindering FITC-dextran diffusion (Fig. 4b, **Supplementary Fig. 5**).

Neutrophils during VVC attribute to the persistent inflammatory response, subsequently boosting the release of cytokines [1, 6, 63]. Overall, we observed that neutrophil influx to the epithelium was associated with exacerbated inflammatory responses. Particularly, pro-inflammatory IL-1 β concentration levels elevated in the presence of neutrophils, in contrast to IL-1 receptor antagonist (Ra) production (Fig. 5d-e). Besides, the neutrophil chemokine IL-8 increased after infection and was particularly significant under neutrophil perfusion (Fig. 5f). On the contrary, GM-CSF concentrations exhibited minimal changes (Fig. 5g). Type I interferon (IFN- α), which has been associated with protective early vaginal epithelial cell responses [14, 70, 71], was significantly increased after infection and further enhanced in the presence of neutrophils (Fig. 5h). Collectively, these data reflect an amplification of pro-inflammatory responses mediated by neutrophils in *C. albicans* infected chips.

Supplementary Fig. 5: Number of neutrophils occupying membrane pores. Average number of neutrophils that occupy the membrane pores were estimated from the distance from the reconstructed membrane pores. A distance of 8 μ m, which corresponds to the thickness of the membrane, was used as a threshold to identify in-pore neutrophils. Data was generated from nine uninfected images and ten infected images obtained from two individual chip experiments. Bars represent the mean + standard error of the mean (SEM) in all panels.

Discussion

In the current study, we present a VVC-on-chip model and demonstrate how it could be leveraged to study innate immune responses relevant to VVC. This humanised model allowed us to recapitulate *C. albicans* infection of the epithelium as well as innate immune responses, particularly neutrophil infiltration, as well as neutrophil-mediated exacerbation of inflammation and inefficient neutrophil-mediated fungal clearance. The inflammatory crosstalk among vulvovaginal epithelial cells,

macrophages, endothelial cells, and circulating neutrophils could also be quantified. This model provides an alternative approach to study VVC compared to conventional *in vitro* and *in vivo* models, with the possibility of dissecting interactions between different cell-types that lead to the hallmark characteristic neutrophil influx and exacerbated inflammatory responses of VVC.

Our results revealed that *C. albicans* vaginal isolate CA3153 could efficiently establish infection of the vulvovaginal epithelium in our VVC-on-chip model. After 24 hours, hyphae induced deep-tissue invasion, demonstrated by fungal CFU that can only be detected after lysis of the epithelium. Extensive fungal growth assessed by CFU plating implies that the vaginal isolate CA3153 performs better in growth and invasion under the context of vaginal infection than the reference strain blood-stream isolate SC5314. It was previously shown that vaginal isolates could adhere better on epithelial cells, potentially by curtailing complosome activity in VECs [72, 73]. These may account for the advantages of CA3153 in growing better on VECs in human vagina-simulating environment.

In our model, the epithelium exhibited a tendency towards reduced cell integrity after infection based on their higher expression of luciferase activity and diffusion of FITC-Dextran. Yet, the timespan used for infection may have been too short in order to detect a substantially compromised epithelial tissue damage using both assays. As an immunopathology, VVC is marked by pronounced inflammatory responses [1]. Our VVC-on-chip model demonstrated a similar pattern, where IL-1 α was significantly increased in response to infection and was further increased by infiltrating neutrophils. With its function as an alarmin, IL-1 α is released upon induction of cell death [68, 69], and has been linked with candidalysin-mediated lysis of VECs [13, 31]. For other pro-inflammatory cytokines, such as, IL-1 β , IL-8, and IFN- α , neutrophil influx played a profound role in the magnitude of the response. This is in accordance with previous studies reporting elevated IL-1 β , and IL-8 concentrations in symptomatic VVC patients together with excessive neutrophil recruitment in the vagina [63]. IL-1 β , as well as the NLRP3 inflammasome complex mediating its processing, have been reported to be instrumental in enhancing the immunopathology in VVC [63, 74, 75]. Importantly, the NLRP3 inflammasome relies on factors released upon tissue damage as a driver of its activation [76]. Meanwhile, genetic variations in NLRP3 are enriched in RVVC patients [77, 78]. On the other hand, epithelial type I interferon responses have been associated with epithelial resistance to infection [14, 71]. It was shown that IFN- α concentrations elevated in the vaginal fluids of VVC patients compared to the control group [79]. Therefore, our VVC-on-chip model can recapitulate cytokine responses observed in clinical (R)VVC. The mechanisms underlying the exacerbation of these inflammatory responses upon neutrophil influx warrant further investigation, as it can provide novel targets for immunomodulatory therapy. In this context, the genetic tractability of the A-431 cell line in the model offers the possibility to dissect the role of epithelial specific responses [53, 80, 81]. Moreover, models based on human-induced pluripotent stem cells could be a promising future direction to allow dissection of individual genes on a holistic level [82–84], as well as modelling the impact of genetic variations associated with susceptibility to VVC [85].

Considering the important contribution of innate immune cells to VVC pathogenesis, we would like to highlight the capacity of our model to mimic robust neutrophil recruitment to infected sites in the

vulvovaginal epithelial compartment. We observed that infiltrating neutrophils did not induce substantial damage to the epithelium based on Nluc release as well as FITC-Dextran diffusion, yet the tissue damage-associated alarmin IL-1 α was increased upon neutrophil infiltration. This discrepancy could be due to potential positive inflammatory feedback loops [68]. It is also possible that there are limitations in detecting epithelial damage using Nluc activity in the chip due to continuous proliferation of epithelial cells, including those that undergo secondary necrosis following apoptosis, partially due to the presence of neutrophils [86–88]. However, another possibility is that the degree of cell death, and release of DAMPs and alarmins are already sufficient to amplify inflammatory responses while cell death assays lack sensitivity in this range. Of note, immunofluorescence microscopy revealed altered morphology of actin cytoskeleton without much destruction of epithelial tissue, thus, extended infection durations or higher fungal burdens may be required in order to detect substantial epithelial cell death. Microscopy using fixable live-dead staining or fluorescent cell death reporters for live-cell imaging are potential approaches to circumvent this limitation and detect localized cell death. Establishing multi-colour live-fluorescent imaging of chip systems would allow quantifications of real-time cell death and tissue damage specifically due to the infection or following immunopathology, which are hard to capture in complex *in vivo* models, whereas a VVC-on-chip model could fill this gap.

Our current image-based analysis of confocal microscopy across different tissue layers suggested several changes upon infection: reorganization of actin cytoskeleton network, increased CD68 expression in macrophages engaging *C. albicans*, and neutrophils engaging in swarming behaviour towards clusters of invading *C. albicans* hyphae. This is in line with previous studies showing formation of actin cuffs and actin reorganization in infected epithelial cells [58, 59], and clusters of hyphae elicit neutrophil swarming [67, 89]. Interestingly, *C. albicans* seemed confined in growth in the presence of neutrophils but CFU-based fungal burden analysis suggests that *C. albicans* were not completely eradicated, which aligns perfectly with previous observations that during VVC, neutrophils failed to clear the infection [6, 61, 90]. These qualitative and quantitative assessments of neutrophil behaviour in our chip model may be able to provide useful insights into how established, as well as experimental therapeutic interventions change the interplay of infection and host responses. The combination of these image-based analyses with our VVC-on-chip model sets the stage for future studies into the complex spatiotemporal dynamics of host-pathogen interactions.

Collectively, the VVC-on-chip model will serve as a complementary model to current *in vivo*, *in vitro*, and *ex vivo* models. We acknowledge the importance of modelling several tissue layers of the female reproductive tract [49, 91], as well as introducing a vaginal microbiota [48]. Our model is specifically designed to capture several hallmarks of VVC immunopathogenesis, such as neutrophil infiltration, potent IL-1-driven inflammatory responses, fungal persistence, and propagation of inflammation by infiltrating neutrophils. In the long-term, our model could serve as a tool to simulate patient-specific predisposing factors to VVC exposure to hormonal treatments (oestrogen), high concentrations of glucose as observed in uncontrolled diabetes, and genetic polymorphisms [82, 92–94]. In this way, our VVC-on-chip model can contribute to studying the heterogeneity of VVC immunopathology and establishing subclusters of different malfunctioning host immune responses.

Materials and methods

Ethical declaration

Human venous whole blood was obtained from healthy volunteers that gave written informed consent. This study was performed following the principles outlined in the Declaration of Helsinki. The blood donation protocol and use of blood were approved by the Jena University Hospital (permission number 2026-4182-BO-A).

Cell maintenance

Authentication of the cell lines was performed by commercial short tandem repeat (STR) profiling (Eurofins Genomic) and checked for mycoplasma contamination using a PCR test kit (PromoKine) following the manufacturer's instructions. NanoLuc® luciferase (Nluc)-expressing A-431 vulvovaginal epithelial cells (VECs; DSMZ no. ACC 91) were kindly provided by the lab of Professor Bernhard Hube and were used to model the vulvovaginal epithelium [53]. The Nluc-expressing A-431 VECs were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated foetal bovine serum (FBS; Bio&Sell) in 75 cm² flasks at 37°C and 5% CO₂ until 80% confluency. Cells were detached using Accutase (Capricorn scientific), counted using a Neubauer counting chamber and the cell concentration was adjusted to 2.5×10^6 /mL before being introduced to the biochips.

hTERT-immortalized human umbilical vein endothelial cells (HUVEC/TERT 2) were purchased from Evercyte (CHT-006-0008) and expanded in 75 cm² flasks in EBM® Endothelial Cell Growth Basal Medium (Lonza) supplemented with EGM® Endothelial Cell Growth Medium SingleQuots® components (Lonza), 10% heat-inactivated FBS and Geneticin (G418; InvivoGen). Cells were detached using trypsin, counted using a Neubauer counting chamber and the cell concentration was adjusted to 2.5×10^6 /mL before being introduced to the biochips.

Immune cell isolation and differentiation

Human peripheral blood mononuclear cells (PBMCs) were collected in EDTA tubes and human serum in coagulation tubes (Sarstedt). Serum was obtained from each donor by centrifugation at $2,000 \times g$ for 10 min at room temperature (RT) and was stored at -20°C until used as a supplement in cell culture medium. PBMCs were isolated by layering 1:2 diluted EDTA blood onto Lymphocyte separation medium (LSM; Capricorn Scientific) and density gradient centrifugation at $700 \times g$ for 30 min with slow acceleration and breaking. The PBMC layer was harvested using a Pasteur pipet and washed twice in ice-cold PBS. Isolated PBMCs were seeded in a cell culture petri dish (Corning) in X-VIVO 15 medium (Lonza) supplemented with Pen/Strep (100 U/mL) and incubated at 37°C and 5% CO₂ (40–50 mL blood per petri dish). To remove non-adherent cells and promote differentiation to macrophages, the medium was exchanged to X-VIVO 15 medium containing 10% serum of the same donor (autologous serum),

supplemented with 10 ng/mL macrophage colony-stimulating factor (M-CSF), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and Pen/Strep (100 U/mL) after 24 hours. The medium was renewed once more after three days during the 7-day period of differentiation before seeding hMDMs into biochips. When the macrophages were differentiated, medium was removed, followed by one wash with PBS. Dulbecco's Phosphate-Buffered Saline (DPBS) was added to facilitate detachment of the differentiated hMDMs by using a cell scraper. Collected macrophages were centrifuged at $300 \times g$ for 5 min at RT. Resuspended in 500 μ L vaginal compartment medium (VCM, RPMI + 10% autologous serum + 1% Pen/Strep + 10 ng/mL GM-CSF and 10 ng/mL M-CSF), hMDMs were counted using a Neubauer counting chamber, and the cell concentration was adjusted to 5×10^5 /mL before being introduced to the biochips.

After density centrifugation human primary neutrophils were isolated from the erythrocyte/granulocyte fraction by hypotonic lysis [95]. To remove erythrocytes, the cell suspension was treated twice with a hypotonic lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3) for 15 min on ice. After each lysis step cells were centrifuged at $300 \times g$ for 10 min at 4°C. Subsequently, neutrophils were harvested, washed twice in PBS. After each lysis step, cells were gently centrifuged at $300 \times g$ for 10 min at 4°C. Isolated neutrophils were resuspended in 1 mL vascular medium – Medium 199 (Gibco) supplemented with 10% FBS, 25 μ g/mL heparin, 7.5 μ g/mL vascular mitogen (ECGS), 5 μ g/mL Vitamin C, 10% autologous human serum, and Pen/Strep (100 U/mL). Neutrophils were counted using a Neubauer counting chamber and the cell concentration was adjusted to 1×10^6 /mL and kept on ice until being introduced into the biochips.

Fungal strains and culture conditions

Candida albicans reference strain SC5314 [60] and vaginal isolate CA3153 [57] were used for this study. Strains were streaked on yeast-peptone-dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and maintained for maximum 4 weeks. Prior to the experiment, a single-cell colony was picked from YPD agar and grown in liquid YPD medium in an orbital shaking incubator for 16 h (180 rpm at 30°C). Yeast cells were collected by centrifugation $10,000 \times g$ for 1 min, washed twice with 1 mL PBS, and counted using a Neubauer counting chamber. The yeast cell concentration was adjusted to 4.2×10^4 /mL in RPMI + Pen/Strep (100 U/mL) to be introduced in the biochip.

Biochip system

Biochips (BC002) were acquired from Dynamic42. Biochip systems are composed of a top and a bottom culture compartment with adjacent channels (Fig. 1). The VVC-on-chip shares similar setup as the intestine-on-chip [44]. Compartments are separated by a polyethylene terephthalate (PET) membrane that has a pore density of 1×10^5 pores/ cm^2 , which are 8 μ m in diameter (TRAKETCH Sabeu, Radeberg, Germany). For the top and bottom compartments, the total volume (including corresponding inlets and outlets) was 290 μ L and 270 μ L, respectively. When perfusion of the biochips was required, medium reservoirs (microfluidic ChipShop) were connected to the chamber inlets before using PharMed BPT thermoplastic elastomer tubing (ISMATEC) to connect the biochips to peristaltic pumps (Harvard Apparatus).

VVC-on-chip model assembly and infection

The VVC-on-chip setup is established by sequential coating and seeding of cells in the respective compartments (Fig. 1). To ensure that hMDMs are fully differentiated on the day that they need to be seeded, PBMCs are isolated and differentiated 1 week prior to seeding (Fig. 1b, **day – 8**). Prior to seeding cells into the biochip to create the VVC-on-chip setup, BC002 biochip membranes were checked for damage, cuts, and unevenness using brightfield microscopy. After quality checks, biochips were sterilized in 70% ethanol for at least 30 min at RT. Biochips were washed twice with sterile distilled water and each compartment was flushed with 500 μ L sterile DPBS with calcium and magnesium, supplemented with Pen/Strep (100 U/mL). Subsequently, top compartments were coated with 250 μ L Collagen A for one hour at 37°C and 5% CO₂. Compartments were flushed once more with 500 μ L DPBS, and then with 200 μ L EBM® Endothelial Cell Growth Basal Medium (Lonza) supplemented with EGM® Endothelial Cell Growth Medium SingleQuots® components (Lonza) supplemented with Pen/Strep (100 U/mL). HUVEC/TERT 2, 200 μ L of 2.5×10^6 /mL, were seeded into the vascular compartments (5×10^5 cells). All compartments were sealed with Luer plugs and the biochips were placed in a glass Petri dish, and incubated at 37°C and 5% CO₂ for 18 h (Fig. 1b, **day – 3**). The epithelial compartment was coated with Collagen A with the same procedure as the vascular compartment. The Nluc-expressing A-431 VECs [53] and hMDMs were combined in VCM with final concentrations of 2.5×10^6 /mL and 5×10^5 /mL, respectively. The epithelial compartments of the VVC-on-chip system were flushed with VCM. The mixed cell suspension was seeded into the biochip (120 μ L) and biochips were placed upside-down to facilitate adherence of the cells onto the bottom part of the membrane in a sterile glass Petri dish and incubated at 37°C and 5% CO₂ for 18 h (Fig. 1b, **day – 2**). The following day, the vascular compartments were subjected to perfusion to differentiate the endothelium in the presence of flow. Before that, the epithelial compartment was carefully flushed with fresh VCM. Afterwards, reservoirs were connected to one outlet of the vascular site and filled with 4 mL EBM® Endothelial Cell Growth Basal Medium (Lonza) supplemented with EGM® Endothelial Cell Growth Medium SingleQuots® components (Lonza) supplemented with Pen/Strep (100 U/mL). A medium pre-filled tube was connected to the reservoir and its corresponding outlet. Perfusion was then initiated with a peristaltic pump (Harvard Apparatus Peristaltic Pump P-70) at a flow rate of 25 μ L/min. Media was pumped from a reservoir into the compartments in a closed circuit (Fig. 1b, **day – 1**). At 24 hpi, the chips were disconnected from the pump, with all tubes and reservoirs removed. To infect the chips, 120 μ L of adjusted *C. albicans* (4.2×10^4 /mL in RPMI-1640 with 100 U/mL Pen/Strep) was loaded in the epithelial compartment and incubated upside down one hour to facilitate *C. albicans* adhesion. Uninfected control epithelial compartments were flushed with RPMI-1640 containing 100 U/mL Pen/Strep instead. After infecting the chips, neutrophils were introduced in the circulatory perfusion of the vascular compartment. The concentration of freshly isolated primary human neutrophils from venous whole blood (same donor as monocyte-derived macrophages) was adjusted to 1×10^6 /mL in vascular medium (Medium 199 (Gibco) supplemented with 10% FBS, 25 μ g/mL heparin, 7.5 μ g/mL vascular mitogen (ECGS), 5 μ g/mL Vitamin C, 10% autologous human serum, and Pen/Strep (100 U/mL)). Afterwards, 4 mL vascular medium

containing 1×10^6 /mL neutrophils was loaded into the reservoir, and neutrophils were perfused and circulated in the vascular compartment at a flow rate of 25 μ L/min for 23 h by the peristaltic pumps.

Samples were taken and saved prior to infection and 24 h post-infection. Media samples (approximately 4 mL) from the reservoirs at the vascular side and from the epithelial compartment (approximately 120 μ L) were collected and centrifuged at $250 \times g$ for 10 min at RT. The supernatants were used in tissue damage assays and remaining supernatants were stored at -80°C until further use. All compartments were washed twice with 250 μ L PBS and the washes were collected for colony-forming units (CFU) plating. Afterward, the chips were either fixed for microscopy or used for membrane integrity assessment.

FITC assay for barrier integrity

Barrier integrity assay was performed as described in Maurer *et al.*, 2019 [45]. Briefly, after washing the compartments 24 hpi, 250 μ L of fluorescein isothiocyanate (FITC)-dextran (4000 g/mol) (Sigma, 1 mg/mL in phenol-free RPMI) was flushed into the vaginal compartment twice. After 1 h incubation inverted at 37°C and 5% CO_2 , media was collected first from the vaginal compartment and then from the vascular compartment. For quantification, a 1:2 standard curve was prepared from 1 mg/mL with 12 dilutions. 50 μ L of each sample and standard was transferred to a black 96-well plate and measured at 490 nm excitation and 520 nm emission.

Chip lysis and colony-forming-unit (CFU) plating

To assess the overall fungal burden, including invasive *Candida*, the resulting pellets from the previous sampling were resuspended in 1 mL PBS, diluted twice 1:10, and 100 μ L were plated on YPD plates. After approximately 2 days of incubation at RT, colonies were counted.

To obtain *Candida albicans* from tissue, lysates were made by flushing 1 mL of 1% Triton X-100 (Sigma) through the vascular site around 30 times and collected afterwards. For the vaginal site, the membrane was excised and placed in a 12-well plate (vaginal cells facing up) with 500 μ L 1% Triton X-100. All cell material was scraped off with pipette tips and collected. The wells were washed again with 500 μ L, and lysates were plated as described above.

Luciferase release cytotoxicity assays

Released luciferase from A-431 Nluc cells was quantified as a measurement of vulvovaginal epithelium damage; 5 μ L of the collected supernatants were transferred into a 96-well white bottom plate and diluted 1:20 in PBS. The luciferase assay solution, 10 μ M coelenterazine (Roth) in PBS, was freshly prepared and 100 μ L was added to each supernatant sample in the plate. Luminescence was measured by a plate reader (Tecan Infinite M200 pro).

Cytokine and MPO quantification

Concentrations of IL-1 α (IL-1F1), IL-1 β (IL-1F2), IL-1Ra (IL-1F3), IL-8 (CXCL8), GM-CSF, and IFN- α in the supernatant of epithelial and vascular compartments were quantified using a human discovery Luminex

assay (R&D Systems) according to the manufacturer's instructions. MPO concentrations were quantified using a duoset ELISA (R&D Systems) according to the manufacturer's instructions.

Immunofluorescence staining and image acquisition

After collecting supernatants and washing, biochips were fixed with Histofix 4% (Carl Roth) for 20 min at RT. After being washed with DPBS, the biochips were stored at 4°C until staining [44]. For the immunofluorescence staining, membranes from the VVC-on-chip were excised and blocked with 0.1% saponin (Sigma-Aldrich) and 1% bovine serum albumin (BSA; Roth) in DPBS for 45 min at RT. Primary antibodies were diluted in 0.1% saponin and 0.1% BSA in DPBS and incubated overnight at 4°C. Macrophages were stained using mouse anti-human CD68 monoclonal antibody (1:100; BD Pharmingen™), neutrophils were stained with a mouse anti-human CD15 antibody (1:100; BioLegend), and *C. albicans* was stained with rabbit anti-*C. albicans* antibody (1:1000; Acris Antibodies). The next day, following three washes with 0.1% BSA and 0.1% saponin in DPBS, membranes were incubated with secondary antibodies in DPBS with 0.1% BSA and 0.1% saponin accordingly – Goat anti-rabbit Alexa Fluor 647 (1:1000; Invitrogen) and donkey anti-mouse Alexa Fluor 555 (1:1000; Invitrogen). In the meantime, nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; 1:2000 in DPBS with 0.1% BSA and 0.1% saponin; Invitrogen) while F-actin was stained using Alexa Fluor 488 Phalloidin stain (1:500; Invitrogen). The membranes were then incubated for one hour at RT and protected from light. Stained membranes were washed with 0.1% BSA and 0.1% saponin in DPBS and mounted on glass slides using ProLong™ Diamond Antifade Mountant (Invitrogen) and covered with a glass coverslip.

Microscopy

Images of the Organ-on-chip components were acquired with a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss Jena) with a 20X/NA 0.55 long working distance (26 mm) objective lens. The following excitation and emission wavelengths were applied – DAPI (Ex/Em: 405 nm/465 nm), AlexaFluor 488 (Ex/Em: 488 nm/517 nm), Alexa Fluor 555 (Ex/Em: 561 nm/568 nm), and Alexa Fluor 647 (Ex/Em: 633 nm/668 nm). Image analysis was performed using ZEN software (Zeiss), and Imaris 11.0.0 and 11.0.1 (Bitplane, Belfast, Northern Ireland). Generation of 3D images was allowed by Z-stack acquisition. Number of z-layers of the stacks ultimately based on the total thickness of the endothelium and *C. albicans* growth on the epithelium. Each stack consisted of approximately 30–40 slices with 1 µm axial slice distance.

Image segmentation

Z stack images acquired by laser scanning confocal microscopy were provided in their native CZI (Carl Zeiss Image) format for quantitative analysis. The images were processed using Imaris 11.0.1 and Imaris 11.0.0 (Bitplane, Belfast, Northern Ireland). The nuclei were segmented as Surfaces objects with the grain size of 0.415 µm and the estimated diameter of 5.00 µm. Spots objects were also created for the nuclei with an estimated diameter of 7.00 µm. Human neutrophils cells were also reconstructed as Surfaces with the grain size set to 1.0 µm and the diameter of largest sphere at 3.00 µm.

The nuclei of the neutrophil were identified based on the overlap between the nuclei and the neutrophil surfaces. In order to improve the fidelity of neutrophil reconstruction, they were also created as Cells objects, with the help of the above described neutrophil nuclei and the neutrophil fluorescence channel. Here the nuclei smoothing filter had a width of 0.208 μm , and the background was subtracted by the Imaris Rolling Ball filter with a width of 0.830 μm . The cell smoothing filter was set to 2.00 μm , and the segmented region was extended to 4.00 μm . To get additional statistical measures for the mean distance of the three nearest neighbours for the neutrophil, we applied a MATLAB script as a custom tool in Imaris.

The actin fibres were recreated in 2 layers based on their z positions. The lower layer was segmented with the surface grain size at 0.2 μm and the diameter of largest sphere of 0.5 μm , and region growing estimated diameter of 5.00 μm . The upper layer was segmented with the surface grain size of 0.4 μm and the diameter of largest sphere of 1.0 μm . For both layers masking channels were created with the fill value inside of 100 and outside 0, as well as filaments with the segment seed point diameter of 0.623 μm and maximum seed point diameter of 2.00 μm , with a maximum gap length of 2.00 μm . Yeast cells and hyphae were segmented as Surfaces objects with a grain size of 0.415 μm and with the diameter of largest sphere set to 1.56 μm . Filament objects were created based on the masked channel of the phalloidin surfaces with the segment seed point diameter of 1.00 μm and maximum gap length of 2.00 μm . For easier segmentation of the membrane pores, the transmitted light channel was inverted and the resulting bright rings were used to create Cells objects, with the smallest diameter set to 6.00 μm and membrane details at 0.415 μm .

Data assembly and plotting

The analysis of the image data provided by Imaris in csv file format was carried out by Python scripts using Python 3.11.10 running in ZooCode, applying the vllm/coder model inside VS Code. The GLM 5.2 [96] (frontier) model was hosted locally on a custom-built GPU server equipped with eight Nvidia H200 GPUs containing 128GB VRAM per GPU. Distance-related graphs were separated into endo- and epithelial sides, based on the object's axial location relative to the nearest membrane pore. Further details of the graphs and the plotting parameters are given in the figure legends.

Experimental design of murine VVC model

8-week-old female C57BL/6J mice were treated subcutaneously with 0.2 mg of β -estradiol 17 valerate (Merck) dissolved in 100 μL of sesame oil (Merck) 24 h prior to vaginal infection as well as on the day of vaginal infection. Oestrogen administration was continued once per week till the end of the experiment to preserve the status of pseudoestrus. Mice were inoculated intravaginally with 20 μL of phosphate-buffered saline (PBS) or PBS containing $2 \times 10^9/\text{mL}$ viable *C. albicans* SC5314 from early-stationary-phase cultures (around 18 h of culture at 37°C on sabouraud dextrose with chloramphenicol). Vaginal lavages were performed with sterile PBS in a total volume of 100 μL with multiple aspiration and agitation. Murine IL-1 β (IL-1F2) and MPO were quantified using DuoSet ELISAs (R&D Systems).

Absorption of developed ELISA plates was measured with microplate reader (Tecan Infinite M-Plex; i-control software).

Murine histology

Mice reproductive tract was collected and fixed in 10% neutral buffered formalin, trimmed in transverse sections to incorporate portions of the uterus, cervix, and vagina and embedded in paraffin. 5 µm-thick sections were cut and placed onto adhesive slides (Superfrost Plus Adhesion, Epredia), and deparaffinated by submerging in a row of xylene (2 × 10 min), 100% ethanol (2 × 1 min), 95% ethanol (1 min), 75% ethanol (1 min), tap and distilled water (1 min each). After brief drying, samples were encircled with an ImmEdge hydrophobic barrier PAP pen (Vector Laboratories) and then washed with PBS containing 0.3% Triton X-100. Slides were blocked with 5% goat serum in PBS with 0.3% Triton X-100 for 90 min. Without prior antigen-retrieval, they were stained with 80 µL primary antibody mix consisting of PBS, 1% BSA, 0.3% Triton X-100, 10 µg/mL anti-*Candida* (polyclonal rabbit, BP1006, Acris Antibodies/OriGene Technologies) and 5 µg/mL AlexaFluor 647-coupled anti Ly6G (clone 1A8, #127610, Biolegend) overnight at 4°C inside a humid chamber. Samples were washed for three times and incubated with secondary stains including 5 µg/mL goat-anti-rabbit IgG AlexaFluor 488 (A-11008, Invitrogen, Thermo Fisher Scientific) and 5 µg/mL DAPI for 90 min at RT in the humid chamber. Samples were embedded in Fluoroshield (Invitrogen) after three washes. Images were acquired on an Axio Observer Z1 (Carl Zeiss Microscopy).

Declarations

Data availability

All data are available in the main text and the supplementary materials, and will be made available upon request.

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Author contributions

KOC: Investigation, Validation, Formal analysis, Writing - Original Draft, Visualization

VEG: Investigation, Validation, Formal analysis, Writing - Original Draft, Supervision

ZC: Investigation, Formal analysis, Supervision, Visualization, Methodology, Software

MK: Investigation, Formal analysis, Visualization, Software

OP: Investigation, Writing - Original Draft

EG: Investigation, Validation

LF: Visualization

TZ: Resources

AD: Investigation, Visualization, Writing - Review & Editing

AKZ: Writing - Original Draft, Project administration

MV: Supervision, Project administration, Writing - Review & Editing

ASM: Funding acquisition, Methodology, Writing - Review & Editing

RAR: Investigation, Writing - Original Draft, Supervision, Methodology, Project administration

DEM: Formal analysis, Writing - Original Draft, Supervision, Visualization, Project administration

MTF: Funding acquisition, Supervision, Resources, Writing - Review & Editing

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Figures

VVC-On-Chip Model

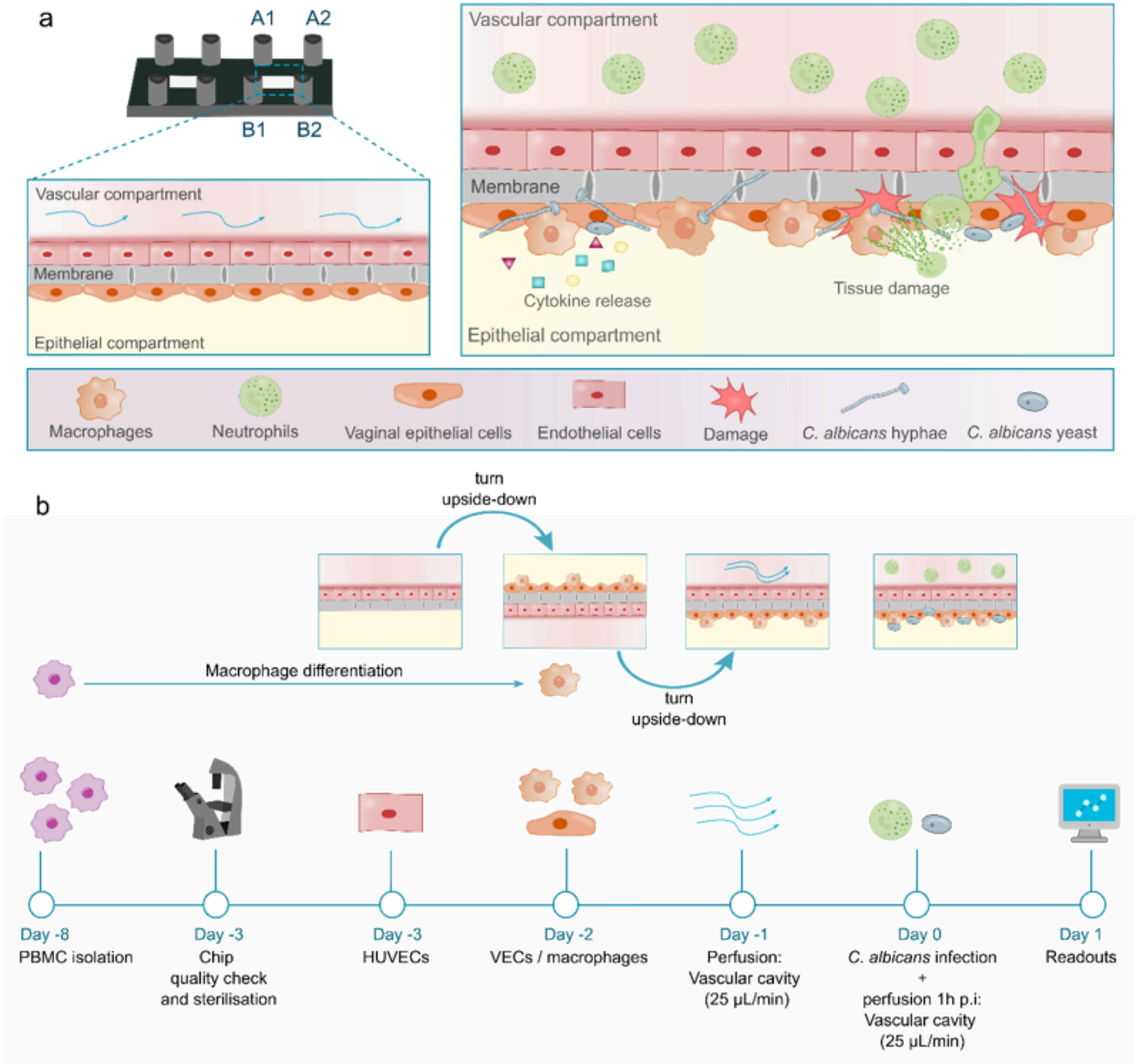


Figure 1

Schematic representation of a VVC-on-chip model. (a, left) The microfluidic biochip has two cavities, each divided into two compartments: a top channel (A) and a bottom channel (B), including two inlets (A1, B1) and two outlets (A2, B2). These compartments are separated by a porous membrane that allows migration of immune cells from the top channel (vascular compartment) to the bottom channel (epithelial compartment). **(a, right)** The interactions among cells cultured in the vascular compartment, such as the endothelial cells (HUVEC/TERT 2) and primary human neutrophils, with vulvovaginal epithelial cells (A-431-Nluc VECs), macrophages (hMDMs), and *C. albicans* seeded in the epithelial

compartment are depicted. **(b)**The workflow of the VVC-on-chip model used to mimic vaginal *C. albicans* infection. Primary human monocytes are isolated on day -8 and differentiated to macrophages. On day -3, HUVEC/TERT 2 are seeded into the vascular compartment. On day -2, A-431-Nluc VECs and hMDMs are cultivated in the epithelial compartment. On day -1, the vascular compartment is perfused to resemble the bloodstream. On day 0, *C. albicans* CA3153 is used to infect the epithelial compartment, followed by perfusion of isolated human neutrophils into the vascular compartment. Following infection, inflammatory crosstalk among cells and tissue integrity are evaluated on day 1 by quantifying cytokine release, Nluc activity, epithelial barrier integrity, myeloperoxidase (MPO) release, neutrophil migration, and fungal burden (colony forming units; CFU).

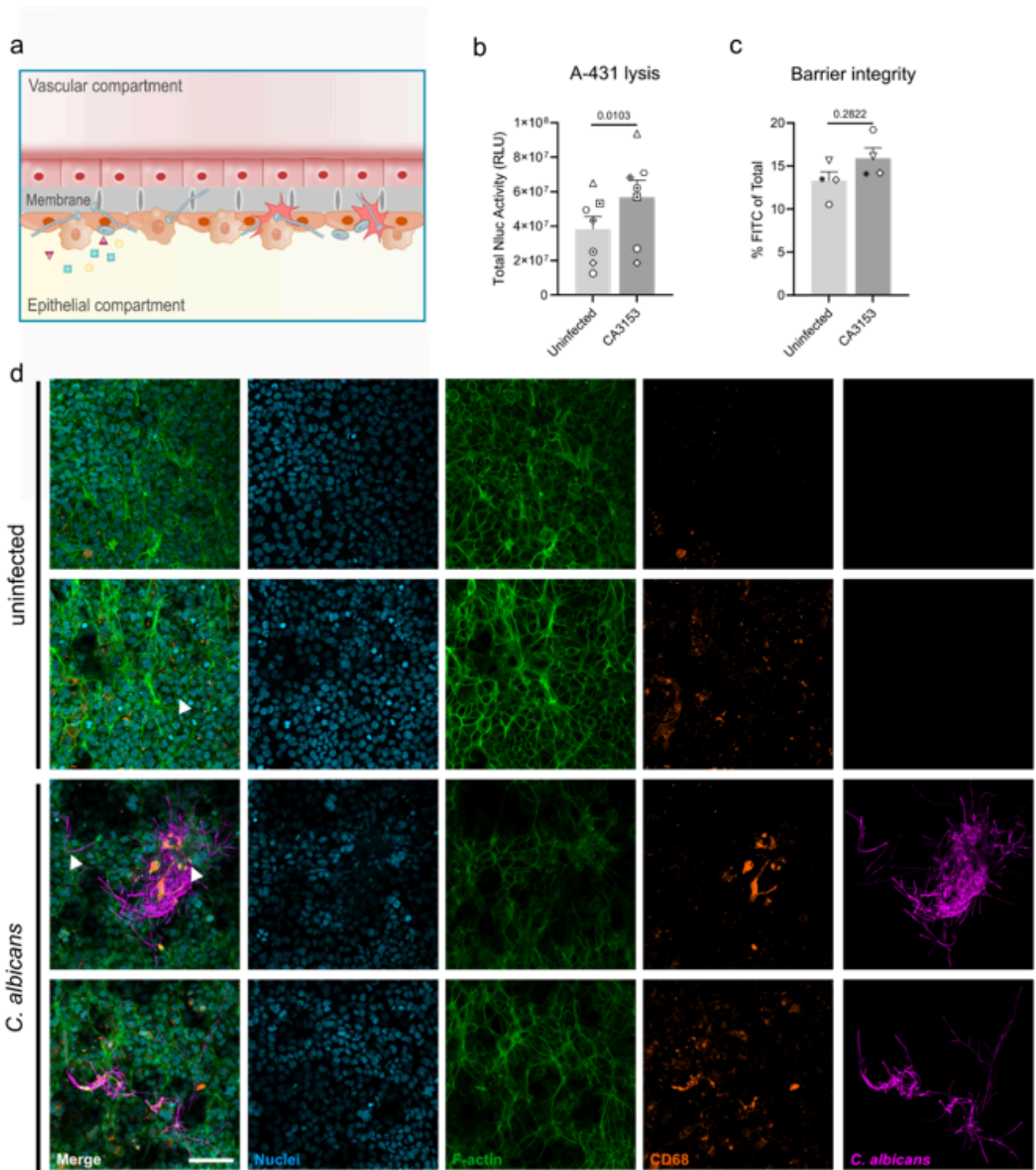


Figure 2

Infection of the VVC-on-chip model. (a) Schematic representation of the biochip including the overall response of vascular cells, vulvovaginal epithelial cells, and macrophages after infection with *C. albicans* (CA3153) 24 hpi. (b) Epithelial damage assessed by release of enzyme Nano-luciferase (Nluc) from Nluc-expressing A-431 VECs after infection ($n = 7$). (c) Integrity of the epithelium determined by the amount of diffused FITC-Dextran from the epithelial compartment to the vascular compartment ($n = 4$).

(d) Representative confocal microscopy of the vulvovaginal epithelium. Arrowheads indicate the regions of interest regarding morphological changes, which are magnified in **Supplementary Figure S2**. Nuclei are stained with DAPI (blue), F-actin is stained with phalloidin (green), human macrophages are stained using anti-CD68 (orange), and *C. albicans* is stained with an anti-*Candida* antibody (magenta). Magnification 20×. Scale bars represent 100 μm. The symbols represent the data from each independent experiment, bars represent the mean + standard error of the mean (SEM), and the numbers represent the *P*-values obtained in each statistical test. Statistical significance was determined using paired t-test (b-c).

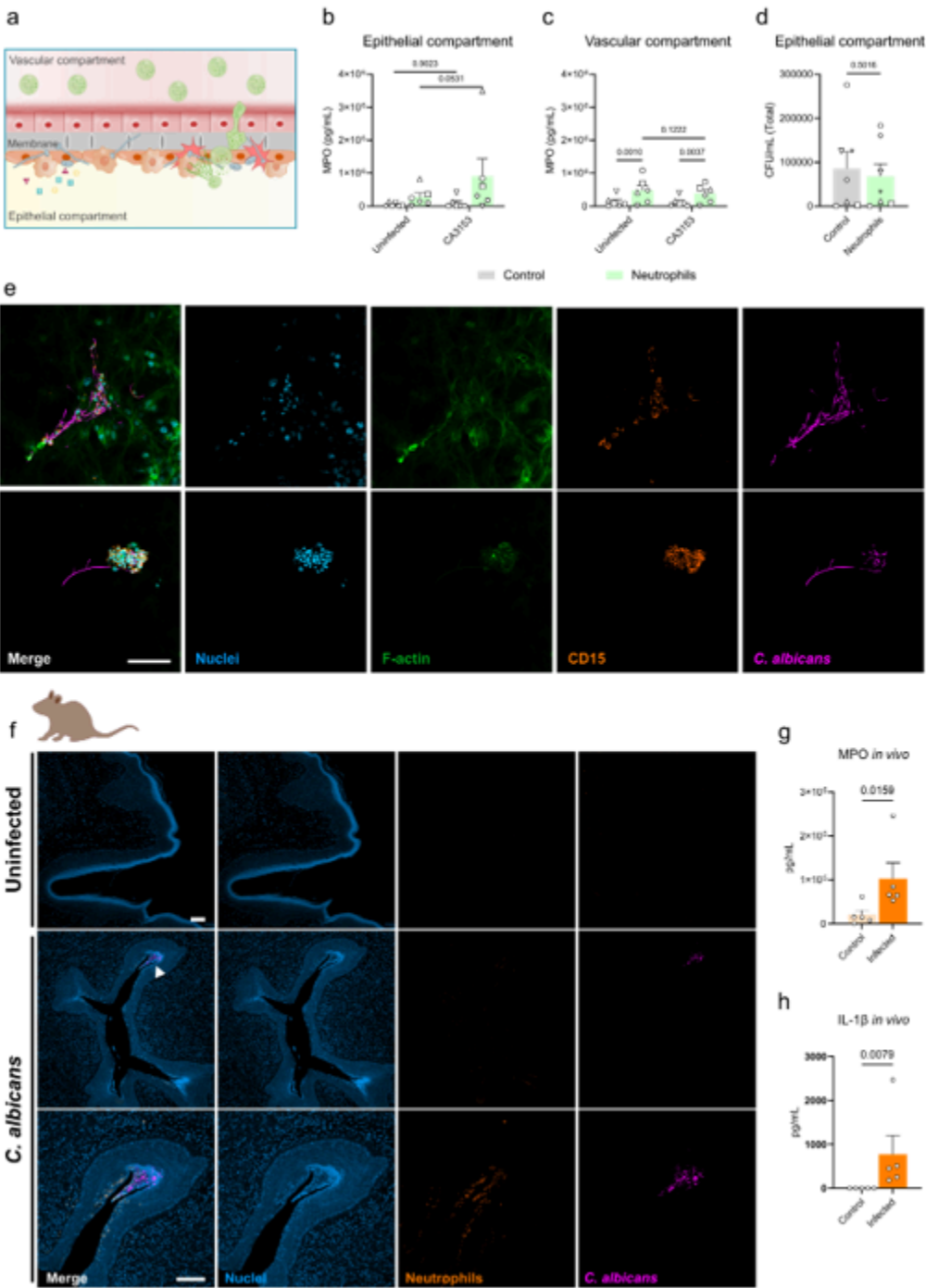


Figure 3

Neutrophil dynamics in a VVC-on-chip infection model. (a) Schematic representation of neutrophil migration from the vascular compartment to the epithelial compartment in response to *C. albicans* CA3153 infection. (b) MPO release detected in the epithelium and (c) in the vascular compartments ($n = 6$). (d) Epithelial fungal burden in the presence and absence of neutrophils measured by quantifying CFU ($n = 7$). (e) Representative confocal microscopy of infected chips perfused with neutrophils. Nuclei are stained with DAPI (blue), F-actin is stained with phalloidin (green), human neutrophils are stained using anti-CD15 (orange), and *C. albicans* is stained with an anti-*Candida* antibody (magenta). Magnification 20 $\times$. Scale bars represent 100 μm . (f) Immunofluorescence of histology sections of vaginal tissue of uninfected mice and mice infected with *C. albicans*. Nuclei were stained with DAPI (blue), neutrophils with anti-Ly6G (orange), and *C. albicans* with anti-*Candida* (magenta). Magnification 10 $\times$; scale bars represent 100 μm (top & middle). Magnification 20 $\times$; scale bars represent 100 μm (bottom, magnified view of the region with white arrowhead in the middle image). (g) MPO and (h) IL-1 β release in vaginal lavages ($n = 5$ uninfected and $n = 5$ infected). The symbols represent the data from each independent experiment (b-d). The dots represent individual mouse (g-h). Bars represent the mean + standard error of the mean (SEM), and the numbers represent the P -values obtained in each statistical test. Statistical significance was determined using paired t-test (e), a two-way ANOVA (c, d), or Mann-Whitney test (h, i).

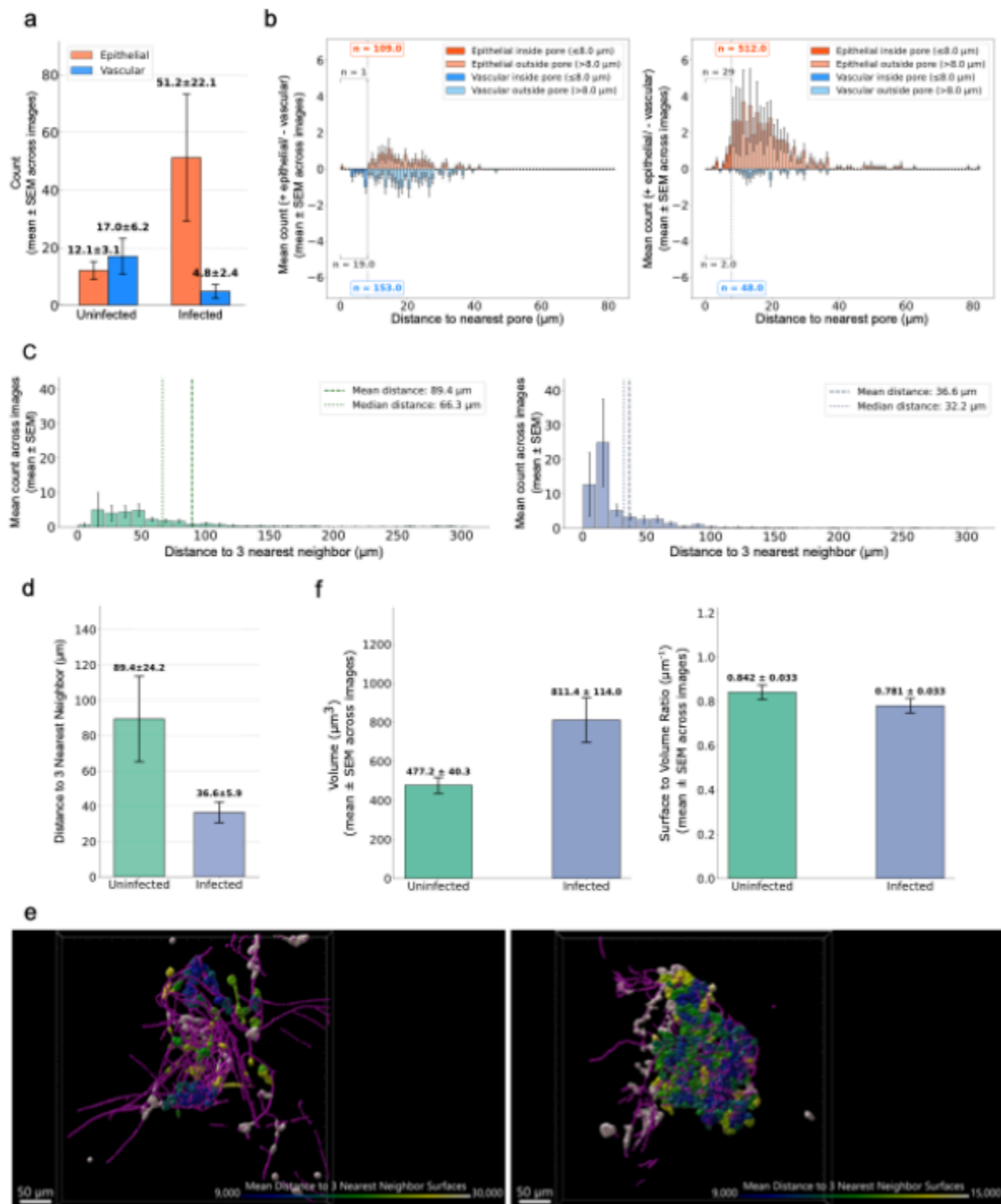


Figure 4

Neutrophil distribution across the VVC-on-chip. (a) Neutrophil distribution per compartment represented by the mean neutrophil count. The orange and blue bars indicate the total number of neutrophils in the epithelial and vascular side, respectively. (b) Neutrophil distance to the nearest membrane pore (left: uninfected; right: infected). The neutrophil number on the epithelial side is plotted positive, whereas on the vascular side is negative. Neutrophils that are inside a membrane pore (i.e., their distance from the membrane is less than 8 μm) are plotted in dark orange for the epithelial side and

dark blue for the vascular side; the vertical dashed lines are placed at 8 μm , to indicate the thickness of the membrane. The numbers in orange and in blue indicate the total number of neutrophils in epithelial and vascular side, respectively. The numbers at the top and bottom part of the plots in grey indicate how many neutrophils are inside the pores on the corresponding sides. **(c)** Mean neutrophil count across images versus the distance to the three nearest neighbours (left: uninfected; right: infected). The plot characterizes the swarming behaviour of the neutrophils in the presence of the fungi by showing much closer neutrophils when they are cluster around the *C. albicans* invading hyphae. Vertical dashed and dotted lines represent mean and median distances, respectively, for each condition, values also indicate in the plot. **(d)** Mean distance to the three nearest neighbour neutrophils. **(e)** Representative 3D reconstruction images of *C. albicans* clustering with recruited neutrophils in the epithelial compartment. **(f)** Neutrophil volume change (left) and neutrophil cell surface to volume ratio (right). Data was generated from nine uninfected images and ten infected images obtained from two individual chip experiments. Bars represent the mean + standard error of the mean (SEM) in all panels.

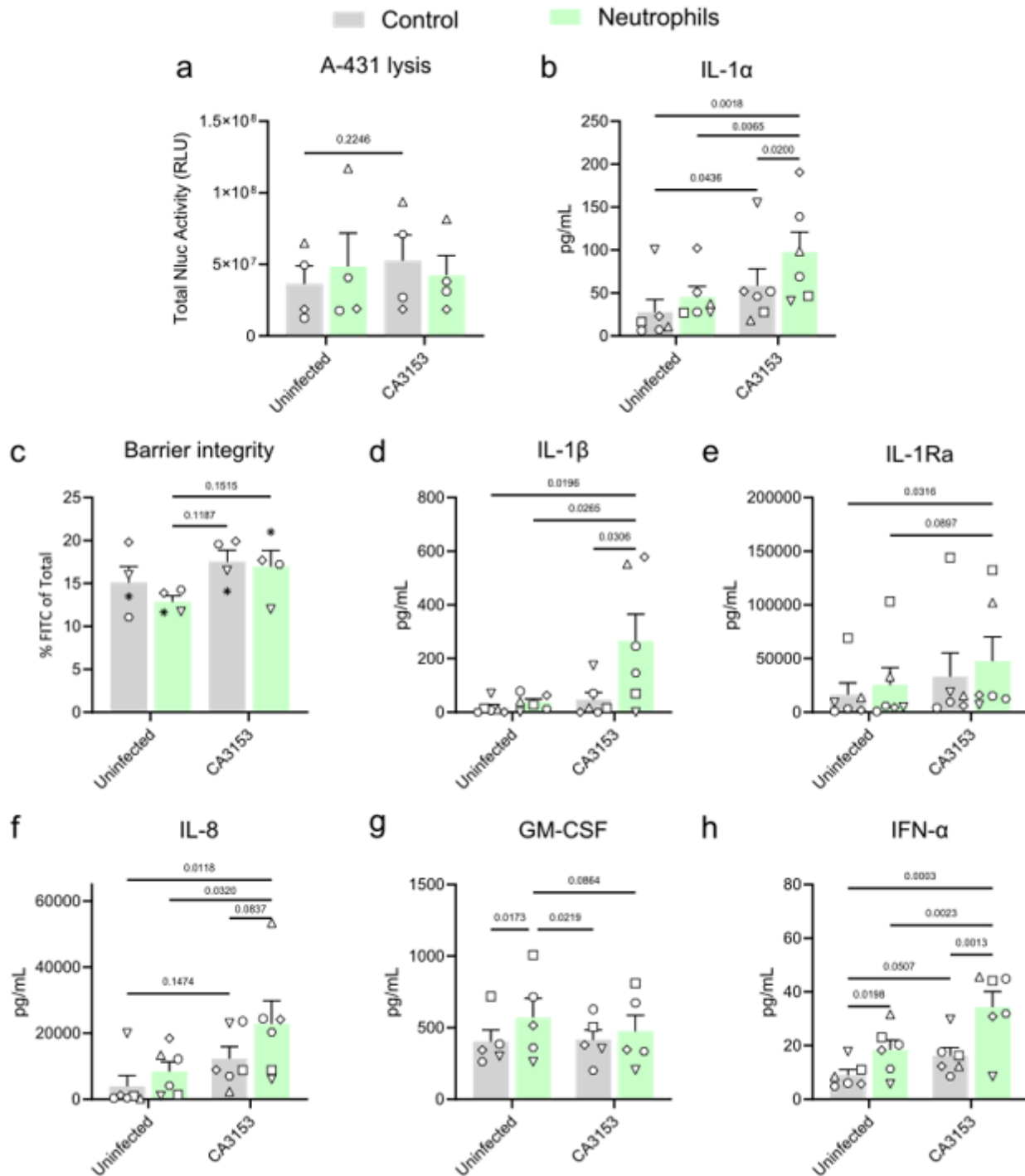


Figure 5

The influence of neutrophils on host cell integrity and inflammatory responses. (a) A-431-Nluc VECs damage assessed by release of enzyme Nano-luciferase (Nluc) after infection in the presence and absence of neutrophils ($n = 4$). (b) IL-1α alarmin release after infection with *C. albicans* CA3153 in the presence and absence of neutrophils ($n = 6$). (c) Barrier integrity determined by the amount of leaked Fluorescein Isothiocyanate-Dextran (FITC) from the epithelium to the vascular compartment following

infection in presence and absence of neutrophils ($n = 4$). **(d-h)** Release of IL-1 β , IL-1Ra, IL-8, GM-CSF, and IFN- α after infection with *C. albicans* CA3153 and in the presence of neutrophils ($n = 6$; GM-CSF $n = 5$). The symbols represent the data from each independent experiment, bars represent the mean + standard error of the mean (SEM), and the numbers represent the P -values obtained in each statistical test. Statistical significance was determined using a two-way ANOVA.

Supplementary Files

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