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Candida **causes an estimated half-billion cases of vulvovaginal candidiasis (VVC) every year. VVC is most commonly caused by *Candida albicans*, which, in this setting, triggers nonprotective neutrophil infiltration, aggressive local inflammation, and symptomatic disease. Despite its prevalence, little is known about the molecular mechanisms underpinning the immunopathology of this fungal infection. In this study, we describe the molecular determinant of VVC immunopathology and a potentially straightforward way to prevent disease. In response to zinc limitation, *C. albicans* releases a trace mineral binding molecule called Pra1 (pH-regulated antigen). Here, we show that the *PRA1* gene is strongly up-regulated during vaginal infections and that its expression positively correlated with proinflammatory cytokine concentrations in women. Genetic deletion of *PRA1* prevented vaginal inflammation in mice, and application of a zinc solution down-regulated expression of the gene and also blocked immunopathology. We also show that treatment of women suffering from recurrent VVC with a zinc gel prevented reinfections. We have therefore identified a key mediator of symptomatic VVC, giving us an opportunity to develop a range of preventative measures for combatting this disease.**

INTRODUCTION

Candida albicans is a commensal member of the human oral, gastrointestinal, and urogenital microbiota. However, this yeast can also cause infections in humans. The most common type of *Candida* infection is vulvovaginal candidiasis (VVC, also known as thrush). VVC affects three-quarters of women of childbearing age, and ~7% of those affected suffer from recurrent infections (four or more vaginal infections per year). Together, this accounts for an estimated half-billion infections every year, which can have an enormous impact on the quality of life and mental health of the 138 million women suffering from recurrent VVC (*1*–*3*).

The symptoms of VVC, which can include itching, burning, discharge, and dyspareunia, are caused by a hyperinflammatory response to *C. albicans* and not necessarily by the presence of fungus itself, which can commensally colonize the vagina. Therefore, understanding the mechanism by which *C. albicans* triggers inflammation could be key to preventing symptomatic infections.

Currently, guidelines for treating recurrent VVC involve 6 months of maintenance therapy with azole antifungals, such as fluconazole. However, after discontinuation, more than half of women suffer from reinfections within the following 6 months (*4*). Moreover, there are reports of clinical isolates that are resistant to azole antifungals (*5*), underscoring the urgent need for additional treatment strategies.

Immunologically, host S100A8/A9 alarmin proteins have been implicated in vaginal inflammation during VVC, which is associated with nonprotective neutrophil infiltration (*6*–*8*); however, no study has yet identified a fungal virulence factor responsible for immunopathology in women. Of note, S100A8/A9 (also known as calprotectin) is known to drive the expression of *C. albicans PRA1* (pH-regulated antigen) (*9*), which encodes a secreted fungal protein with several immune-modulatory properties, including complement modulation and neutrophil chemotaxis (*10*–*12*). We have shown that the primary function of *C. albicans* Pra1 is to capture zinc from the environment and, as such, is regulated by this essential trace mineral (*13*); the transcriptional induction of *PRA1* by calprotectin can be repressed with zinc (*9*).

Here, we demonstrate that *C. albicans PRA1* is expressed in colonized and infected women and is required for inflammatory responses ex vivo as well as in mouse models of vaginal candidiasis. We show that zinc prevents inflammation in our experimental models and prevents reinfection in women who had been suffering from recurrent VVC.

RESULTS

Expression of *PRA1* in women correlates with inflammatory cytokines

Vaginal swab samples were collected from *C. albicans*–colonized and –infected (n = 17) and *C. albicans*–negative (n = 12) women and assessed for pH, calprotectin, inflammatory cytokines interleukin-1β (IL-1β) and IL-8, *Candida* fungal burden, and expression of *PRA1*, as well as the well-established virulence genes *ECE1* (extent of cell elongation), *SAP6* (secreted aspartyl protease), and *HWP1* (hyphal wall protein) (tables S1 and S2). *C. albicans*–colonized and –infected women exhibited significantly higher pH than noncolonized individuals ($P < 0.0001$). Noncolonized pH ranged from

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3.5 to 4.5 (mean, pH 3.75), whereas *C. albicans*–colonized and –infected pH ranged from 4.2 to 5.1 (mean, pH 4.57) (Fig. 1A). Calprotectin concentrations did not differ significantly between groups ($P > 0.05$); however, a far narrower distribution was observed in uninfected individuals (Fig. 1B). In contrast, the proinflammatory cytokines IL-1β and IL-8 were significantly higher in *C. albicans*–colonized and –infected samples than in negative controls ($P < 0.001$ in both cases; Fig. 1, C and D).

We then excluded the negative samples from our dataset and performed a Pearson correlation analysis of all clinical parameters. *PRA1* was highly expressed in 7 of 17 samples, likely as a result of local zinc restriction and the presence of host zinc-binding proteins. A positive relationship between the inflammatory cytokine IL-8 and *PRA1* expression was observed, whereas the relationship between *PRA1* and IL-1β was not significant (Fig. 1, E to G). Of all the parameters measured, the strongest relationship observed was between *PRA1* and IL-8 (Fig. 1, E to G). Although *ECE1* was more highly expressed in some samples compared with in vitro yeast culture conditions, we did not observe a strong relationship between its transcript abundance and molecular markers of inflammation (Fig. 1E and fig. S1).

We next sought to address a regulatory paradox: The human vagina is acidic, and the *PRA1* gene was originally named because it is expressed at neutral/alkaline pH (*14*). We have since demonstrated that the function of Pra1 is to scavenge zinc, and as such, the gene is also under tight regulatory control by the availability of this essential micronutrient (*13*).

To test whether *PRA1* expression can be zinc-regulated during vaginal infection, we developed an epithelial tissue culture model using the human vaginal epithelial cell line A-431. These cells have been used extensively to study *Candida* pathogenicity; however, the commercially available culture medium is neutral/alkaline. We selected a tissue culture medium without zinc supplementation, and then, to mimic the environment of the human vagina more closely, we acidified it to pH 5.0. This had no obvious effect on the vaginal epithelia, and during a 16-hour coincubation with *C. albicans*, the pH did not change more than 0.8 units (fig. S2). After infection of the vaginal epithelia in this acidified medium, *PRA1* exhibited similar expression patterns as at pH 7. We reasoned that this was due to the low zinc content of the medium. Medium supplementation with 25 μM zinc sulfate fully repressed *PRA1* expression by *C. albicans* at both neutral and acidic pH (Fig. 1H). Therefore, although *PRA1* remains a pH-regulated gene (*14*), its regulation by zinc appears to dominate in this vaginal tissue culture model (Fig. 1I). We can effectively switch off *PRA1* expression by the addition of relatively low amounts of zinc.

Candida PRA1 is associated with neutrophil attraction

To further explore a role for Pra1 in vaginal inflammation, we used an ex vivo human blood model of neutrophil chemotaxis (Fig. 2A). All experiments used neutrophils isolated freshly from human blood; the gating strategy can be found in fig. S3. *C. albicans* wild type (WT), *PRA1* deletion mutant (*pra1*Δ), and genetic revertant (*pra1*Δ + *PRA1*) were incubated in RPMI tissue culture medium for 5 days. Under these conditions, all strains grew equally, and the pH remained neutral alkaline (fig. S4). *PRA1* was expressed as determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR; Fig. 2B) and Western blot (fig. S4). The culture filtrate from WT *C. albicans* drove robust neutrophil recruitment comparable to a proinflammatory IL-8–positive control (Fig. 2C). Deletion of *PRA1* abrogated neutrophil chemotaxis, and genetic complementation of the *pra1*Δ mutant restored *PRA1* expression and neutrophil chemotaxis (Fig. 2, B and C).

These data demonstrate that Pra1 is essential for neutrophil infiltration in the ex vivo human blood chemotaxis model. To complement this approach, we designed an alternative model for studying *C. albicans*–neutrophil interaction. Individual *C. albicans* cells were incubated on ibidi-VIII well microscopy slides in tissue culture medium for 10 hours until they had formed hyphal microcolonies. Human neutrophils were then added, incubated for a further 3 hours, and the slides were washed and stained to visualize both fungi and immune cells. The number of neutrophils within the same field of 40 microcolonies per strain was enumerated. *PRA1*-expressing *C. albicans* microcolonies were associated with significantly higher numbers of neutrophils (Fig. 2D).

We next examined the phylogenetic and genetic relationship between *Candida PRA1* and VVC immunopathology. The *PRA1* gene arose in the last common ancestor of the Dikarya (*13*) and is present in most extant fungal lineages (*15*). The Dikarya is the major subkingdom of fungi and encompasses ~98% of known species (*16*), including most pathogens, as well as industrially relevant and food-producing species. However, certain species have lost the gene. This is true of *Candida glabrata*, which is the second most common cause of vaginal yeast infections (Fig. 3A) (*13*, *17*). *C. glabrata* vaginal infections are much rarer, and their clinical pathology has not been reported.

Neutrophils from uninfected *C. albicans*– or *C. glabrata*–infected women ($n = 12$ per group) were enumerated. The number of neutrophils per field ranged from 0 to 3 in uninfected samples (mean, 1.08); this was significantly higher in *C. albicans*–infected samples ($P < 0.0001$), ranging from 2 to 20 (mean: 9.25) (Fig. 3B). In contrast, *C. glabrata*–infected samples were associated with low numbers of neutrophils (ranging from 0 to 6, mean: 2.25).

The association between *C. albicans* VVC and neutrophil infiltrate is in line with numerous previous studies (*8*). We are not aware of previous comparison between neutrophil numbers in *C. albicans* versus *C. glabrata* in clinical vaginal samples. Low neutrophil numbers in *C. glabrata*–infected women are consistent with observations made by one study that reported no signs of inflammation in a murine model of *C. glabrata* vaginal infection (*18*). It would therefore appear that vaginal *C. glabrata* infections in both mice and humans are associated with low neutrophil infiltrate.

To test whether there is a functional relationship between these phylogenetic and clinical observations, we took a molecular genetics approach. *C. albicans* and *C. glabrata* clinical isolates and laboratory strains were cultured in low-zinc cell culture medium as before, and the filter-sterilized supernatant was tested for chemotaxis in our ex vivo human neutrophil model. Again, *C. albicans* WT strains' culture filtrate promoted robust neutrophil recruitment comparable to that of the IL-8–positive control. In contrast, neutrophils did not migrate toward *C. glabrata* supernatant (Fig. 3C). We then placed a codon-optimized copy of *PRA1* from *C. albicans* under the control of the *C. glabrata GPD* promoter and introduced it into two independent *C. glabrata* genetic backgrounds; this resulted in the expression of *PRA1* in this otherwise *PRA1*-null species (fig. S5A). The culture supernatants from these modified *C. glabrata* cells recruited neutrophils at levels comparable to those of *C. albicans* (Fig. 3C and fig. S5B). These data show that *PRA1* expression

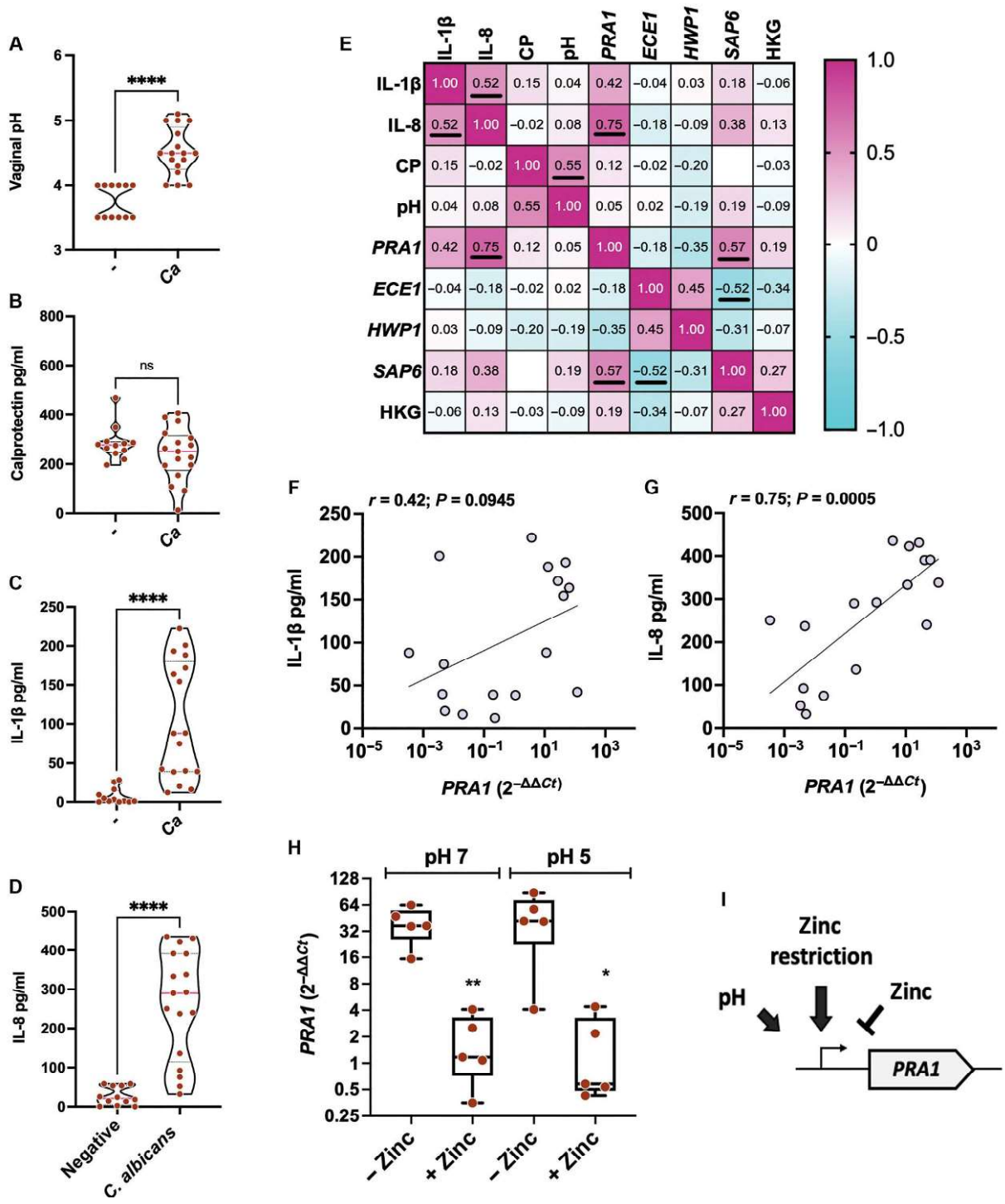

Fig. 1. *PRA1* zinc scavenger gene expression correlates with inflammatory cytokine concentrations during *C. albicans* vaginal colonization and infection in humans. (**A** to **D**) Vaginal swabs from uninfected ($n = 12$) and *C. albicans*–infected or –colonized ($n = 17$) women were assessed for pH (A), calprotectin (B), IL-1β (C), and IL-8 (D). (**E**) Pearson correlation matrix of the *C. albicans*–infected samples showing the relationships between host parameters [displayed in (A) to (D)] and expression of the indicated fungal genes as determined by qRT-PCR ($2^{-\Delta\Delta Ct}$); the mean RNA (pg/ml) of two housekeeping genes (HKG), *ACT1* and *CEF3*, was used as an indication of fungal burden. (**F** and **G**) Correlations between *PRA1* expression in women and cytokines IL-1β (F) and IL-8 (G) [*r* values from (E) and *P* values are displayed on graph]. (**H**) *PRA1* expression in response to low zinc during vaginal epithelial tissue culture infection at both neutral and acidic pH. *C. albicans* (clinical isolate SC5314) was incubated on A-431 vaginal epithelial cells for 16 hours in RPMI at pH 7 or adjusted to pH 5, with or without 25 μM $ZnSO_4$. Whiskers show minimum to maximum of all points. (**I**) Model showing the transcriptional control of *PRA1* in *C. albicans*. Statistical tests: (A to D) Mann-Whitney test. (E) Pearson *r* correlation displaying *r* values. Positive correlations are highlighted in magenta, and negative correlations are highlighted in cyan. Significant ($P < 0.05$) *r* values are underlined in the matrix. (H) *t* test between vehicle- and zinc-treated samples. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$; ns, not significant.

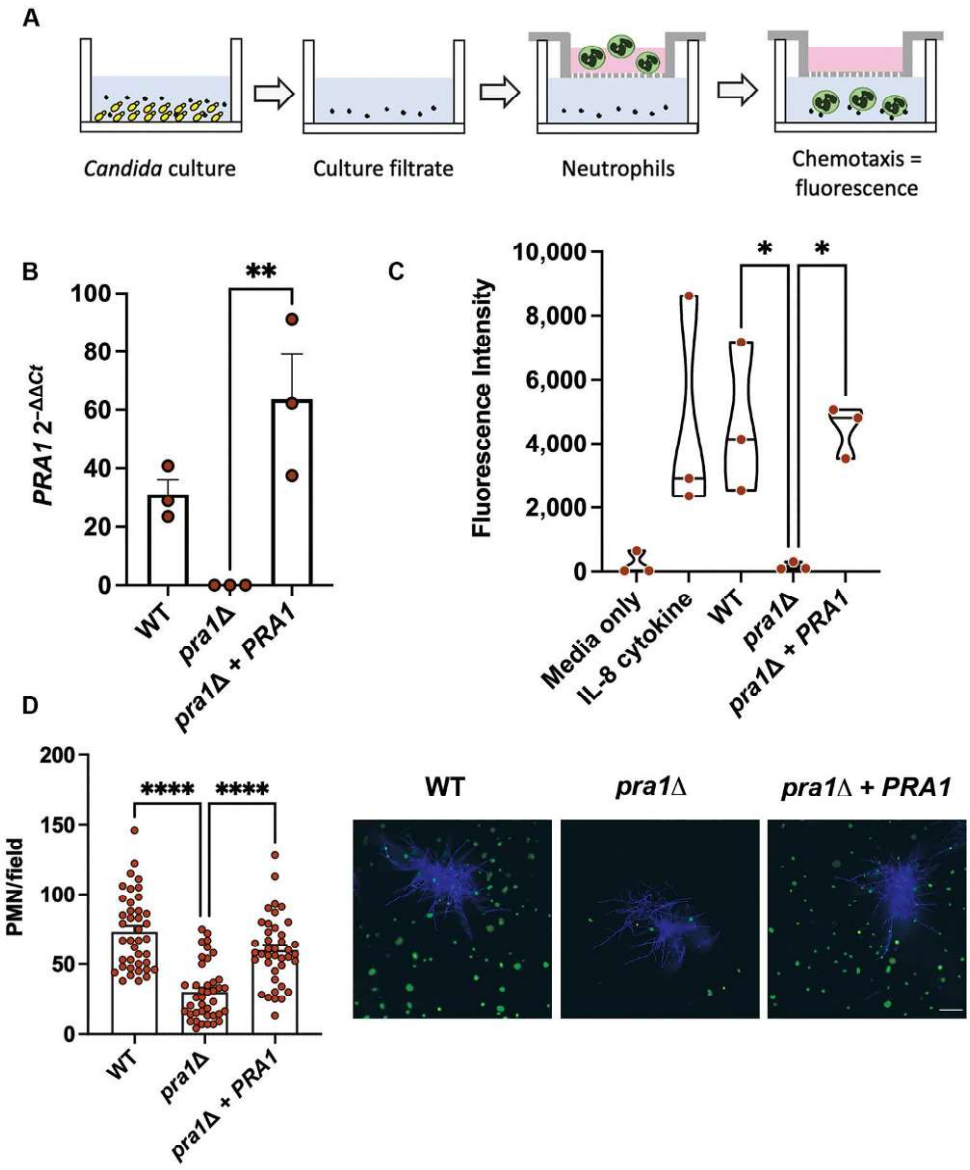

Fig. 2. *PRA1* is essential for neutrophil attraction. (**A**) Schematic overview of the neutrophil chemotaxis assay. *Candida* culture filtrate was added to the lower compartment of the chemotaxis plate. Fluorescently labeled neutrophils were added to the upper compartment, and chemotaxis was assessed by measuring fluorescence in the lower compartment after migration of neutrophils through the membrane. (**B**) *C. albicans* wild-type (WT), *pra1*Δ, and *pra1*Δ + *PRA1* strains were incubated for 5 days in RPMI tissue culture media, after which *PRA1* transcription was assessed by qRT-PCR ($n = 3$). (**C**) *PRA1* involvement in neutrophil chemotaxis. After the 5-day incubation period, culture filtrate, unconditioned media (RPMI), or media containing neutrophil chemotactic factor IL-8 (100 ng/ml) were added to the lower compartment of the chemotaxis plate. Freshly isolated human neutrophils were fluorescently labeled with calcein and added to the upper compartment. After 2-hour incubation, neutrophil chemotaxis was determined by measuring the fluorescence intensity (at 485/530 nm) in the lower compartment ($n = 3$). (**D**) As an alternative model, individual *C. albicans* yeast cells (~20 cells per well) were incubated in ibidi-VIII well microscopy slides in RPMI culture medium for 10 hours to induce microcolony development. Human neutrophils were added and incubated for a further 3 hours. Samples were fixed, and fungi were stained with Calcofluor white (blue) and neutrophils with Sytox Green. Forty individual microcolonies per strain (from two independent experiments) were imaged, and the number of neutrophils per field was enumerated. Representative micrographs show the greater number of neutrophils in proximity to *PRA1*$^+$ *C. albicans* microcolonies. Scale bar, 150 μm. Statistical tests: (B and C) one-way analysis of variance (ANOVA) with Tukey post hoc test [excluding positive and negative control in (C)]. (D) Kruskal-Wallis test. Error bars show SEM. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

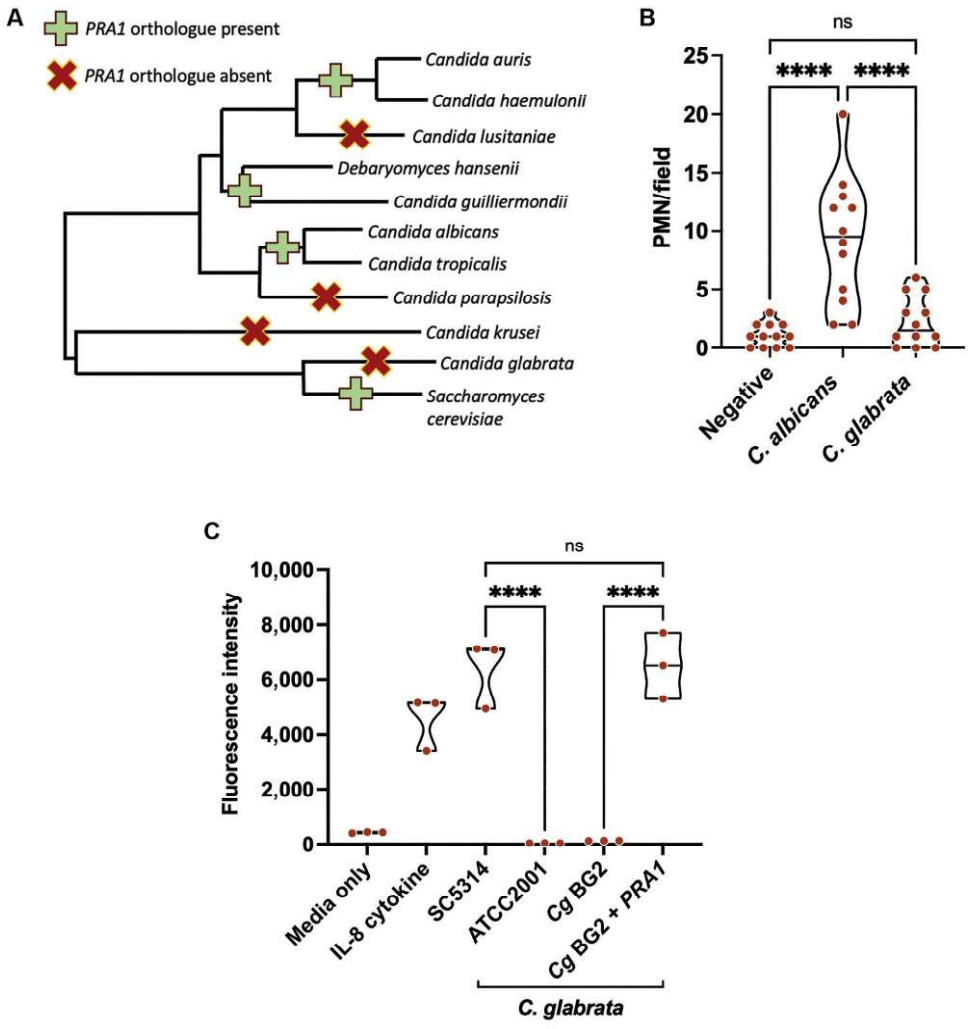

Fig. 3. *Candida PRA1* genetic status influences neutrophil attraction. (**A**) The *PRA1* gene has been lost by certain *Candida* species, including *C. glabrata*. A phylogenetic tree was drawn on the basis of Munoz *et al.* (*17*), and the presence or absence of *PRA1* orthologues were determined by BLASTp. (**B**) Vaginal swabs from uninfected, *C. albicans*–infected, and *C. glabrata*–infected women were evaluated for neutrophil infiltrate. $n = 12$ samples per group. (**C**) *PRA1* neutrophil chemotaxis assay. *C. albicans PRA1* was codon-optimized for expression in *C. glabrata*, placed downstream of the *C. glabrata GPD2* promoter, and transformed into *C. glabrata* BG2. Conditioned culture filtrate of *C. albicans* and *C. glabrata* strains with and without *PRA1* was used in a chemotaxis assay as outlined in Fig. 2. Unconditioned media (RPMI), media containing neutrophil chemotactic factor IL-8 (100 ng/ml), or the indicated culture filtrates were added to the lower compartment of the chemotaxis plate. Freshly isolated human neutrophils were fluorescently labeled with calcein and added to the upper compartment. After 2-hour incubation, neutrophil chemotaxis was determined by measuring fluorescence intensity (at 485/530 nm) in the lower compartment. $n = 3$. Statistical tests: (B and C) One-way ANOVA with Tukey post hoc test. ****$P < 0.0001$.

can "switch on" a neutrophil chemotactic response toward an otherwise unrecognized yeast.

PRA1 is required for vaginal inflammation in vivo

To test whether Pra1 plays a functional role in vaginal inflammation in vivo, we used the well-established estradiol-treated mouse model of VVC. We used outbred CD-1 mice to try to better capture host genetic diversity. Vaginal infection with *C. albicans* resulted in a strong inflammatory response, including elevated concentrations of proinflammatory cytokines IL-1β and CxCL-2 (a murine cytokine with similarities to human IL-8) (Fig. 4, A and B), as well as neutrophil infiltration (Fig. 4C and fig. S6). Deletion of *PRA1* in *C. albicans* did not affect vaginal fungal burden as assessed by measuring colony-forming units, and WT and *pra1*Δ exhibited both yeast and hyphal morphologies (Fig. 4D and fig. S7). However, deletion of *PRA1* blocked the production of both inflammatory cytokines and neutrophil infiltrate (Fig. 4) but did not affect calprotectin concentrations (fig. S8). Therefore, in the absence of *PRA1*, *C. albicans* is able to colonize the murine vagina without provoking inflammation.

Zinc prevents immunopathology in vivo

Our observations suggest a connection between Pra1 and vaginal inflammation in the clinical setting and in our experimental infection models of VVC. Because the zinc-scavenging function of Pra1 is known (*13*), we reasoned that we may be able to prevent the symptoms of VVC with zinc supplementation. Of note, in humans, oral zinc supplementation does not affect zinc abundance in cervicovaginal lavage fluid (*19*).

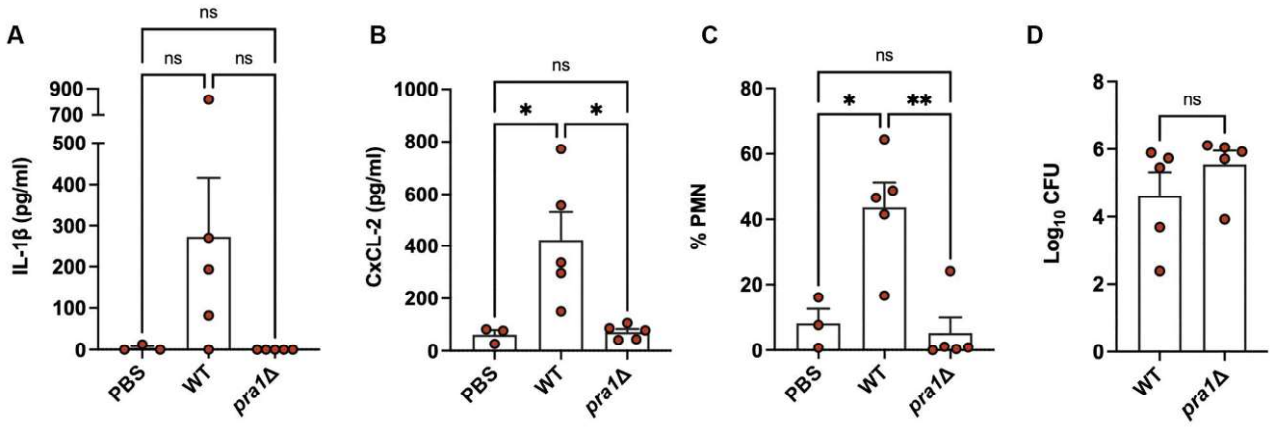

Fig. 4. Deletion of *PRA1* prevents inflammation in a mouse model of VVC. (**A** to **D**) Mice were vaginally infected with *C. albicans* WT or *PRA1*-deletion mutant (*pra1*Δ). Twenty-four hours after infection, vaginal lavage fluid was collected and assessed for the presence of the proinflammatory cytokines IL-1β (A) and CxCL-2 (which has similarities to human IL-8; B) by enzyme-linked immunosorbent assay (ELISA). The presence of neutrophil (PMN) infiltration by flow cytometry (C) and fungal burden by plating colony-forming units (CFU, D) were also assessed. Statistical tests: (A to C) One-way ANOVA with Tukey post hoc test. (D) Unpaired *t* test. Error bars = SEM. $*P<0.05$; $**P < 0.01$.

Therefore, using our mouse VVC infection model, we vaginally administered a zinc sulfate solution (10 μl at 25 μM) at the point of infection and 8 hours after infection. We chose this concentration because it is nontoxic to both microbe and host (*20*, *21*). Similar to *PRA1* genetic deletion, this micronutrient supplementation did not adversely affect *C. albicans* colonization of the mouse vagina or fungal morphology (fig. S7). Zinc treatment increased the numbers of colony-forming units recovered from mice (Fig. 5A). We have previously shown that zinc can reduce *C. albicans* adhesion to surfaces, which may perturb fungal burden determination by the colony-forming unit method (*22*) and therefore assessed fungal burden by measuring the expression of housekeeping genes *ACT1* and *CEF1*. This analysis revealed no significant difference in fungal colonization in the control versus zinc-treated mice ($P > 0.05$; Fig. 5B). In contrast, zinc administration down-regulated *C. albicans PRA1* expression in vivo by 2.6-fold (Fig. 5C). Zinc treatment of *C. albicans*–infected mice abrogated the production of both inflammatory cytokines (IL-1β and CxCL-2) (Fig. 5, D and E) and reduced neutrophil infiltration to that observed in uninfected animals (Fig. 5F) without affecting calprotectin concentrations (fig. S9). Gene expression of IL-1β and CxCL-2 (Fig. 5, G and H), as well as CxCL-1, CxCL-5, and IL-6 (Fig. 5, I to K), was down-regulated in response to zinc treatment. Therefore, administration of relatively low amounts of zinc can down-regulate *PRA1* in the mouse vagina and prevent inflammation, which is the hallmark pathology of VVC in humans.

To test whether zinc can prevent inflammation in humans, we first returned to our ex vivo blood model. *C. albicans* was incubated for 5 days in tissue culture medium with or without zinc supplementation. Zinc resulted in more than 300-fold down-regulation of the *PRA1* gene (Fig. 5L) and prevented chemotaxis of human neutrophils (Fig. 5M). On the basis of our molecular understanding of zinc acquisition by *C. albicans* and our observations on the link between *PRA1* and vaginal inflammation in vitro, ex vivo, in vivo, and in the clinical setting, we performed a retrospective pilot study on the effect of zinc on vaginal infections in women.

Juvia is a moisturizing gel that is used to treat vaginal dryness (*20*, *23*). It contains 20 μM zinc sulfate heptahydrate, a concentration very similar to what we used in our experimental models of VVC. Full details of the study can be found in Materials and Methods. We recruited 10 women who were suffering from recurrent vaginal infections, defined as suffering from at least one infection every 3 months. The participants were asked to intravaginally self-apply 2 ml of the zinc-containing gel nightly for 2 weeks and then twice per week. Treatment failure was defined as recurrence within the first 3 months. Of the eight participants who completed the study, six had been suffering from recurrent VVC and two had been suffering from recurrent bacterial vaginosis (RBV). The zinc gel had no effect on bacterial vaginosis (Fig. 5N). *PRA1* is unique to the fungal kingdom (*13*, *15*), and our current model of vaginal inflammation does not encompass bacterial infections. In contrast, five of six women (83%) who had previously been burdened with recurrent VVC did not suffer a reinfection within the 3-month course of our study (Fig. 5N).

DISCUSSION

Our work has revealed an important intersection between innate and nutritional immunity. Zinc restriction (nutritional immunity) drives the expression of the fungal zinc scavenging protein Pra1. Although the zincophore plays an important role in micronutrient scavenging for the fungus, innate immunity has evolved to recognize and target this protein. Because the expression of nutrient scavenging proteins is a hardwired response to limitation of their substrate, this is a phenomenon wherein nutritional immunity creates an environment that induces expression of a neutrophil chemoattractant by the pathogen.

Inflammation is often critical for the resolution of fungal infections, and the loss of *PRA1* by certain pathogenic species may represent an important step in their evolution. The *PRA1* gene emerged in the last common ancestor of the Dikarya (the major subkingdom of fungi) and, as such, is present in most sequenced fungi on the planet (*13*, *15*). Therefore, our observations also raise important questions on the impact of *PRA1* locus status on the evolution of human fungal pathogens in general. Outside the setting of VVC, neutrophils can play important roles in the control and killing of

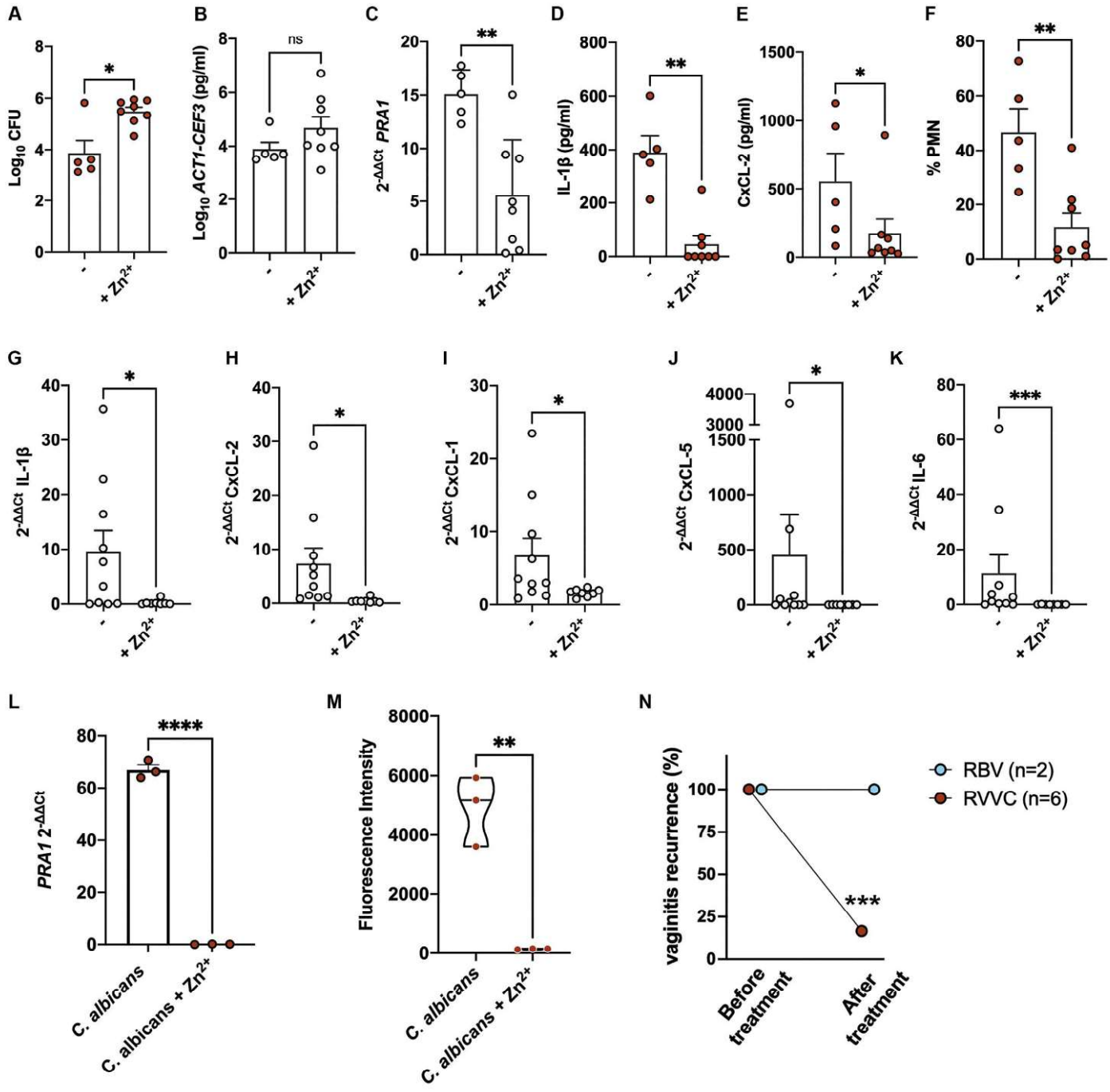

Fig. 5. Zinc down-regulates *PRA1* and prevents vaginal candidiasis. Mice were vaginally infected with *C. albicans* WT and administered with vehicle control or with zinc sulfate ($+Zn^{2+}$, 10 μl at 25 μM) at the time of infection and 8 hours after infection. After 24 hours, vaginal lavage fluid [red circles, (A) and (D) to (F)] or organs [gray circles, (B) and (C) and (G) to (K)] were collected. (**A** to **C**) Fungal burden was determined by measuring colony-forming units (A) or expression of housekeeping genes *ACT1* and *CEF3* (B); *PRA1* expression was evaluated by qRT-PCR (C). (**D** to **F**) Luminal cytokines [IL-1β and CxCL-2, (D) and (E)] and neutrophils (PMN, F) were determined by ELISA and flow cytometry, respectively. (**G** to **K**) Vaginal tissue–associated expression of proinflammatory cytokines IL-1β (G), CxCL-2 (H), CxCL-1 (I), CxCL-5 (J), and IL-6 (K) was measured by qRT-PCR. *C. albicans* clinical isolate SC5314 was incubated for 5 days in tissue culture medium with or without zinc supplementation and *PRA1* gene expression measured by qRT-PCR (**L**). Culture filtrates were added to the lower well and calcein-labeled human neutrophils to the upper well of the chemotaxis system, and neutrophil migration was assessed by measuring fluorescence at 485/530 nm in the lower compartment after 2-hour incubation (**M**). In a retrospective study, eight women who had been suffering from recurrent vaginal infections used a zinc (20 μM)–containing gel. Rates of reinfection for recurrent bacterial vaginitis (RBV, $n = 2$) and for recurrent vulvovaginal candidiasis (RVVC) were assessed after the 3-month study (**N**). Statistical tests: (A to K) Mann-Whitney test. (L and M) Unpaired *t* test. (N) Paired *t* test. Error bars = SEM. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; ****$P < 0.0001$.

fungal pathogens. The loss of the *PRA1* gene by extant pathogenic species such as *C. glabrata* may therefore have had interesting effects on their interaction with our innate immune system in other disease settings. Given the evolutionary distance between *C. albicans* and *C. glabrata*, differences in *PRA1* locus status, and the notable differences in disease pathology observed in this study, we suggest that vaginal yeast infections caused by *C. albicans* and *C. glabrata* be considered fundamentally distinct diseases.

VVC is characterized by nonprotective inflammation, and our observations provide evidence that vaginal inflammation is driven, at least in part, by Pra1. Our study is limited by the small number of participants, and a larger clinical trial would be needed to confirm these results; however, our findings could provide a simple means and a defined molecular mechanism to prevent VVC. Because *C. albicans* Pra1 expression can be effectively dampened by low concentrations of zinc, this study suggests a potentially beneficial use for zinc in preventing the immunopathology associated with VVC. This could be particularly useful in cases of recurrent VVC, which affects 138 million women and is currently difficult to treat.

MATERIALS AND METHODS

Study design

The objective of our study was to assess the contribution of the *C. albicans* Pra1 protein to vaginal inflammation. For this, we assessed clinical parameters of women colonized and infected with *Candida* as well as the efficacy of a zinc-containing gel on preventing recurrence of clinical vaginal candidiasis. We also used genetically manipulated *Candida* strains, gene expression, and tissue culture and ex vivo and in vivo infection models to understand the mechanism by which *C. albicans* causes inflammation in vaginal tissue and how zinc treatment prevents this inflammation.

Each in vitro and ex vivo experiment was performed in at least triplicate. A pilot study of mice was conducted to reduce the number of animals used, estimate variability among animals, and evaluate procedures and their effects. Mouse experiments were not blinded until sample collection. All in vitro and ex vivo experiments and sample measurements were blinded. The number of samples and the number of experimental replicates for each experiment are reported in figure captions. The minimum number of human samples collected was decided during the writing of the project and before ethical approval. All data are included in each figure, including outliers.

Ethics statement

All methods were performed in accordance with relevant guidelines and regulations. For ex vivo chemotaxis with human neutrophils, blood samples were donated from healthy volunteers recruited from the Medical Research Council (MRC) Centre for Medical Mycology at the University of Exeter (Exeter, UK). All participants provided written informed consent and donated a maximum of 100 ml of blood. This project was reviewed and approved by the University of Exeter College of Life and Environmental Sciences (CLES)–Biosciences Research Ethics Committee (UECLES-Biosciences REC reference no.: eCLESBio000371 V5).

All women recruited from a hospital in the Umbria region (Italy) and from a hospital in the Friuli Venezia Giulia region (Italy) signed informed consent in accordance with the Declaration of Helsinki. Local Ethical Committee CEAS (Comitato Etico delle Aziende Sanitarie, Umbria, Italy) approval was received for the Umbria region study (VAG1 n. 2652/15) on 1 February 2016. Local Ethical Committee CEUR (comitato etico unico regionale, Friuli Venezia Giulia, Italy) approval was received for the Friuli Venezia Giulia region study (RC 13/18-2715) on 1 June 2018. The zinc gel study was approved by the University of Debrecen Regional and Institutional Committee of Science and Research Ethics (approval no. 5912-2021), and all enrolled patients signed informed consent.

CD1 female mice were provided from Charles River UK and maintained in the specific-pathogen–free facilities of the University of Exeter. All animals were acclimatized for at least 1 week before starting experiments and were randomly assigned to experimental groups. Experiments were not blinded. All animal use was approved and in compliance with local university animal research ethical regulations and a UK Home Office project license (P6A6F95B5). The physical condition of the animals was checked at least once daily until the end of each experiment.

Vaginal human sample collection

The data reported here are from two different hospitals and were collected during two different periods.

Group 1: The first group of women enrolled in this study attended a hospital in the Umbria region (Italy) between 2016 and 2017. The group consisted of 36 nonpregnant, nondiabetic women, aged 21 to 40 years. These samples were used for neutrophil influx evaluation. Seven of them were also used to assess host- and pathogen-related parameters, as detailed below. Twelve women were negative for *Candida* isolation, 12 women were positive for *C. albicans* isolation, and 12 women were positive for *C. glabrata* isolation from a vaginal swab. Vaginal pH was measured directly from the vaginal swab by pH-Fix strips (Macherey-Nagel GmbH & Co.) and was in the range of 3.5 to 4.0 for noncolonized women and in the range of 4.0 to 5.0 for *Candida*-colonized women. No individuals presented any other vaginal coinfection (bacterial vaginosis or vaginitis). A vaginal swab was obtained from each participant, soaked in 1 ml of saline, and the presence of *Candida* was evaluated by plating the swabs on CHROMagar*Candida* (VWR International PBI) and performing a matrix-assisted laser desorption/ionization–time-of-flight test (Biomérieux S.A.). One hundred microliters was examined under a light microscope (Olympus) to evaluate the presence of neutrophils (PMN) on the basis of morphology. The number of polymorphonuclear (PMN) cells was counted in four fields at ×40 magnification and expressed as average number of PMN per field.

Group 2: The second group of women enrolled at a Hospital in the Friuli Venezia Giulia region (Italy) consisted of 22 nonpregnant, nondiabetic women, aged 18 to 50 years, between June and September 2021. Ten women were negative for *Candida* isolation, and 12 women were positive for *C. albicans* isolation and molecular identification. At enrollment, the women referred the eventual presence of symptoms (itching, burning, or abnormal discharge), and at gynecological examination, clinical findings (abnormal vaginal discharge or erythema) of vulvovaginal infection were evaluated. Two vaginal swabs were obtained from each participant and soaked in 1 ml of saline. Immediately after, one sample was stained with hematoxylin and eosin to analyze the vaginal microbiota, neutrophil influx, *Candida* colonization, *Candida* morphology, presence or absence of lactobacilli, and presence or absence of any other types of bacteria. Using these parameters, a Nugent score

was assigned to each sample to evaluate the grade of vaginal dysmicrobism. The second swab was used to perform both *Candida* isolation on CHROMagar *Candida* (VWR International PBI) and DNA extraction for a multiplex PCR analysis to confirm *Candida* species (Allplex Candidiasis Assay Seegene) and the presence of other bacteria responsible for vaginitis, vaginosis (Allplex bacterial vaginosis/vaginitis assay, Seegen), or any other sexually transmitted infections (Neoplex 7 STI, Genematrix). All women involved in the current study had no coinfections other than *C. albicans*.

Vaginal swabs obtained from these 29 participants (7 from the first hospital in Umbria and 22 from the second in Friuli Venezia Giulia) were centrifuged, and the supernatant was used to quantify IL-1β, IL-8, and calprotectin concentrations as described in the Supplementary Materials. Cell pellets were used to quantify the expression of *PRA1*, *ECE1*, *SAP6*, and *HWP1*. *ACT1* and *CEF3* were used as housekeeping genes and also to approximate the fungal burden for each woman. RNA extraction and gene expression were performed as reported above. Briefly, *Candida* RNA was extracted, treated with deoxyribonuclease and quantified, and 500 ng of RNA from each sample was converted to cDNA. Two different strategies were tried to evaluate *C. albicans* gene expression from the whole human vaginal swab. First, cDNA was immediately checked via qRT-PCR, but housekeeping genes were not immediately detected because most of the cDNA in each sample was from the host. For this reason, the cDNA was preamplified for 15 cycles in a normal PCR reaction using the AmpliTaq Gold DNA Polymerase Kit (Thermo Fisher Scientific) before performing real-time quantitative PCR using the same amount of sample for all the genes tested. All *Candida* primers used are reported in table S4.

Zinc-containing vaginal hydrogel pilot study

A retrospective cohort study was performed with the enrollment of 10 women with a history of recurrent vaginal infections at the outpatient clinic of the Department of Obstetrics and Gynecology, University of Debrecen, Hungary. Recurrent vaginal infection included RBV and recurrent vulvovaginal candidiasis (RVVC). Enrollment criteria included the presence of RBV or RVVC, and recurrent vaginal infection was defined as three or more vaginal infections in the last 12 months [American College of Obstetricians and Gynecologists (ACOG) Practice Bulletin]. Exclusion criteria were a postmenopausal state, defined as individuals who had at least 12 consecutive months of amenorrhea without any other obvious reason or who had consistently elevated follicle-stimulating hormone blood concentrations of 30 mIU/ml or higher; local or systemic hormone therapy within the past 6 months; cytological atypia; prior radiation treatment; a history of breast, ovarian, or other gynecological cancer; pelvic organ prolapse >stage 2; or recent use (3 months) of any vaginal product or douching. Women were asked to use a commercially available zinc-containing vaginal hydrogel (JUVIA vaginal gel, Fempharma LLC) as prophylactic treatment. The key gel ingredients are water, hydroxyethyl cellulose, 20 μM zinc sulfate, and lactic acid. The hydrogel was self-applied intravaginally via a vaginal applicator. Participants were asked to place 2 ml of gel into the vagina nightly for two consecutive weeks and, after that, twice per week. Women were asked to return to the clinic if any symptoms of vaginal infection were present for evaluation. Patients underwent a detailed gynecological exam at enrollment and the follow-up visit. At the first visit, general gynecological and medical history was taken, including age, body mass index, previous pregnancies, deliveries or operations, menstruation cycle, hormonal therapy, current relationship status, sexual partners in the last 12 months, and episodes of vaginitis over the previous 12 months. Demographic and pertinent clinical information was recorded prospectively and stored in a dedicated database.

Statistical analysis

Before performing statistical analyses, GraphPad Prism 9.0 software was used to test the normal distribution of each dataset. On the basis of the distribution (normal or not normally distributed) the appropriate statistical test was chosen and performed as described in each figure caption. GraphPad Prism 9.0 software was used to perform most statistical analyses apart from the zinc gel study in Fig. 5N, where Microsoft Excel 2019 was used. Statistical tests and P values are described in the manuscript and figure legends. $P < 0.05$ was considered significant.

Supplementary Materials

This PDF file includes:

Materials and Methods
Figs. S1 to S9
Tables S1 to S5
References (*13*, *24*–*31*)

Other Supplementary Material for this manuscript includes the following:

Data file S1
MDAR Reproducibility Checklist

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