Pathogens and Disease, 75, 2017, ftx096

doi: 10.1093/femspd/ftx096
Advance Access Publication Date: 30 August 2017
Research Article

RESEARCH ARTICLE

Contribution of TLR2 pathway in the pathogenesis of vulvovaginal candidiasis

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One sentence summary: Lack of TLR2 immune receptor signaling predisposes mice to increased fungal burden and massive fungal invasion of vaginal tract. This fact promotes an exacerbated expression of innate mediators that contribute to immunopathogenesis of vulvovaginal candidiasis.

Editor: Christophe d'Enfert

ABSTRACT

Candida albicans is the prevalent etiological agent in acute vulvovaginal infection and the most severe chronic condition known as recurrent vulvovaginal candidiasis (VVC). A critical role of local innate immunity in defense and pathogenesis of vaginal infection by *Candida* is proposed. The fungal recognition by the innate immune receptor is an essential step for the induction of local responses including cytokines and antimicrobial peptides (AMPs) production for host protection. Using TLR2-deficient mice, we characterized the early innate immune response during VVC. Intravaginal challenge of TLR2$^{-/-}$ mice with *C. albicans* demonstrated that in response to the initial massive penetration, a strong local inflammatory reaction with recruitment of polymorphonuclear neutrophils was developed. Both interleukin 1β (IL1β)—regarded as the hallmark of VVC immunopathogenesis—and IL6 were increased in vaginal lavage. Murine beta defensin 1 (mBD1), a constitutive AMP with fungicidal and chemotactic activity, was significantly upregulated in wild type (WT) animals in response to infection. Interestingly, in the absence of TLR2 recognition, levels of mBD1 RNA more than twice higher than those in WT infected animals were observed. Interestingly, our results demonstrate that TLR2 signaling is important to control the fungal burden in the vaginal tract. These finding provide new evidence about the role of this innate receptor during VVC.

Keywords: vulvovaginal candidiasis; TLR2; cytokines; antimicrobial peptides; beta defensin 1

Received: 24 April 2017; **Accepted:** 29 July 2017

INTRODUCTION

Vaginal infection by *Candida* species, known as vulvovaginal candidiasis (VVC) is an acute inflammatory disease that affects 75% of women in reproductive age at least once in their lives. Besides, a significant percentage between 6% and 8%, experience recurrent episodes of VVC (RVVC) (Sobel 1985, 1993, 2007; Cassone 2015). This distressing condition is characterized by at least four episodes of VVC per year (Sobel 2015). A recent study, reported by Foxman *et al*., estimated that RVVC affects approximately 138 million women worldwide annually (Foxman *et al*. 2013; Sobel 2015). Different predisposing risk factors such as pregnancy, oral contraceptives, diabetes mellitus, long-term broad-spectrum antibiotic treatment or steroid and immunosuppressive therapies have been reported as responsible for increasing the susceptibility to VVC (Achkar and Fries 2010). Interestingly, a high percentage of RVVC is idiopathic, suggesting that genetic errors may be involved in the etiology of the mycosis in these patients. Despite the widespread prevalence of *Candida* vaginitis, little is known about the factors that determine which women will undergo the transition from sporadic VVC to RVVC (Cassone 2015; Sobel 2015).

Over the past few years, clinical and experimental evidences have revealed the critical role of local innate immunity in both defense and pathogenesis of vaginal infection by *Candida*. In the vaginal tract, epithelial cells provide the first barrier against the fungus and are actively involved both in the recognition of pathogenic phenotype of fungus and in the initiation of a local response through the release of antimicrobial peptides (AMPs), alarmins, chemokines and some cytokines (Moyes *et al*. 2010; Netea and Kullberg 2010; Pericolini *et al*. 2015; Yano *et al*. 2010). Among them, AMPs are a crucial component of the host defense at mucosal epithelia because of their ability to exert antimicrobial and anticandidacidal activity and chemotactic properties (Hans and Madaan Hans 2014). Pattern recognition receptors (PRRs) in innate immune cells sense molecular moieties of fungus and thereafter induce intracellular signals that stimulate the production of effector molecules (Becker *et al*. 2015). The secretion of proinflammatory cytokines, such as TNFα, interleukin 1β (IL1β) and IL6, are also involved in the activation and recruitment of other immune populations. This activated microenvironment promotes the migration of polymorphonuclear neutrophils (PMNs) to the lamina propria and the vaginal lumen (Peters *et al*. 2014b; Yano, Noverr and Fidel 2012). Failure to adequately control the different elements, such as loss of recognition by PRRs, increased fungal burden, and expression of hyphae-associated virulence factors, elicits inflammatory signaling, thus resulting in the continued stimulation and amplification of innate immune effectors, contributing to characteristic immunopathology of *Candida* vaginitis (De Luca *et al*. 2013; Peters *et al*. 2014a; Romani 2011).

Several genetic errors and polymorphisms predisposing to RVVC are related to PRRs or their molecular activation pathways, including single nucleotide polymorphisms (SNPs) in gene coding for *mannose-binding lectin*, *CLEC7A*, *CARD9*, *NLPR3* inflammasome complex, and, more recently reported, *TLR2* Pro631His polymorphism (Drummond and Lionakis 2016; Ferwerda 2009; Glocker *et al*. 2009; Jaeger *et al*. 2016, 2013; Rosentul *et al*. 2014). TLR2 is an important PRR for *Candida albicans* recognition, activating innate immune responses alone or in synergy with Dectin-1 (Becker *et al*. 2015). The contribution of TLR2 activation in the protection against this fungus has been evaluated in animal models and is controversial. TLR2-deficient mice were reported to have an increased susceptibility to disseminated candidiasis that might be attributed to a decreased secretion of TNFα and impaired recruitment of PMN (Bellocchio *et al*. 2004). Tessarolli *et al*. reported that in the absence of the TLR2 receptor, neutrophil recruitment, activation and survival decreased after peritoneal infection with *C. albicans* (Tessarolli *et al*. 2010). TLR2 has also been proposed in gut protection against colonization and endogenous invasion by *C. albicans* (Choteau *et al*. 2017; Prieto *et al*. 2016). In contrast, other authors have shown that TLR2$^{-/-}$ mice are more resistant to systemic candidiasis exhibiting increased chemotaxis and IFNγ secretion. Interestingly, the recruitment of monocytes was enhanced in TLR2$^{-/-}$ mice, promoting an increased candidacidal activity and clearance of fungus (Netea *et al*. 2004). On the other hand, working in a well-characterized model of *C. albicans* infection, we reported that signals triggered by TLR2 contributed not only to mount a robust and protective response in the liver of infected animals, but also participated in the induction of cell death promoting tissue damage and favoring the infection (Renna *et al*. 2006, 2012, 2015). TLR2 is widely expressed in the organism, including the female reproductive tract (Fazeli, Bruce and Anumba 2005; Hickey, Fahey and Wira 2013). At present, the contribution of this innate receptor in the local response during *C. albicans* vaginal infection has not been explored (Romani 2011; Peters *et al*. 2014b; Rosentul *et al*. 2014).

In the current study, we evaluated TLR2 role in murine VVC. We used mice with a selective deficiency of this receptor to explore the local changes in the vaginal tract through the evaluation of fungal burden, invasiveness, inflammatory response and cytokine profile during the course of the infection. Since at present the role of mouse β-defensin 1 (BD1), a constitutive AMP with anticandidacidal and chemoattractant activity, has been little explored in VVC, we report here new and interesting evidence about this molecule in wild-type (WT) and TLR2-deficient mice.

MATERIAL AND METHODS

Animals

Experimental groups, comprising 8–10-week-old female WT C57BL/6J mice, and knockout mice with targeted disruption of TLR2 (TLR2$^{-/-}$), were maintained under a standard light cycle (12 h light/dark) and were allowed free access to water and food. C57BL/6J mice were purchased from Faculty of Veterinary Science, National University of La Plata, Argentina and TLR2$^{-/-}$ mice on the C57BL/6 background were from The Jackson Laboratory, Bar Harbor, ME, USA.

All animal experiments were approved and conducted in accordance with the guidelines of the Animal Experimentation Ethics Committee, Faculty of Chemical Sciences, National University of Cordoba (Permit Number EUNC0045378/2010/RD939), in strict accordance with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance number A5802-01).

Candida albicans

Candida albicans ATCC 36801 was used in all experiments (Carvalho *et al*. 2012; Yano, Noverr and Fidel 2012; de Freitas Araujo *et al*. 2013). Yeast cells were kept on Sabouraud Dextrose Agar (SDA; Britania, Argentina) at room temperature (RT). *Candida*'s inoculum used in the experiments was standardized by counting 5 $\times$ 10^6 yeast in phosphate-buffered saline (PBS) on a

Neubauer hemocytometer. The number of viable yeast was checked on SDA.

Vaginal infection

WT and TLR2$^{-/-}$ mice were treated subcutaneously with 0.2 mg of β-estradiol 17-valerate (Sigma Chemical Co.) dissolved in 100 μl of sesame oil (Sigma) on days (D)-6 and -3 before vaginal infection on D0. On D2 and 4 post infection (pi) they received estradiol injections to maintain pseudoestrus. The estrogenized mice were inoculated intravaginally (i.v.) with 20 μl of *C. albicans* suspension (infected group) or PBS instead (uninfected group). Animals without any treatment were used as controls (unestrogenized group) (Yano and Fidel 2011).

Colony-forming units assay

The time course of infection was monitored in individual mice by culturing 100 μl of serially diluted (1:10) vaginal lavages on SDA. Vaginal lavages were conducted using 70 μl of sterile PBS with repeated aspiration and agitation. Colony-forming units (CFUs) were counted after incubation at RT for 48 h and expressed as CFU/mL of vaginal lavage. Quantitative counts of CFU in lavage fluids were evaluated (Yano and Fidel 2011; de Freitas Araujo *et al.* 2013).

Cytospin

To show the main cell populations present in vaginal lavage, samples were adjusted at a concentration of 5×10^4 cells/100 μL by counting on a Neubauer hemocytometer and were cytocentrifuged at 500 rpm for 5 min using the Shandon Elliot cytospin. Then, samples were stained with May-Grünwald Giemsa (MGG) and observed with a NIKON ECLIPSE microscope (Renna *et al.* 2015; De Luca *et al.* 2013). PMNs were identified by their morphology, staining appearance and characteristic trilobed nuclei. For each smear, PMNs were manually counted in five nonadjacent fields by standard light microscopy using a 40× objective. PMN counts were averaged per field. Values are reported as mean PMN counts per group ± standard errors of the means (SEM) (De Luca *et al.* 2013; Peters *et al.* 2014a; Yano *et al.* 2014; Bruno *et al.* 2015).

Histological analysis

For histology, the vaginas were removed and immediately fixed in 10% neutral buffered formalin (Biopack, Argentina) for 24 h. The vaginas were dehydrated, embedded in paraffin, sectioned into 3–4 μm and stained with periodic acid-Schiff reagent. Histology sections were observed using a NIKON ECLIPSE microscope (de Freitas Araujo *et al.* 2013).

Invasiveness and inflammation score

Five visual fields of quintile sections were examined at ×400 magnification. The extent of invasiveness and inflammation was evaluated by a modified method based on the report of Zhang *et al.* (2013), and the average scores of five fields were recorded in each section. Invasiveness extent was scored as Score 0, meaning no *Candida* in the epithelial layer; Score 1, 1–5 *Candida*; Score 2, 6–10 *Candida*; Score 3, 11–15 *Candida*; Score 4, 16–20 *Candida*; Score 5, 21–25 *Candida*; Score 6, 26–30 *Candida*; Score 7, 31–35 *Candida* and Score 8, >36 *Candida*. Inflammation extent was scored as Score 0 or absent, meaning no PMNs; Score 1 or mild, meaning 1–10 PMNs or 1–3 microabscesses in the epithelial layer and a few PMNs in the submucosa; Score 2 or moderate, meaning 11–20 PMNs or 4–6 microabscesses in the epithelial layer and some PMNs in the submucosa; and Score 3 or severe, meaning >20 neutrophils or >6 microabscesses or large abscess formation in the epithelial layer and numerous PMNs in the submucosa.

Immunohistochemical analysis

Immunohistochemical staining was performed as described previously (Garcia *et al.* 2015). The sections were briefly incubated overnight, after being blocked, at 4°C with an antibody-recognizing BD1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with bound antibodies being detected using an anti-rabbit biotin-labeled antibody (Vector Laboratories, Burlingame, CA, USA) in 1% PBS/BSA. The sections were then incubated with ABC complex (VECTASTAIN Vector Labs, Southfield, MI, USA). Diaminobenzidine (Sigma-Aldrich) was used as a chromogen substrate. Histology sections were observed by using a NIKON ECLIPSE microscope.

Cytokine immunoassays

The level of cytokines in the vaginal lavage was quantified using TNFα, IL6, IL1β (BD OptEIA) and TGFβ (BD reagents) ELISA tests as described elsewhere (Renna *et al.* 2012). Absorbance was measured using BIO-RAD microplate reader, and concentrations extrapolated from standard curves. Data were expressed as picogram per milliliter.

Real-time PCR

Real-time PCR was used to evaluate the levels of mRNA BD (Morampudi, Braun and D'Souza 2011; Bauer *et al.* 2012; Erhart *et al.* 2011) and CXCL1 (Ermann *et al.* 2014) using the StepOne System (Life technologies) and SYBR® Select Master Mix (Life Technologies). Vaginas were lysed and total RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit (Sigma), and then reverse transcribed with the Reverse Transcriptase M-MLV (Promega), according to the manufacturer's directions. The PCR primers used were: murine beta defensin 1 (mBD1) Fw 5, AGGTGTTGGCATTCTCACAAG, Rev 5, GCTTATCTGGTTTACAGGTTCCC; CXCL1 Fw 5, CTGGGATTCACCTCAAGAACATC, Rev 5, CAGGGTCAAGGCAAGCCTC and Rplp0 Fw 5, GGGCATCACCACGAAAATCTC, Rev 5, CTGCCGTTGTCAAACACCT, as housekeeping gene. The PCR programme used was: 95°C 15 s, 60°C 30 s, 72°C 30 s, 40 cycles.

Statistical analysis

Data were expressed as means ± SEM. Differences between group means or ratios were assessed using one-way or two-way ANOVA followed by Bonferroni test for multiple comparisons. A P value < 0.05 was considered statistically significant. GraphPad Prism version 5.0 for Windows (GraphPad Software, USA) was employed. All experiments were repeated at least twice to test the reproducibility of results.

RESULTS

Susceptibility to vaginal infection by *Candida albicans* in WT and TLR2$^{-/-}$ animals

In order to characterize the role of TLR2 in murine VVC, we intravaginally infected C57BL/6 (WT) and TLR2$^{-/-}$ mice with *Candida albicans* blastospores and evaluated the pattern of susceptibility and/or resistance to infection in terms of local fungal burden, morphotype presence in vaginal lavage and fungal

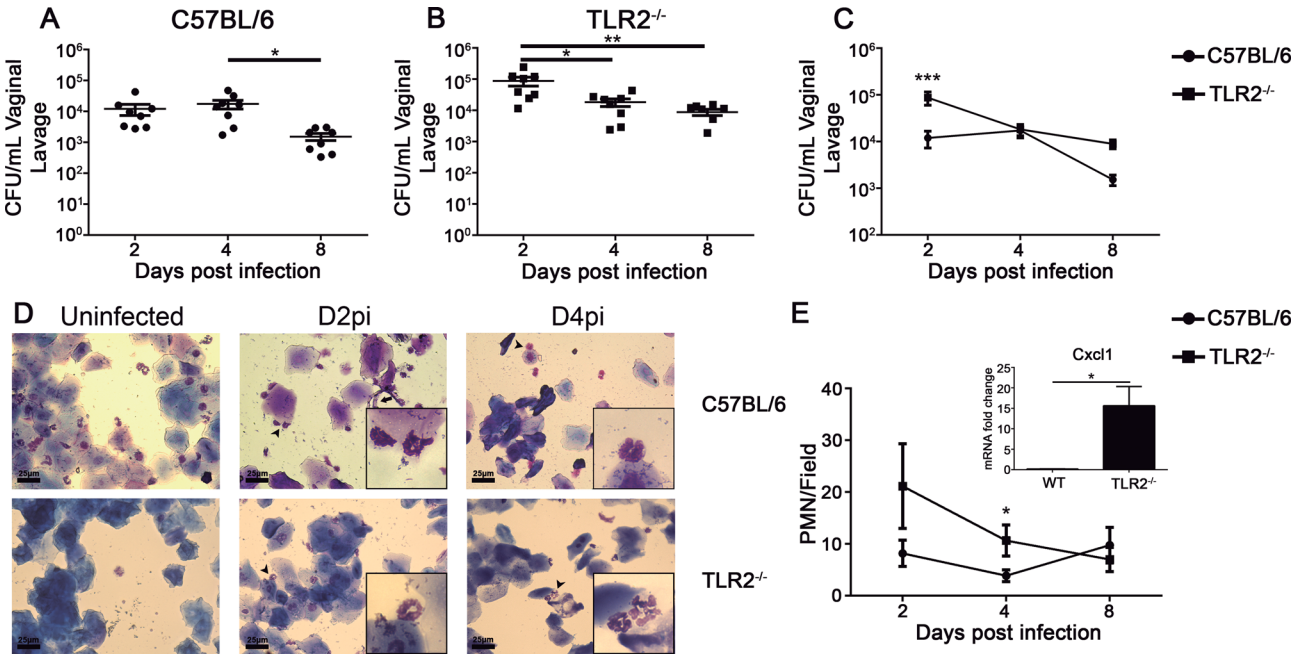

Figure 1. Susceptibility to vaginal infection by *C. albicans* in WT and TLR2$^{-/-}$ animals. **(A)** Fungal growth (CFU ± SEM) at D2, 4 and 8 pi in vaginal lavage of C57BL/6 or **(B)** TLR2$^{-/-}$ mice infected i.v. with 5 × 10^6 cells of *C. albicans* ATCC 36801. Each point represents an individual mouse. Data are representative of three independent experiments. **(C)** Fungal growth (CFU ± SEM) at D2, 4 and 8 pi in vaginal lavage of C57BL/6 (circles) or TLR2$^{-/-}$ (squares) mice infected i.v. with 5 × 10^6 cells of *C. albicans* ATCC 36801. n ≥ 7. **(D)** Cytospin preparations of cellular fractions in vaginal lavage from uninfected or infected i.v. with 5 × 10^6 cells of *C. albicans* ATCC 36801 C57BL/6 (up) or TLR2$^{-/-}$ (down) mice at D2 or D4 pi. Cytospins were MGG-stained. Images are shown at ×400 and ×1000 magnification in the insets. **(E)** PMNs in cytospin preparations of cellular fraction in vaginal lavage from infected i.v. with 5×10^6 cells of *C. albicans* ATCC 36801 C57BL/6 (circles) or TLR2$^{-/-}$ (squares) mice at D2, 4 and 8 pi. Vaginal PMNs were quantified in 10 high-powered fields (×400 magnification) per mouse, and values were averaged. n ≥ 7. Cxcl1 mRNA expression of infected mice at D4 pi was quantified by real-time PCR. The expression was normalized to that of the Rplp0 gene and expressed as the fold increase over expression in uninfected mice. n ≥ 4. Results are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

clearance. Animals were estrogen-treated and inoculated with 5 × 10^6 yeast (ATTC 36801) (de Freitas Araujo *et al.* 2013), and on D2, 4 and 8 pi vaginal lavages were obtained. Microscopic analysis of cells recovered from the vaginal cavity from all mice groups showed abundant keratinized epithelial cells. Early after the infection (D2 and D4), PMNs recruited into vaginal lumen were observed in both WT and TLR2$^{-/-}$ infected animals' smears, while in uninfected mice few to no PMNs were detected (Fig. 1D). The PMNs showed conserved nuclear morphology (inset Fig. 1D). PMN recruitment significantly increased in TLR2$^{-/-}$ mice when compared with WT animals at D4 of infection ($P < 0.05$) (Fig. 1E). In agreement with this result, the levels of neutrophil chemoattractant, CXCL1, also exhibited an upregulated expression in TLR2$^{-/-}$ mice ($P < 0.05$) (inset Fig. 1E). Hyphae of *C. albicans* were visualized in fresh and MGG stains in both groups of infected animals, evidencing the presence of fungal growth and invasive morphotype.

WT and TLR2$^{-/-}$ mice were robustly colonized by *C. albicans* and the infection remained along the evaluated period in both strains, but the course of the mycosis was not equal among different mice backgrounds. WT infected animals showed similar colonization on D2 and D4 pi, with a marked and significant decrease of the fungal burden at D8 pi ($P < 0.05$) (Fig. 1A). On the other hand, TLR2$^{-/-}$ mice exhibited the maximum colonization at D2, and a progressive decrease in CFUs number throughout the study was observed ($P < 0.05$) (Fig. 1B). The comparative analysis of *C. albicans* vaginal infection between both mouse strains revealed that, early in the infection (D2), the fungal burden in the absence of TLR2 receptor was significantly higher than in WT mice ($P < 0.05$). At the other two time points evaluated (D4 and D8), no significant changes in the fungal growth between groups could be detected. Similar results were obtained when animals were inoculated with minor infective doses of fungus (5 × 10^5 yeast, data not shown). The relative fungal clearance between D2 and the last day of study (D8) in both strains of mice was also calculated, and similar results were obtained. While WT animals removed about 87% of fungal burden, TLR2$^{-/-}$ mice exhibited a clearance of 92%.

Fungal invasion and inflammatory response in WT and TLR2$^{-/-}$ animals during VVC

CFUs quantification from vaginal lavage is the standard technique for assessment of vaginal fungal burden, but it does not reflect the epithelial invasion by *C. albicans*, which is the fact that finally leads to cell damage and the activation of host innate immune response. In order to explore the outcome of infection in WT and TLR2$^{-/-}$ mice, we performed histological studies and determined both invasiveness and inflammatory score during the course of VVC.

The histological study in both groups of uninfected animals, showed an intact mucosa and absence of inflammatory infiltrate (Fig. 2A). At D2 pi, tissue architecture was maintained and numerous pseudohyphae and blastoconidia adhered to the stratified epithelium were visualized in WT infected animals, while in TLR2$^{-/-}$ infected mice a significant invasion of the vaginal epithelium by *C. albicans* was observed. A massive presence of invasive fungal morphotype penetrated the epithelium and reached deeper layers; also in the vaginal lumen, large keratin debris was observed. The invasiveness score in TLR2$^{-/-}$ infected mice was higher than WT and significant differences between groups

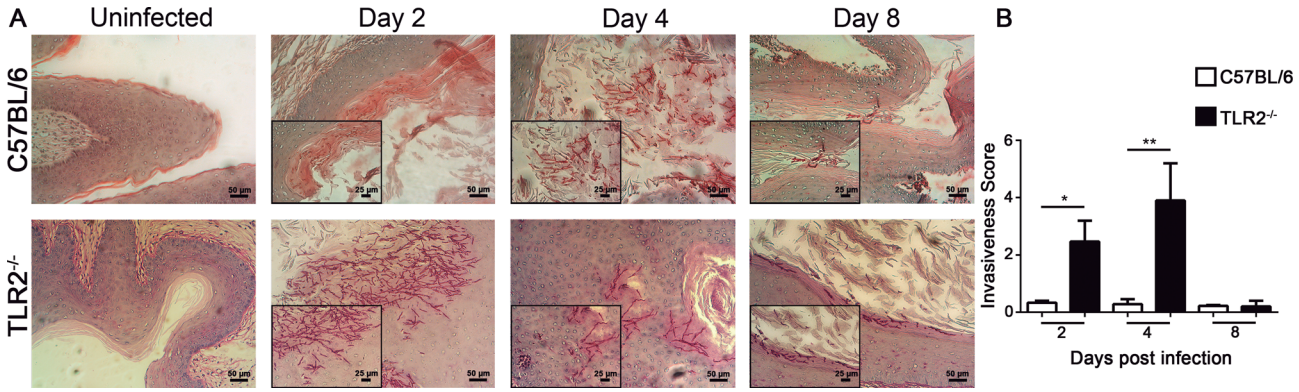

Figure 2. Fungal vaginal invasion in WT and TLR2$^{-/-}$ animals during VVC. **(A)** Periodic acid-Schiff-stained vaginal sections from uninfected or infected at D2, 4 and 8 pi of C57BL/6 (up) or TLR2$^{-/-}$ (down) mice i.v. inoculated with 5×10^6 cells of *C. albicans* ATCC 36801, showing the presence of the infective form of the pathogen. Images are shown at ×200 and ×400 magnifications in the insets. Representative images from three experiments are shown. **(B)** Invasiveness score of C57BL/6 and TLR2$^{-/-}$ histological slides. Five visual fields were examined at ×400 magnification. The extent of infection was evaluated by a modified method based on the report of Zhang *et al.* (2013), and the average scores of five fields were recorded in each section. Invasiveness extent was scored as Score 0, meaning no *Candida* in the epithelial layer; Score 1, 1–5 *Candida*; Score 2, 6–10 *Candida*; Score 3, 11–15 *Candida*; Score 4, 16–20 *Candida*; Score 5, 21–25 *Candida*; Score 6, 26–30 *Candida*; Score 7, 31–35 *Candida* and Score 8, >36 *Candida*. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

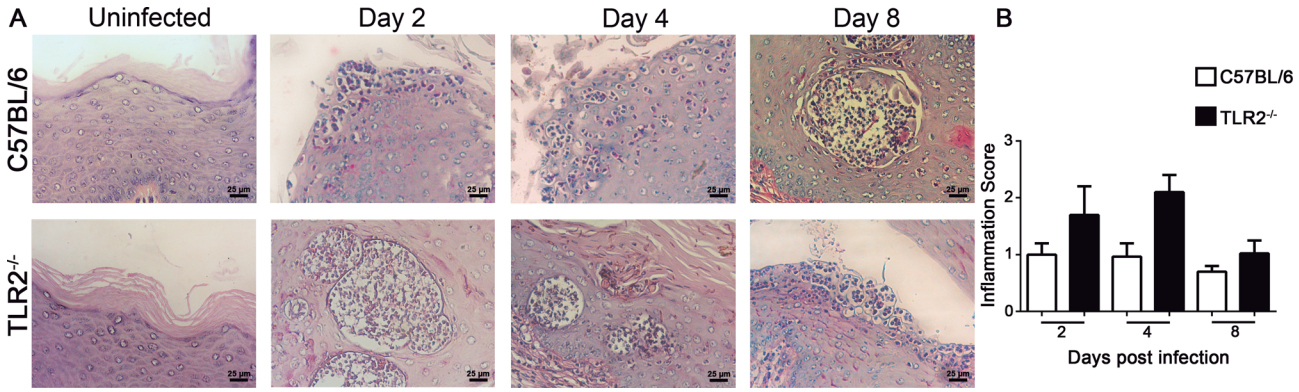

Figure 3. Inflammatory response in WT and TLR2$^{-/-}$ animals during VVC. **(A)** Periodic acid-Schiff-stained vaginal sections from infected at D2, 4 and 8 pi of C57BL/6 (up) or TLR2$^{-/-}$ (down) mice infected i.v. with 5×10^6 cells of *C. albicans* ATCC 36801, showing the presence of inflammatory infiltrating cells in the tissue. Images are shown at ×400 magnification. Representative images from three experiments are shown. **(B)** Inflammation score of C57BL/6 and TLR2$^{-/-}$ histological slides. Five visual fields were examined at ×400 magnification. Inflammation extent was scored as Score 0 or absent, meaning no PMNs; Score 1 or mild, meaning 1–10 PMNs neutrophils or 1–3 microabscesses in the epithelial layer and a few PMNs neutrophils in the submucosa; Score 2 or moderate, meaning 11–20 PMNs or 4–6 microabscesses in the epithelial layer and some PMNs in the submucosa; and Score 3 or severe, meaning >20 PMNs or >6 microabscesses or large abscess formation in the epithelial layer and numerous PMNs in the submucosa.

were obtained ($P < 0.05$) (Fig. 2B). At D4, in WT infected animals, abundant hyphae of *C. albicans* were present in profuse desquamated vaginal cells, and some fungi were identified in the epithelium. In TLR2$^{-/-}$ infected mice, many hyphae were detected in deeper epithelial layers, and the invasiveness score reached the highest value, with differences compared with WT infected animals ($P < 0.01$). At the end of the study, while WT animals showed a few fungal forms in the cornified epithelium, in TLR2$^{-/-}$ mice, frequent *C. albicans* hyphae were observed. At this time (D8) the invasiveness score was similar in both groups.

The histological analysis performed in WT animals on D2 pi evidenced PMNs infiltration and characteristic leukocyte microabscesses. A progressive increase in the number of inflammatory infiltrate and subcorneal microabscesses were observed at D4 pi. At the end of the study, large abscesses around *C. albicans* hyphae could be visualized, disperse in the epithelia (Fig. 3A). The inflammatory score in WT infected animals (1.0 ± 0.3) did not show significant changes during the course of vaginal infection (Fig. 3B). Interestingly, the analysis of tissue reaction in TLR2$^{-/-}$ infected animals showed a strong activation of inflammatory response with large abscesses and numerous PMNs in the epithelial layer at D2. The inflammatory score at D2 and D4 reached values between 1.7 ± 0.6 and 2.1 ± 0.4. Subcorneal microabscesses and PMNs infiltration were observed at the end of the study.

Taken together, these results reveal that, in response to massive and deep mucosal invasion by *C. albicans*, TLR2$^{-/-}$ animals were able to orchestrate a robust tissue reaction.

Local cytokine profiles in WT and TLR2$^{-/-}$ animals during VVC

The different findings during VVC in WT and TLR2-deficient mice suggest that distinct cytokine profiles may be developed in the vaginal microenvironment, following TLR2 engagement. We measured the local production of proinflammatory cytokines IL1β, TNFα and IL6, and anti-inflammatory cytokine TGFβ that has a crucial role in the vaginal homeostasis. We found that IL1β levels were higher in TLR2$^{-/-}$ infected mice at D4 and D8

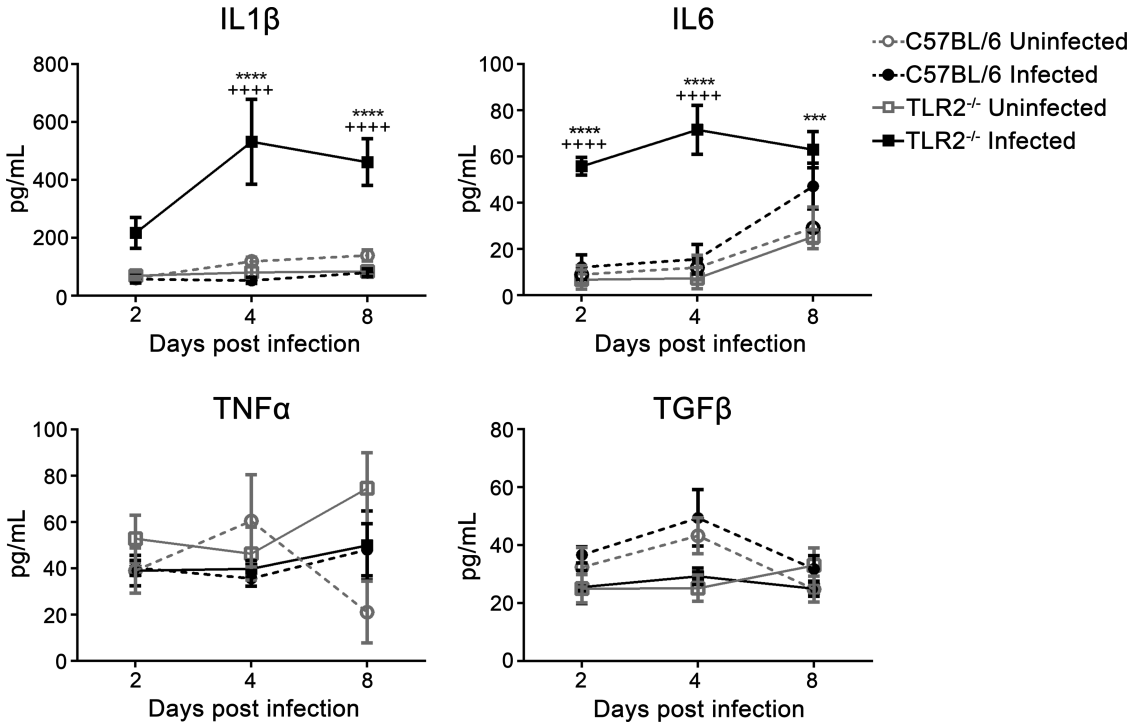

Figure 4. Local cytokine profiles in WT and TLR2$^{-/-}$ animals during VVC. Evaluation of IL1β, IL6, TNFα and TGFβ concentration by ELISA test on supernatants of vaginal lavage obtained from uninfected C57BL/6 (grey circles, dotted line) or TLR2$^{-/-}$ (grey squares) and infected at D2, 4 and 8 pi of C57BL/6 (black circles, dotted line) or TLR2$^{-/-}$ (black squares) mice i.v. inoculated with 5×10^6 cells of *C. albicans* ATCC 36801. Results are expressed as mean ± SEM (n = 8 mice, 4 mice for each of two independent experiments). $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, $^{++++}P < 0.0001$ (C57BL/6 infected mice vs TLR2$^{-/-}$ infected mice); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (TLR2$^{-/-}$ infected mice vs TLR2$^{-/-}$ uninfected mice).

pi compared to both, TLR2$^{-/-}$ estrogenized animals and WT infected mice ($P < 0.001$) (Fig. 4). *Candida albicans* was unable to stimulate significant release of IL1β in the vaginal lumen in WT mice. Interestingly at D2 pi, IL6 concentration reached significant values in the vaginal lavage of TLR2$^{-/-}$ infected mice and remained high during the course of infection, showing significant differences with TLR2$^{-/-}$ uninfected animals and WT infected mice ($P < 0.001$). No significant changes between groups were observed when the levels of TNFα in vaginal lumen were compared. The anti-inflammatory cytokine TGFβ did not exhibit significant variation during the course of vaginal infection (Fig. 4).

BD1 production in WT and TLR2$^{-/-}$ after vaginal infection with *Candida albicans*

Antimicrobial peptides like beta-defensins (BDs) are a crucial component of the host defenses at the epithelial mucosa. BDs exert antimicrobial and chemoattractant activities. Human BD1 (mouse BD1-homologous) is constitutively expressed in normal tissue (Suarez-Carmona *et al.* 2015). To test the ability of *C. albicans* to modulate local expression of mBD1, we evaluated in WT and TLR2$^{-/-}$ infected mice the levels of mBD1 transcripts by qPCR in vaginal homogenates and protein expression by immunohistochemistry (Fig. 5). In accordance with the early activity of this AMP, and our previous kinetic studies, *C. albicans* was able to upregulate the expression of constitutive mBD1 mRNA in the vaginal tract of WT infected animals compared with WT unestrogenized ($P < 0.05$) at D2 pi. Immunohistological analysis of vaginal tissues showed a basal expression of mBD1 in unestrogenized animals, a low stain in uninfected mice and a clear and positive immunostaining in epithelial cells, infiltrating PMNs and in corneal microabscesses in infected animals (Fig. 5B). Interestingly, in absence of TLR2 signals, the level of mBD1 mRNA was significantly upregulated when compared with TLR2$^{-/-}$ unestrogenized animals ($P < 0.05$). In this group, mBD1 also exhibited a constitutive expression in the vaginal tract and estrogen treatment produced a small increase. After *C. albicans* infection an upregulated expression of mBD1 was observed both in the mucosa and infiltrating PMNs.

Interestingly, the levels of mBD1 transcripts were higher in TLR2-deficient mice infected with the fungus, evidencing that TLR2 engagement is not required for the local production of this important AMP. Additionally, this result illustrates the ability of TLR2$^{-/-}$ animals to upregulate an early mediator involved in recruitment and amplification of local inflammation during *Candida* vaginitis.

DISCUSSION

VVC and RVVC are two forms of diseases caused by *Candida albicans* affecting a high number of women in reproductive age. Over the past few years, this ancient disease has experimented a paradigm shift in its pathogenesis, based on experimental and clinical evidence (Peters *et al.* 2014b; Sobel 2015). Different studies reveal that *Candida* vaginitis results from an immunopathological response governed by host innate immunity, where robust PMN recruitment and production of inflammatory effectors are actively involved (Yano *et al.* 2010, 2014; Peters *et al.* 2014a). While some degree of inflammation is required for protection against *C. albicans* invasion, progressive inflammation worsens the disease and ultimately prevents pathogen eradication (Zelante *et al.* 2009; Romani 2011). In spite of a lot of effort, an

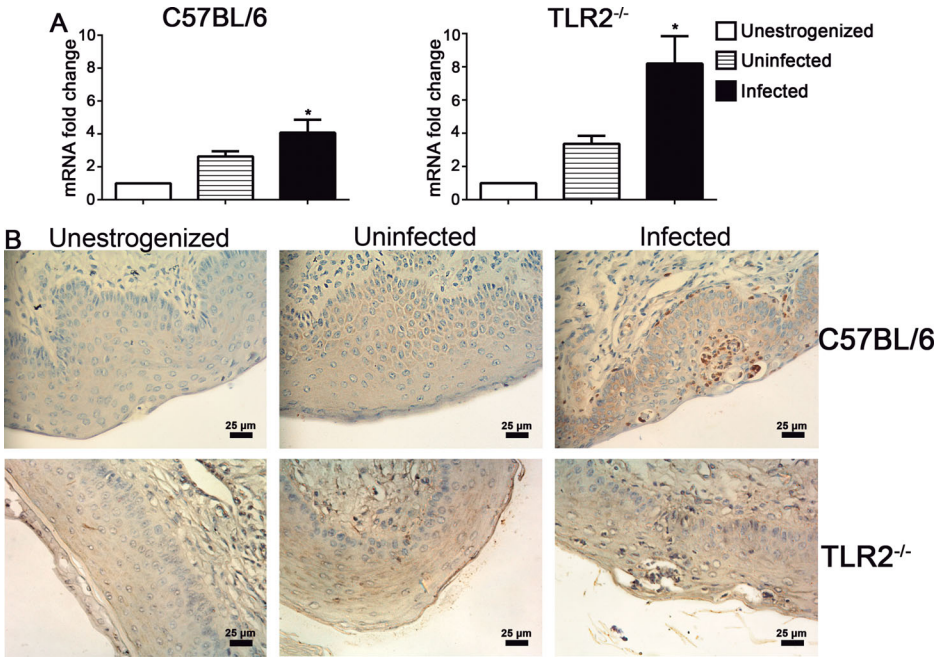

Figure 5. BD1 production in WT and TLR2$^{-/-}$ after vaginal infection with *C. albicans*. **(A)** mBD1 mRNA expression by real-time PCR in vaginal tissue from unestrogenized, uninfected or infected at D2 pi of C57BL/6 or TLR2$^{-/-}$ mice i.v. inoculated with 5×10^6 cells of *C. albicans* ATCC 36801. *$P < 0.05$ (infected vs unestrogenized mice). **(B)** Vaginal tissue sections from unestrogenized, uninfected or infected at D2 pi of C57BL/6 (up) or TLR2$^{-/-}$ (down) mice i.v. inoculated with 5×10^6 cells of *C. albicans* ATCC 36801 were stained with anti-mBD1 antibody. Positive cells stained brown with diaminobenzidine against the blue hematoxylin counter staining. Images are shown at ×400 magnification.

explanatory mechanism involved in this mycosis pathogenesis has remained undefined. In this paper, we provide novel evidence about the contribution of the innate receptor TLR2 in the recognition and progression of local response during vaginal infection in an experimental model of VVC.

Our results show that the absence of TLR2-mediated signaling predisposes to an increased initial vaginal fungal burden (D2), followed by an efficient clearance but without total pathogen eradication, reaching similar values to WT animals at the end of the study. The evaluation of CFUs only offers a partial view of the local phenomenon. It is well known that yeast-to-hypha morphogenetic switch in *C. albicans* is the major virulence factor of this fungal pathogen, as hyphae formation is associated with an elevated secretion of hydrolytic enzymes, increased adherence to host surfaces and direct invasion of tissue layers (Mayer, Wilson and Hube 2013). In our study, the ability of *C. albicans* to invade and infect deeper epithelial layers can be evaluated by microscopic observation and assessed by invasiveness score throughout the course of VVC in both strains of animals. While in WT animals the fungus was adhered to the cornified epithelium, in TLR2$^{-/-}$ animals the epithelial barrier could easily be crossed by *C. albicans* hyphae and massive presence of invasive fungus morphotype reached deeper stratus (Fig. 2). Tissue damage and fungi presence are correlated with an increase in innate immune signaling during vaginitis. Recently, Peters *et al.*, through the intravaginal inoculation of mice with *C. albicans* strains deleted for transcriptional regulators controlling the yeast-to-hyphae switch, revealed a crucial role for morphogenetic signaling through the Efg1 and the Bcr1 pathways. They also demonstrated that the inflammatory responses observed during murine infection are dependent on fungal morphogenesis pathway, suggesting the association between morphogenetic changes in the pathogen and the activation of local response in the host (Peters *et al.* 2014a).

The results obtained demonstrate that TLR2 signaling is important to control the fungal burden in vaginal tract. In response to the initial massive penetration occurred during the early step of infection (D2 and D4 pi), a strong local inflammatory reaction characterized by the presence of large abscesses in the epithelium upregulated expression of CXCL1 neutrophil chemoattractant and increased recruitment of PMNs (D4) was observed in the absence of a TLR2 signal. In WT animals an efficient and complete fungal recognition prevented a deeper invasion and orchestrated a moderated and progressive tissue inflammatory response that successfully controlled the local infection. This indicate that the higher inflammatory response observed in TLR2$^{-/-}$ infected mice compared with WT animals was the consequence of an increased stimulation due to a higher fungal burden and tissue damage in these animals. In the vaginal microenvironment, constitutive and conserved levels of the cytokine TGFβ are involved in both the local tolerance and anti-inflammatory response, which preserve the homeostasis of genital tract (Wira *et al.* 2005). Interestingly, the *C. albicans* strain used in this work did not induce a significant production of inflammatory cytokines IL1β, TNFα and IL6 in WT mice; however, moderated levels of TGFβ were detected in the vaginal cavity. One possible explanation is that in this strictly regulated niche, the signal delivered by this strain was unable to reach the basal threshold needed to trigger the inflammasome activation and TNFα and IL6 pathways required to break the local tolerance (Drummond *et al.* 2014; van de Veerdonk, Joosten and Netea 2015). In connection with this, it has been reported that the cytokine production is fungal strain-dependent during systemic *Candida* infection (Marakalala *et al.* 2013) and at the vaginal level, the IL1β production is also associated with *C. albicans* strain (Peters *et al.* 2014a; Bruno *et al.* 2015). Conversely, in the absence of TLR2 recognition, intravaginal levels of IL6 were upregulated throughout the course of infection and the local production of IL1β reached high

values at D4 and D8 pi. The TLR2-signal was a crucial event in the control of fungal growth. In TLR2$^{-/-}$ mice the increased inflammatory response was directly generated in response to increased fungal burden and tissue damage through multiple host receptors involved in *C. albicans* recognition. Other members of the TLR family, C-type lectins and NOD receptors in this deficient host can sense the fungal presence and activate the local response, as previously reported in several models of *C. albicans* infection (De Luca *et al*. 2013; Drummond *et al*. 2014; Becker *et al*. 2015; Drummond and Lionakis 2016; Miro *et al*. 2016, 2017). Taken together, our results reflect that while the presence of TLR2 in WT animals could contribute to the protection of this mucosa tract controlling the fungal burden and preventing the massive fungal invasion, in their absence, *C. albicans* can penetrate the epithelia and promote the release of two key inflammatory mediators, IL-1β and IL-6, involved in PMNs recruitment, tissue reaction and amplification of local response.

The inflammasomes are intracellular receptors involved in IL1β and IL18 production. The essential role of the NLRP3 inflammasome during the course of *C. albicans* infection has been well documented (Hise *et al*. 2009; Latz, Xiao and Stutz 2013; van de Veerdonk, Joosten and Netea 2015). The hyphal form of *C. albicans* is associated with cellular damage and secretion of the major inflammasome effector IL1β during VVC (Peters *et al*. 2014a). Bruno *et al*. working with Nlrp3$^{-/-}$ mice intravaginally infected with *C. albicans*, demonstrated severely reduced levels of PMNs, alarmins and inflammatory cytokines, including IL1β in vaginal lavage fluid during VVC. They also showed that the local administration of WT mice with a potent NLRP3 inflammasome inhibitor (glyburide), reduced PMN infiltration and IL1β, down to levels similar to those observed in Nlrp3$^{-/-}$ mice, providing evidences of a direct link between NLRP3-signaling with IL1β secretion to *C. albicans* during vaginitis (Pietrella *et al*. 2013; Gabrielli *et al*. 2015). These authors and others agree that IL1β is a crucial mediator involved in VVC immunopathogenesis (Pietrella *et al*. 2013; Gabrielli *et al*. 2015).

In the mucosal surfaces, the release of inflammatory mediators from epithelial cells is a crucial event for the generation of protective host responses, including the production of direct AMPs as well as the recruitment of inflammatory cells. Members of this AMP superfamily, defensins, are small (4–6 kDa) cationic peptides with antimicrobial and chemotactic activity that direct immune effector cells to the site of infection (Machado and Ottolini 2015; Suarez-Carmona *et al*. 2015). Human BD1 is constitutively expressed in uninflamed normal tissue, such as urogenital, gastrointestinal and respiratory tracts, and considered the most important AMP in epithelial defense against infection (Liu *et al*. 1997; O'Neil *et al*. 2000; Zhu *et al*. 2003; Prado-Montes de Oca 2010). Several reports documented that insufficient hBD1 expression leads to poor innate immunity response against infectious microorganisms (Prado-Montes de Oca 2010). The homologue of hBD1 have been identified in mice as mBD1 (Bals, Goldman and Wilson 1998) and recently, direct fungicidal activity by recombinant mBD1 was reported (Wang *et al*. 2010). The role of this mBD has been explored during oral infection by *Candida*. Tomalka *et al*. recently reported that mBD1-deficient mice exhibited increased mucosal and systemic fungal burdens during the early stages of fungal infection. They also reported impaired upregulation of inflammatory cytokines and chemokines and a profound defect in PMN infiltration to mucosal sites of *Candida* infection in the mBD1$^{-/-}$ mice as compared with WT mice (Tomalka *et al*. 2015). While little is known about the expression of mBD1 during vaginal infection, the present work provides evidence of mRNA and protein increase in epithelial cells and PMNs recruited to the vaginal tract during acute fungal infection. In the vaginal tract, the basal expression of these molecules can be detected in both strain of animals, and, in agreement with reports (Hickey, Fahey and Wira 2013). The estrogen treatment induces a low increase of mBD1. More interestingly, in TLR2-deficient mice, the exacerbated fungal burden and massive hyphae mucosal invasion (D2), triggered the local transcription of levels of mBD1 RNA, more than twice as high as those observed in WT infected animals. To our knowledge, this is the first report evidencing the upregulated expression of this constitutive AMP in the absence of TLR2 engagement.

It is well established that the mechanism involved in the protection against the fungus is different during systemic or mucosal infection (Drummond *et al*. 2014; Miro *et al*. 2016, 2017). Recently Rosentul *et al*. reported that the TLR2 Pro631His polymorphism was associated with an almost three-fold increase in susceptibility to RVVC. Only two individuals bearing the mutation exhibited a decreased production of IFNγ and IL17 when peripheral blood mononuclear cells were exposed to heat-killed *C. albicans* (Rosentul *et al*. 2014). The authors indicated that a definitive conclusion about the immune dysfunction associated to TLR2 SNP could not be drawn due to the low number of patients with functional studies. In our mouse model of VVC, genetic disruption of TLR2 favored the early fungus invasion to deep mucosa and promoted robust PMN infiltration, increase of local production of inflammatory cytokines and upregulated expression of constitutive AMP, mBD1 with antifungal and chemoattractant activity. However, the pathogen cannot be totally removed from vaginal lumen, representing a permanent stimulation source of local response. This paper demonstrates for the first time that ablation of TLR2 signaling predisposes mice to exacerbated expression of innate mediators contributing to immunopathogenesisof VVC.

FUNDING

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica—FONCyT [grant number PICT-2012-2949]; Secretaría de Ciencia y Tecnología—SECyT-UNC [grant number 30720150100934CB]; Consejo Nacional de Investigaciones Científicas y Técnicas—CONICET [grant number PIP-112 201501 00652 CO] and Ministerio de Ciencia y Técnica de la Provincia de Córdoba [grant number 000113/2011].

Conflict of interest. None declared.

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