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Cytokines in the host response to *Candida* vaginitis: Identifying a role for non-classical immune mediators, S100 alarmins

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Abstract

Vulvovaginal candidiasis (VVC), caused by *Candida albicans*, affects a significant number of women during their reproductive years. More than two decades of research have been focused on the mechanisms associated with susceptibility or resistance to symptomatic infection. Adaptive immunity by Th1-type $CD4^+$ T cells and downstream cytokine responses are considered the predominant host defense mechanisms against mucosal *Candida* infections. However, numerous clinical and animal studies have indicated no or limited protective role of cells and cytokines of the Th1 or Th2 lineage against vaginal infection. The role for Th17 is only now begun to be investigated in-depth for VVC with results already showing significant controversy. On the other hand, a clinical live-challenge study and an established animal model have shown that a symptomatic condition is intimately associated with the vaginal infiltration of polymorphonuclear leukocytes (PMNs) but with no effect on vaginal fungal burden. Subsequent studies identified S100A8 and S100A9 Alarmins as key chemotactic mediators of the acute PMN response. These chemotactic danger signals appear to be secreted by vaginal epithelial cells upon interaction and early adherence of *Candida*. Thus, instead of a putative immunodeficiency against *Candida* involving classical immune cells and cytokines of the adaptive response, the pathological inflammation in VVC is now considered a consequence of a non-productive innate response initiated by non-classical immune mediators.

Keywords

Candida albicans; vaginitis; epithelial cells; inflammation; S100A8; S100A9

1. Introduction

Vulvovaginal candidiasis (VVC) is a prevalent infection caused by *Candida* species that affects approximately 75% of healthy women during their childbearing age [1]. *Candida* is a dimorphic fungal commensal organism of the gastrointestinal and genitourinary tracts where several *Candida* species colonize in healthy individuals [1]. Among those, *C. albicans* is the most common cause of diagnosed VVC cases (80-90%) [1]. Recurrent VVC (RVVC, three or more VVC episodes per year) is known to affect a separate population of 5-8% of menarchal women [2]. Both acute VVC and RVVC can be attributed to exogenous factors

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that may modulate host responses to *Candida* or may directly alter the growth of the organism as a consequence of environmental changes. Such exogenous factors include disturbance in reproductive hormone levels due to pregnancy, high-estrogen contraceptive usage or hormone replacement therapies, antibiotic usage, and uncontrolled diabetes [1]. In most acute VVC cases, the disease subsides once these predisposing factors are eliminated and/or antifungal therapy is used. However, for many women who suffer from RVVC, episodes are idiopathic and antifungal treatment does not prevent recurrence [2].

As a result of exposure to *Candida* early in life, most immunocompetent individuals have developed adaptive immunity toward *Candida* evidenced by serum/mucosal antibody production, and *in vitro* T-cell responses and related cytokine production (reviewed in [3]). These *Candida*-specific host immune responses are generally considered critical to generate protection at the mucosal-*Candida* interface and keep commensal *Candida* from converting into an opportunistic pathogen. In addition to VVC/RVVC, mucosal *Candida* infections can occur in other anatomical sites as seen in oropharyngeal candididiasis (OPC), esophageal and gastrointestinal candidiasis, or chronic mucocutaneous candidiasis (CMC). Unlike VVC/RVVC, these forms of disease predominantly occur in individuals with an immune deficiency (e.g. patients with HIV/AIDS, post-chemotherapy treatments) when $CD4^+$ T cells and subsequent production of effector cytokines become reduced. Of those, it was originally hypothesized that Th1 cytokines, mainly IL-12 and IFN-γ, play an important role in mediating protective host defense against *Candida* at mucosal sites. IL-12 is a key cytokine produced by antigen-presenting cells (APCs) that initiates Th1-type cell-mediated immunity (CMI) hallmarked by IFN-γ-driven proinflammatory responses toward *Candida* [4, 5]. Thus, intact Th1 responses promote resistance to *Candida* in the immunocompetent host. In the event of Th2-skewed responses to *Candida*, however, the development of Th2 cells by IL-4 signaling and subsequent cytokine production (e.g. IL-10) are known to dampen the Th1-mediated protection, leading to susceptibility to *Candida* infection [4]. Although *Candida* can elicit an antibody response at systemic (IgG) and local (IgG and IgA) levels, no strong protective role for Th2-type humoral immunity by neutralizing *Candida*-specific antibodies has been demonstrated in humans or animal models [6, 7]. These findings indicate that the balance between the two arms of adaptive immunity is required for effective clearance of *Candida* from infected mucosal sites. The discovery of Th17 cells, however, has added a new perspective to the Th1-protective/Th2-nonprotective paradigm of mucosal immune responses to *Candida*. Th17 cells predominantly produce IL-17 and IL-22 that act on other innate immune cells such as epithelial and stromal cells to induce production of proinflammatory cytokines, chemokines and antimicrobial proteins [8]. Until recently, Th17 cells had been misinterpreted as Th1 cells due to the common subunit in their key cytokines for differentiation, IL-23 and IL-12, respectively. A protective role of IL-23/Th17 in OPC, rather than IL-12/Th1, was elegantly demonstrated by Conti *et al*. using IL-$23p19^{-/-}$ and IL-$12p35^{-/-}$ mice [9]. There is an increasing body of evidence showing a strong anti-*Candida* response by Th17 cells in OPC, CMC as well as disseminated candidiasis [9-11]. In gastrointestinal candidiasis, however, the activation of Th17 has been reported to lead to pathology of the disease by eliciting exacerbated inflammation and impaired immune resistance to *Candida* [12]. Furthermore, an epidemiological study on dectin-1 mutations found in a small group of individuals with familial recurrent candidiasis suggested a link between a defective Th17 response and susceptibility to mucocutaneous *Candida* infections, including RVVC, possibly due to the lack of dectin-1-mediated Th17 activation [13]. Yet, a protective role for Th17 immunity remains a subject of controversy as clinical evidence by others implicated otherwise [14].

In this review, we highlight current knowledge on cytokines and cells involved in host immune mechanisms against VVC and describe recent advances in identification of secretory immune mediators that may play a key role in the immunopathogenesis of VVC.

2. Review of studies evaluating the role of classical cytokines involved in adaptive immunity during vaginal candidiasis

2.1. Th1 responses

Early clinical reports implicated that mucosal candidiasis occurred predominantly in individuals with T cell immunodeficiency, and a strong role for CMI by Th1 cells against *Candida* was demonstrated by various experimental models [15-20]. Based on these observations, the original consensus was that resistance to mucosal infections, including VVC, was mediated by Th1-type CMI, while susceptibility was associated with Th2-type humoral responses [21]. However, highly variable results were obtained from clinical studies evaluating RVVC patients for systemic *Candida*-specific CMI where evidence for both normal and defective cellular immunity was reported [22-24]. These inconsistent results for a role of systemic CMI were further accompanied by additional clinical observations indicating that 1) women with RVVC did not exhibit increased susceptibility to other forms of mucosal candidiasis (e.g. OPC, CMC) and 2) incidence of RVVC in immunocompromised women (e.g. HIV^+/AIDS) was equivalent to that in the healthy population [2, 25-27]. Together, a new hypothesis was proposed that mechanisms for resistance and susceptibility to infection lie within local immune dysfunctions at the vaginal mucosa rather than deficiency in systemic CMI.

Subsequent studies focused on local CMI by evaluating vaginal T cells and cytokines in vaginal secretions during infection. The majority of the studies conducted in humans and mice showed a sparse yet unique resident T cell composition in the vaginal mucosa and a lack of any T cell infiltration into the vaginal mucosa during infection [28-33]. These findings were further supported by low/undetectable levels of Th1 cytokines, namely IL-2, IL-12 and IFN-γ, in the vagina of inoculated mice [29]. In clinical studies, on the other hand, women exhibited constitutive Th1/Th0 cytokines in the vagina irrespective of the infection status [34, 35]. In both models, other proinflammatory cytokines and chemokines, such as MCP-1, IL-1α, TNF-α, MIP-1α, MCP-1, IL-8 (or MIP-2 in mice), were also unaffected during infection and had no influence on vaginal fungal burden [30, 34]. Nonetheless, the absence of Th1 responses to the vaginal presence of *Candida* became prominent although a few cases of contrary data have challenged this hypothesis. One is a rat vaginitis model in which an accumulation of vaginal $CD4^+$ T cells in the lamina propria and epithelium occurred following inoculation [36]. These vaginal lymphocytes also showed *in vitro* proliferative activity in response to mannoprotein of *C. albicans*, and vaginal fluid of inoculated rats contained high levels of IL-12 during primary infection followed by the production of IL-2 and IFN-γ during the subsequent infections [37]. The other case is a reconstituted human vaginal epithelium (RHVE) model in which infection of the tissue with *C.albicans* induced strong proinflammatory cytokine and chemokine responses [38]. However, the conflicting results from the experimental rat vaginitis and the *in vitro* infection of RHVE with previous human studies may raise questions about the clinical relevance of the models.

As evidence for the lack of CMI against VVC accumulated, a series of animal studies were conducted to test the hypothesis that CMI at the vaginal mucosa is suppressed in the host while detectable *Candida*-specific Th1 immunity is present at the systemic level. Data from these studies showed constitutive expression of TGF-β in vaginal tissues of mice, which was further increased as a result of pseudoestrus and inoculation with *C. albicans* [29]. A constitutive elevation of TGF-β was also observed in humans irrespective of the infection status, while other Th1 and Th2 cytokines remained at low levels [34]. Thus, the involvement of TGF-β, a potent immunoregulatory cytokine, could explain the lack of CMI in the vagina despite the presence of systemic CMI against *Candida*. Further analyses of

resident vaginal T cells revealed an increased number of γδ T cells in the vaginal mucosa of mice, and γδ T cell-deficiency showed increased resistance to vaginal infection [39]. This could represent another immunoregulatory mechanism involving γδ T cell-mediated induction of immune tolerance toward the vaginal presence of *Candida*. Subsequently, studies were designed to evaluate cell adhesion molecules on the vaginal epithelium and homing receptor expression on T cells within the draining lymph nodes. Although vaginal cell adhesion molecule expression was elevated following inoculation with *Candida*, T cells expressing the reciprocal homing receptors in the draining lymph nodes were reduced in inoculated mice, suggesting a means to limit *Candida*-specific T cell infiltration into the vagina [40]. Finally, in studies evaluating dendritic cells (DCs) in the vagina, the draining lymph nodes of mice inoculated with *Candida* showed the vast predominance of plasmacytoid DCs over myeloid DCs, characterized by the absence of cellular activation markers and lack of upregulation in co-stimulatory molecules [41]. This suggested a potential involvement of DCs in the initiation of immunoregulation toward *Candida* through the tolerogenic action of pDCs. Together, the lack of vaginal CMI against *Candida* has been postulated to be the result of a symbiotic interaction between the fungus and the vaginal tissue, which maintains tolerogenic responses to avoid a chronic inflammatory reaction toward commensal organisms at a reproductive site.

2.2. Th2 responses

Some of the pioneering studies conducted in an attempt to elucidate the factors associated with susceptibility to RVVC focused on putative deficiencies in *Candida*-specific antibody responses. However, no study could demonstrate any major difference in *Candida*-specific antibody levels in blood or vaginal secretions of those with RVVC compared to control women [6, 7]. Likewise, analyses of Th2 cytokines in clinical studies showed only a small amount of IL-4, IL-5 and IL-13, if detected, in vaginal fluid with no effect on fungal burden [34]. Contrary to these findings, Babula, *et al*. showed increased vaginal IL-4 with evidence of a polymorphism in IL-4 in women with RVVC, possibly resulting in reduced production of anticandidal compounds such as nitric oxide and mannose-binding lectin (MBL) [42]. A similar controversy has been shown in a mouse model where one study showed IL-4 and IL-10 to be extremely low in the vagina under uninoculated or inoculated conditions [29], while another study showed that increased IL-4 and IL-10 were associated with higher fungal burden and severity in pathology [43]. In the rat model, no IL-4 or IL-5 was detected in the vaginal fluid following inoculation [37], although a unique subset of B cells ($CD5^+$) was identified following *Candida* antigenic stimulation [36]. Subsequent studies in rats showed that adoptive transfer of these B cells from *Candida*-immunized rats to naïve animals resulted in a significant reduction in vaginal fungal burden. In addition, *in vitro* production of antibodies specific to *Candida*-mannoprotein (MP), together with secretion of several inflammatory and anti-inflammatory cytokines by the B cells, suggested a protective role of anti-MP antibody producing B cells against vaginal infection [44]. Antibody-mediated protection against vaginitis was further evidenced through the protective immunization of naïve rats with vaginal fluid from immunized rats containing an anti-secreted aspartyl proteinase (SAP) antibody [45]. Although it would be interesting to investigate the presence of anti-MP or anti-SAP antibodies in humans, it is likely that the antibody response could be a species-specific outcome in rats based on the clinical data showing no antibody deficiency in RVVC women and the absence of protective *Candida*-specific antibody production in inoculated mice. On the other hand, studies by Han *et al*. showed that administration of isolated/purified anti-mannan IgM and IgG3 antibodies resulted in protection against vaginitis in mice [46]. This finding suggests that although such antibody-mediated protection may not naturally occur in mice or humans, protection could be elicited if protective monoclonal antibodies against a specific *Candida* immunogen could be identified and induced in abundance. This implication is further supported by the concept

proposed by Casadevall regarding a pool of “protective”, “nonprotective” and “indifferent” antibodies induced by any one immunogen, with those present in the highest concentration predominating the overall immunological outcome (i.e. protection, pathology or no effect) against infection [47]. Thus, the lack of protective antibody responses in humans and mice could be explained by the low abundance of ‘protective’ antibodies whose concentration may not be high enough to elicit protection in the vaginal microenvironment, whereas such ‘protective’ antibodies are produced at high concentrations in rats. Designing vaccine strategies using specific *Candida* immunogens to promote ‘protective’ vaginal antibody production in experimental or clinical settings would be a great advancement in the development of potential immunotherapeutics for VVC/RVVC.

Another hypothesis previously proposed was that susceptibility to RVVC was associated with an allergic reaction triggered either by *Candida*-specific IgE production [48, 49] or through histamine-induced prostaglandin E_2, resulting in the suppression of Th1-type CMI [50]. Limited clinical studies evaluating systemic and vaginal IgE showed that some women with acute VVC or cured VVC had elevated concentrations of IL-13 and IgE in the vaginal fluid suggestive of an allergic response in the pathogenesis of VVC [51]. Overall, it appears that only a small minority of RVVC cases are the consequence of vaginal IgE hypersensitivity [52]. In contrast, there appears to be some precedent for hyper IgE syndrome in OPC [53].

2.3. Th17 responses

Our understanding of adaptive immune responses to mucosal *Candida* infections advanced dramatically in the last few years, particularly by the addition of the Th17 axis of immunity into the conventional Th1/Th2 paradigm. The activation of Th17 cells and downstream cytokines, particularly IL-17 and IL-22, signals epithelial and stromal cells of infected mucosa to elicit proinflammatory responses [8]. Inflammatory mediators induced by IL-17 and/or IL-22 signaling include epithelial-derived antimicrobial proteins such as β-defensins and S100 proteins [54]. The resulting Th17-dependent inflammation via regulation of innate immune cells, mainly PMNs, has been shown to mediate rapid clearance of *Candida* in OPC and CMC, as well as disseminated candidiasis and *Candida*-induced skin abscesses [9-11, 55]. Despite the strong protective role in *Candida* infections, data from the gastrointestinal candidiasis model showed that a skewed Th17 response could result in tissue inflammatory pathology and exacerbated infection [12]. Therefore, Th17 immunity appears to have dichotomous effects in response to the same pathogen depending on the site of infection.

In the female genital tract, Th17 immunity has been shown to provide protection against *Neisseria gonorrhoeae* and *Chlamydia muridarum* infections in mice through the induction of PMN migration to the site of infection [56, 57]. In *Candida* vaginitis, there are only a few documented studies that evaluated systemic and local Th17 cytokine responses in women with RVVC. One was a study describing an association of dectin-1 deficiency with susceptibility to familial recurrent mucocutaneous candidiasis, including RVVC, reported by Ferwerda *et al* [13]. Data showed that PBMCs from dectin-$1^{-/-}$ individuals produced reduced IL-6 and IL-17 in response to a *Candida* β-glucan challenge, suggesting a protective role for a systemic Th17 response against *Candida*. However, these results are inconsistent with other clinical observations where no Th17 cytokine deficiency was found in blood or vaginal fluids of RVVC patients [13, 14]. In another study, Lev-Sagie *et al*. reported that IL-6, IL-12 and IL-23 were detected only in vaginal fluids from a small subset of RVVC women, implicating a lack of a major role for either Th1 or Th17 immunity [14]. Thus, this hypothesis requires additional studies under a more defined setting in order to properly investigate host immunological factors associated with RVVC. In animal models, Pietrella *et al*. recently demonstrated in mice that depletion of Th17 cells by halofuginone, an inhibitor of TGF-β signaling, significantly reduced IL-17 production by vaginal $CD4^+$ T cells. The

study also showed that the depletion of Th17 cells resulted in exacerbated vaginal infection, possibly due to reduced production of antimicrobial peptides β-defensin (BD)-2 and BD-3 by vaginal epithelial cells [58]. However, the study suffers somewhat from weak IL-17-mediated protection and only a partial effect of halofuginone on vaginal Th17 cells. In contrast to these results, recent data from our laboratory using IL-23p19$^{-/-}$, IL-22$^{-/-}$ and IL-17 receptor A (IL-17RA)$^{-/-}$ mice showed little to no differences between each other or to wild-type mice in vaginal fungal burden or PMN infiltration following inoculation with *Candida* (Fidel, submitted). The animal study also revealed a negligible amount of IL-17 and IL-22, if detected, in the vaginal fluid of both wild-type and Th17-deficient mice irrespective of infection or inflammatory status. Thus, at the present time, the role of the Th17 axis in VVC remains controversial. A summary of the roles for various cytokines (Th1, Th2, Th17, proinflammatory, and regulatory) and chemokines investigated to date is illustrated in Table 1. Interestingly, note that no cytokine is reported unequivocally to play a role in protection.

3. Paradigm shift for VVC – Role for innate immunity

3.1. Early studies of innate immunity and the development of a human live challenge protocol

Since efforts to gain evidence for adaptive immune mechanisms against VVC continued to show a lack of protection by CMI or antibody responses, studies focused on characterizing innate immune factors that may be associated with host resistance against vaginitis. Vaginal innate immune cell populations were first evaluated in the mouse model in which PMNs were identified to be the predominant cell type among vaginal leukocytes [30]. Despite the strong phagocytic activity against *Candida*, studies showed that the presence of vaginal PMNs during infection was erratic, and had no effect on clearing vaginal fungal burden [28, 59]. Neutropenia, on the other hand, reduced inflammation associated with vaginitis in mice [59]. Other innate immune cells present in the vaginal cavity such as macrophages and natural killer cells were also evaluated and were found only in small numbers, if any [60, 61]. Subsequent studies evaluating roles of epithelial cells revealed the ability of vaginal epithelial cells isolated from mice, humans and macaques to inhibit the growth of *C. albicans in vitro* [61-63]. In vaginal and oral epithelial cells, the mechanism of action involved direct cell contact with *Candida* but no role for soluble factors [61, 64]. In addition, the antifungal activity was fungistatic rather than fungicidal, which could be elicited by intact, not necessarily live, epithelial cells [65]. We recently reported our discovery that a likely candidate for this anti-*Candida* activity is Annexin A1, a molecule expressed on the surface of epithelial cells and known to be involved in regulating growth pathways in *C. albicans* [66]. In a study evaluating vaginal epithelial cells from women with RVVC, the anti-*Candida* activity was significantly reduced in cells from RVVC women compared to those from a control group [67]. Together, these data strongly suggest that vaginal epithelial cells may play a key role as the first line of defense at the vaginal mucosa, and the anti-*Candida* activity appears to be an innate mechanism to control the growth of *Candida* in a non-inflammatory fashion. Other molecules that act against *Candida* in the oral cavity, such as defensins or histatins [68, 69], have not been studied at any depth in VVC. The only study, to date, is a preliminary evaluation of several innate host factors (i.e. lysozyme, myeloperoxidase, C5a) in lavage fluid of women with VVC/RVVC where no one factor was shown in higher or lower levels during symptomatic infection (Fidel, unpublished observation).

In the subsequent years, we have made dramatic progress in our understanding of host immune responses against VVC with the development of a human live challenge model. In the live challenge study, resistant (no previous VVC episode) or susceptible (with infrequent VVC episodes) women were evaluated for the natural history of infection following

intravaginal inoculation with live *C. albicans*. Results revealed that protection, as evidenced by asymptomatic vaginal colonization with *Candida*, occurred in the absence of any inflammatory response, while symptomatic infection was accompanied by a heavy vaginal cellular infiltrate consisting of PMNs (Figure 1). In addition, PMN infiltration scores assessed from these women positively correlated with vaginal fungal burden although the hyphal (pathogenic) form of *Candida* could be present in both asymptomatic and symptomatic conditions [70]. Finally, when tested in an *in vitro* PMN migration assay, vaginal lavage fluid from women with symptomatic infection showed increased PMN chemotactic activity compared to lavage fluid from those with asymptomatic colonization, suggesting a chemotactic factor(s) (e.g. cytokines, chemokines) had been produced and secreted into the vaginal cavity in response to the *Candida* challenge [70]. Notably, evaluation of common proinflammatory cytokines and chemokines (G-CSF, TNF-α, IL-1, IL-6, IL-8 and IL-17) in lavage fluids from the study failed to identify candidates for the PMN chemotactic factors (reported in [71]). Moreover, *Candida* itself fails to directly induce PMN chemotaxis or through any organism-derived factors (Fidel, unpublished observation). Hence, organism virulence or virulence factors alone do not appear to be a major player in the PMN response. These important data from the live challenge study reshaped our knowledge based on the conventional dogma regarding Th1-resistance/Th2-susceptibility to mucosal *Candida* infections. Furthermore, based on the evidence showing the strong involvement of innate components of host immune responses during VVC, the paradigm has shifted into the current concept that both resistance and susceptibility to VVC are associated with innate immunity.

To explain the relationship between host susceptibility to VVC and the vaginal presence of *Candidą* the hypothesis was proposed that symptomatic infection is acquired when the number of *Candida* organisms achieves a threshold which varies between women depending on the sensitivity of vaginal epithelial cells and may ultimately determine the clinical outcome (reviewed in [72]). Based on the hypothesis, we postulate that 1) in women with RVVC, vaginal epithelial cells are extremely sensitive to *Candida* and respond by secreting a danger signal that promote PMN infiltration after exposure to low numbers of *Candida*. These women are susceptible to recurrence by small increases in organism numbers (i.e., shortly following disruption or cessation of maintenance antifungal therapy. 2) In women with infrequent history of VVC due to any of the known predisposing factors (i.e., oral contraceptives, hormone replacement therapy, antibiotic usage, etc.), vaginal epithelial cells are less sensitive to *Candida* and have a higher threshold for *Candida*. These women remain asymptomatic following exposure to moderate numbers of *Candida*, but if the numbers rise following the use of antibiotics or estrogen, the threshold will be breached and a similar response will result in a symptomatic condition. 3) In women with no history of VVC, their vaginal epithelial cells are insensitive to even large numbers of *Candida* and hence the threshold is rarely breached. Therefore, the PMN response rarely, if ever, occurs and these women remain asymptomatic. At present, it is unknown what factors are involved in establishing the level of epithelial cell sensitivity to *Candida*, but is presumed to be a genetic predisposition. Indeed, some polymorphisms have been identified in women with RVVC [13, 42], On the other hand, no differences in susceptibility to infection have been demonstrated for the various haplotypic strains of mice [71, 73-75].

3.2. Dissecting the vaginal PMN response in animal studies

In parallel with the early clinical studies examining innate immune factors against VVC, the mouse model also showed no role for the erratic presence of vaginal PMNs against *Candida* [28, 59, 71]. Although PMNs were identified to be the most predominant leukocyte population in vaginal lavage fluid irrespective of time post-inoculation, their erratic presence was presumed to be a product of the pseudo-estrus condition [76, 77].

Consequently, however, a series of studies was conducted to formally classify the PMN response in various conditions. Similar to clinical observations, data showed that a heavy vaginal PMN migration occurred in a subset of inoculated animals irrespective of the haplotype or the duration of infection without affecting fungal burden [71]. Of note, previous studies testing effects of estrogen concentrations, inocula and strains of *C. albicans* also showed similar PMN infiltration patterns [76-78]. In light of symptomatic/asymptomatic conditions observed in inoculated women from the live challenge study, the high PMN and low PMN responses in mice were classified to be simulating the symptomatic and asymptomatic conditions of VVC, respectively (Figure 1) [71]. Although previous attempts to quantify clinical signs and symptoms of vaginitis in mice (e.g. redness, swelling, irritation, scratching) were unsuccessful and therefore could not be used as correlates to the PMN infiltration, vaginal PMNs levels appear to be rigid criteria based on the association with the clinical symptomatology of VVC. Accordingly, when tested in an *in vitro* PMN migration assay, results showed vaginal lavage fluids from mice under a high PMN (symptomatic) condition had increased PMN chemotactic activity compared to those from a low PMN (asymptomatic) condition, suggesting the presence of a similar chemotactic factor(s) secreted in response to *Candida* [71]. Taking into account previous reports showing a lack of strong vaginal cytokine/chemokine responses in humans and mice in VVC, identification of this putative PMN chemotactic factor(s) was crucial to further uncovering immune processes associated with susceptibility to symptomatic disease. The mouse model of VVC was then exploited to further dissect the innate mechanisms associated with the immunopathology of VVC.

4. Immune mediators involved in the immunopathogenesis of VVC

4.1. Identification of PMN chemotactic factors – S100A8 and S100A9 alarmins

Subsequent animal studies by Yano *et al*. incorporated proteomic approaches in search of a putative factor(s) responsible for the inflammatory response during the symptomatic condition [71]. First, vaginal lavage fluid from mice inoculated with *C. albicans* was evaluated for protein expression patterns. Based on the PMN-associated symptomatology criteria, two distinct proteins at 10 kDa and 14 kDa were identified by differential expression that positively correlated with the levels of vaginal PMNs. These proteins were further analyzed by mass spectrometry for protein identification and showed a significant match for S100A8 (10 kDa) and S100A9 (14 kDa) calcium-binding proteins. Confirming the initial protein identification, detection by western blots and ELISA showed high abundance of S100A8 and S100A9 in vaginal lavage fluid from inoculated mice with high PMNs, but were extremely low in fluids from those with low PMNs. Consistently, evaluation of vaginal tissues by immunohistochemistry showed elevated S100 protein expression localized at the apical surface of vaginal epithelium of mice with high PMNs, while the expression was dramatically reduced in tissues from those with low PMNs. This observation was further supported by increased S100 expression in vaginal epithelial cells at the mRNA levels, confirming epithelial cells as a primary source of S100 proteins produced under the symptomatic condition. Finally, neutralization of S100A8, but not S100A9, reduced the PMN chemotactic activity of vaginal lavage fluid in an *in vitro* migration assay, suggesting a role for S100A8 in mediating PMN migration during vaginal infection. Hence, at least in the mouse model, S100A8 appears to be produced by vaginal epithelial cells following interaction with *Candida*, and may play a key role as a PMN chemotactic factor that initiate the inflammatory response during symptomatic VVC.

S100A8 and S100A9 are small molecular weight calcium- and zinc-binding proteins of the S100 family and are also known as calgranulin A, myeloid-related protein (MRP) 8, chemotactic protein (CP) 10, and calgranulin B, MRP-14, respectively. S100A8 and S100A9 are found as monomers or heterodimeric complex called calprotectin, which has been shown

to elicit antimicrobial properties to various microbial pathogens including *C. albicans* [55, 79, 80]. These proteins also serve as potent chemoattractants for PMNs and participate in inflammatory processes. Due to their involvement as endogenous danger-signaling chemoattractant molecules, S100A8 and S100A9 have been termed “alarmins”, a subgroup of endogenous damage-associated molecular patterns (DAMPs) eliciting host immune responses in similar mechanisms as exogenous pathogen-associated molecular patterns (PAMPs) [81]. S100 alarmins are produced by phagocytes, monocytes, epithelial cells and endothelial cells and released at sites of inflammation [82-86]. In contrast to other mucosal sites where S100 alarmins are readily detected as calprotectin [55, 79, 80], data from the mouse study showed that S100A8 and S100A9 were mostly present as monomers in the vaginal secretions [71]. In addition, vaginal S100 alarmins exhibited no antimicrobial effect on *C. albicans* as evidenced by the fact that vaginal fungal burden remained unaffected by the levels of S100 alarmins detected in the vagina. However, the magnitude of PMN infiltration positively correlated with the amount of vaginal S100 alarmins [71]. Although vaginal epithelial cells appear to be a primary source of S100 alarmins during experimental VVC, PMNs also likely contribute to the S100 production once recruited into the vaginal mucosa. A proposed hypothesis is that PMNs recruited in response to the initial epithelial cell-derived S100 alarmins may initiate a second wave of S100 production, amplifying the PMN response as part of positive feedback mechanism. In addition, S100 alarmins have been implicated to stimulate innate immune cells via pattern recognition receptors such as Toll-like receptors (TLRs) and receptor for advanced glycation endproducts (RAGE), further eliciting proinflammatory responses [87]. However, specific receptors for S100A8 and S100A9 remain elusive. The possible mechanism for the effects of vaginal S100 alarmins on PMN migration during VVC is depicted in Figure 2.

Despite their well-characterized biochemical properties, the discovery of the link between S100A8 and S100A9 alarmins and vaginal infection is relatively new, with only a few previously documented studies. One is a clinical study reported by Hashemi *et al*. showing cervico-vaginal fluids containing HIV^+ monocytes had more immunoreactive S100A8 than those without [88]. These studies also showed that human recombinant S100A8 enhanced HIV production by a human monocytic cell line up to 40-fold compared to untreated cells. Although potential inflammatory properties of vaginal S100A8 were not evaluated in this study, the results provided primary evidence for the vaginal presence of S100A8 and S100A9 and their involvement as HIV-inducing, rather than antimicrobial factors within the vaginal tract. Another study to consider is a histological evaluation of S100A8 and S100A9 expression on epidermal keratinocytes from various types of clinical tissue specimens, including vulva and vaginal mucosa [89]. In this study, Abtin *et al*. demonstrated constitutive upregulation of both S100 alarmins in the upper layers of the genital epithelia, possibly due to the constant interaction with microbiota. Furthermore, the S100A8 and S100A9 protein production was shown to be induced by bacterial flagellin through a TLR5-dependent mechanism in human foreskin-derived keratinocytes. Although the authors found a lack of S100 induction by these cells when challenged with *C. albicans* culture supernatant, it would be interesting to test whether the S100 induction occurs by a similar mechanism in the vaginal epithelial cells and to investigate the bioactivity (*i.e.* antimicrobial and/or chemotactic properties) of S100A8 and S100A9 in the vaginal mucosa in humans.

4.2. Role of S100A8 and S100A9 alarmins in inflammatory diseases

As seen in the mouse model of VVC, there is an increasing body of evidence showing the association of S100A8 and S100A9 alarmins with other inflammatory conditions (Table 2). The S100 alarmins were reported to be elevated in tumor cells, particularly in skin and epidermal carcinomas with localized inflammation [90, 91]. Effects of S100 alarmins on tumor cells also include a tumor-promoting activity involving induction of cell proliferation

of colonic tumor cells via NF-κB activation [92]. Additional studies showed that S100A9$^{-/-}$ mice (which lack both functional S100A8 and S100A9 proteins) exhibited reduced growth of lymphomas and sarcoma, while S100A8 and S100A9 were upregulated in splenocytes from tumor-bearing wild-type mice [93]. In cases of infectious diseases, data showed that S100A9$^{-/-}$ mice, having intact TLR4 signaling, had reduced inflammatory responses toward LPS stimulation [94]. Subsequently, addition of extracellular S100A8/S100A9 rescued the impaired phenotype, suggesting a critical role for initiation of S100 alarmin-mediated inflammation following a bacterial challenge. Moreover, a clinical study showed a clear reduction in antimicrobial activity of calprotectin (S100A8/S100A9 heterodimer) against *C. albicans* in HIV^{+}OPC^{+} patients [95], implicating a protective role of S100 alarmins against OPC. This antifungal effect of S100A8 appears to be elicited by a Zn^{2+}-chelating property of the molecule whereby the metal ion necessary for the fungal growth becomes depleted from the extracellular microenvironment [96]. The association of S100 alarmins with autoimmune diseases has been long known in various forms of inflammatory conditions. In rheumatoid arthritis, patients exhibit elevated S100A8 and S100A9 levels in both serum and synovial fluid [97, 98] and S100A8/S100A9-producing phagocytes were detected at sites of cartilage destruction [99]. Moreover, S100A9$^{-/-}$ mice displayed reduced leukocyte infiltration and joint inflammation in the experimental antigen-induced arthritis, while wild-type mice showed upregulation of S100 alarmins accompanied by severe signs of the disease [100]. In the gastrointestinal tract, accumulation of S100-positive phagocytes was observed at the site of inflamed colonic tissues from patients with Crohn's disease and ulcerative colitis, but not in healthy mucosa of the same individuals [101-103]. In agreement with the finding in the experimental VVC study, S100 alarmins were produced and secreted by intestinal epithelial cells at the site of inflammation [104]. All the evidence described above strongly implicates the involvement of S100 alarmins in the induction of disease immunopathology as seen in tissue injury and dysfunction at the various mucosal sites. The regulation of leukocyte dynamics, and microbial growth in some cases, by S100 alarmins within mucosal tissues is quite intriguing and undoubtedly will be the subject of more intense research in several other forms of inflammatory, degenerative and malignant diseases.

5. Future directions and perspectives - S100A8 and S100A9 alarmins as potential target for diagnostics and therapeutic intervention

In diseases discussed above, S100 alarmins can be detected in serum and mucosal secretions and have been shown to correlate with either the presence or absence of symptomatic disease, including VVC, OPC, inflammatory bowel disease (IBD), rheumatoid arthritis, and several cancers [71, 90, 91, 95, 97, 98, 105-108]. Furthermore, the levels of S100 alarmins in cervical mucus were unaffected during different phases of menstrual cycle and have been proposed for a biomarker of inflammation in the lower female genital tract [109]. Hence, there is a great potential that vaginal S100 alarmins may serve as a diagnostic tool for early detection of an onset of symptomatic VVC or predicting disease relapse.

There is also a wide range of possibilities for S100 alarmins as novel targets for therapeutic interventions. In a clinical study on patients with sepsis, S100 alarmin levels were decreased in sera from surviving individuals during recovery, whereas nonsurvivors exhibited high S100 serum levels [110], suggesting that monitoring and controlling serum S100 alarmins may lead to improved prognosis of the disease. Interestingly, when observed at the tissue levels in a mouse model of streptococcal pneumonia, blockage of S100 alarmins by neutralizing antibodies inhibited phagocyte migration into the alveoli, but not accumulation within the lung tissue, without affecting other chemokine levels [111]. Furthermore, studies using a mouse model of IBD revealed that blockage of carboxylated glycans, putative binding partners of S100 alarmins, by specific antibodies, reduced colonic inflammation and

associated tumorigenesis [112]. S100 alarmins were also shown to bind TLR4 on other surrounding cells, including PMNs themselves, and further amplify the inflammatory reaction [94, 113]. Based on the clear implication of S100 alarmins as mediators of pathological inflammation during VVC, neutralizing soluble S100 alarmins or inhibiting their secretion into the vagina could inhibit the acute PMN response and ameliorate the symptoms of infection. Thus, S100 alarmin-targeted immunotherapies may represent a novel treatment option to cure acute VVC and prevent recurrent infection without relying on the conventional antifungal regimens.

6. Conclusions

Current treatment regimens for acute VVC/RVVC are dominated by azole derivatives via topical or oral administration [2]. Although the incidence of antifungal drug resistance is rare in RVVC, the static nature of conventional antifungal therapies often leads to repeated infections caused by relapse of surviving *Candida*. Thus, eliciting well-balanced host responses between protective and tolerogenic immunity to *Candida* that works in concert is important to maintain a healthy vaginal microenvironment.

Despite more than two decades of intense research, studies are yet ongoing to answer this fundamental question: what is the underlying immunological factor(s) that allow/create susceptibility to RVVC? Current knowledge on host immune mechanisms against VVC presented herein suggest non-classical immune mediators, S100A8 and S100A9 alarmins, are critical for the immunopathological inflammation and accompanying symptoms of *Candida* vaginitis. However, as the role of S100 alarmins in human VVC/RVVC remains elusive, additional clinical studies are needed to gain more insight into the mechanisms of local innate immune responses in human VVC/RVVC before exploiting S100 alarmins as a target for clinical intervention.

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Abbreviations

VVC	vulvovaginal candidiasis
RVVC	recurrent vulvovaginal candidiasis
Th	T helper
PMNs	polymorphonuclear leukocytes
OPC	oropharyngeal candidiasis
CMC	chronic mucocutaneous candidiasis
CMI	cell-mediated immunity
IFN-γ	interferon-gamma
DCs	dendritic cells
MBL	mannose-binding lectin
MP	mannoprotein
SAP	secreted aspartyl proteinase
PBMCs	peripheral blood mononuclear cells

BD	defensin
MRP	myeloid-related protein
CP	chemotactic protein
DAMP	damage-associated molecular pattern
PAMP	pathogen-associated molecular pattern
TLR	toll-like receptor
RAGE	receptor for advanced glycation endproduct
LPS	lipopolysaccharide
IBD	inflammatory bowel disease

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Highlights

- Cytokines and cells of adaptive immunity do not appear to mediate protection against VVC.
- Symptomatic VVC is associated with vaginal PMN influx and ensuing non-protective inflammation.
- Epithelial cell-derived S100 alarmins appear to induce the PMN-associated immunopathology.
- S100A8 and S100A9 alarmins may serve as novel targets for therapies to treat or prevent VVC.

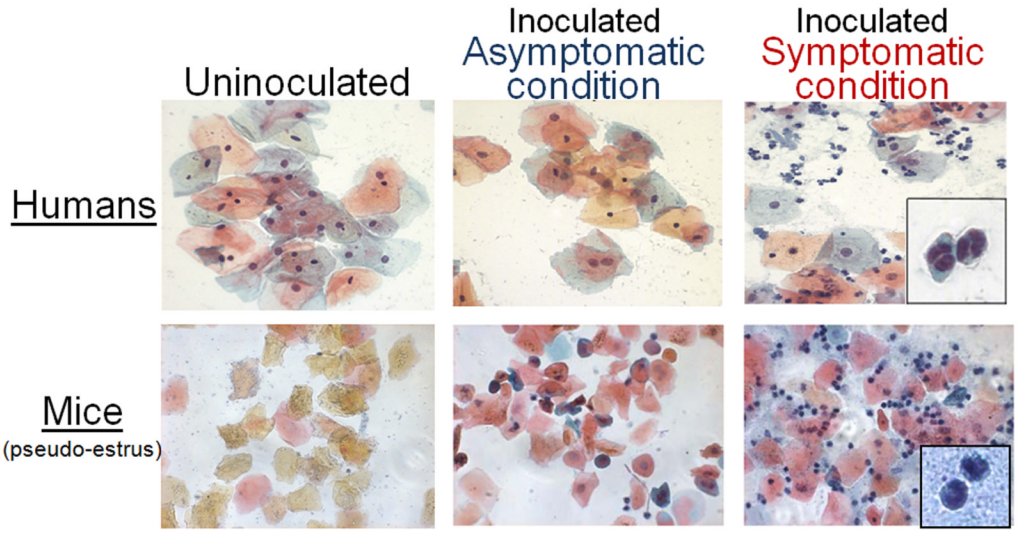

Figure 1.

Presence of PMNs in vaginal lavage fluid following inoculation with *C. albicans*. (A) Vaginal smears collected on day 7 post-inoculation from women who acquired a symptomatic infection (symptomatic condition), became asymptomatically colonized (asymptomatic condition) or uninoculated women. (B) Vaginal smears collected on day 7 post-inoculation from estrogen-treated inoculated mice with high PMNs (symptomatic condition), low PMNs (asymptomatic condition) or uninoculated mice. Smear samples were preserved and stained using the Papanicolaou technique. Images are shown at X400 magnification. Inserts show a high-magnified view of PMNs at 120% of X1,000 magnification. Partially reproduced with permission from ASM Press.

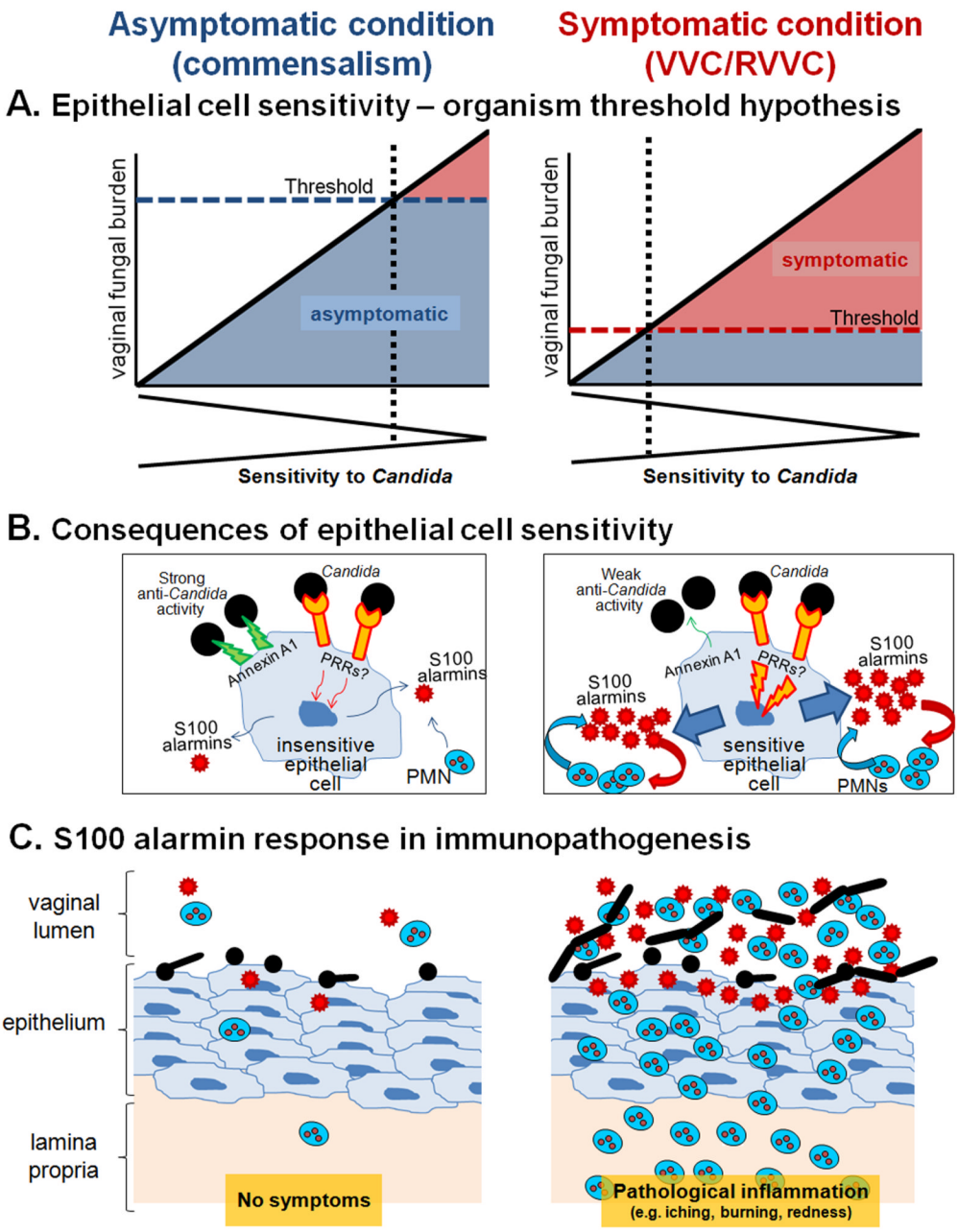

Figure 2.

Schematic diagrams representing the mechanism for the effects of vaginal S100 alarmins on PMN migration during VVC. (A) Epithelial cell sensitivity – organism threshold hypothesis. In women with no history of VVC (left panel), their vaginal epithelial cells are insensitive to *Candida*. These women remain asymptomatic as even in the presence of high numbers for *Candida* (threshold number to initiate pathological response rarely breached). In women with RVVC (right panel), vaginal epithelial cells are extremely sensitive to *Candida*. These women are susceptible to symptomatic infection following exposure to even small numbers of *Candida* (low organism threshold). The thresholds represent an arbitrary organism number of the upper limit for vaginal fungal burden that would initiate symptomatic

infection. (B) Consequences of epithelial cell sensitivity. Under asymptomatic condition (left panel), vaginal epithelial cells are insensitive to *Candida* and remain unstimulated following interaction with *Candida*. In turn, PMN migration does not occur in the absence of S100 alarmin production. Strong cell-surface Annexin A1-dependent (proposed based on oral epithelial cells) antifungal activity provides non-inflammatory means to maintain *Candida* at the commensal state. Under symptomatic conditions (right panel), vaginal epithelial cells are extremely sensitive to *Candida* and exert weak antifungal activity through Annexin A1. Epithelial cells become activated upon recognition of *Candida* via unidentified PRRs. S100 alarmins are secreted as danger signals toward which vaginal PMNs migrate through vaginal epithelium. Once in the vaginal epithelium, recruited PMNs also produce S100 alarmins as part of positive feedback mechanism to further amplify the PMN response. (C) S100 alarmin response in immunopathogenesis. PMN infiltration remains minimal in the absence of S100 alarmin production by vaginal epithelial cells, therefore, no symptom occurs (left panel). In contrast, high concentrations of S100 alarmins in vaginal epithelium trigger PMN migration to the vaginal cavity, resulting in pathological inflammation associated with the symptoms of infection. The inflammatory process enhances *Candida* growth and hyphal formation (right panel).

Table 1

Summary of vaginal cytokines and chemokines evaluated during vaginal candidiasis

	Cytokine	Reported outcome in VVC/RVVC			References
		Humans	Mice	Rats	
Th1	IL-2	P−	P+	P+	[37, 43, 44, 52, 114]
	IL-12	P−	P−	P+	[34, 37, 52, 114]
	IFN-γ	P−, S+	P−	P+	[34, 37, 51, 52]
Th2	IL-4	P−, S+	S+	P−	[34, 37, 42, 43, 51, 115]
	IL-5	P−		P−	[37, 115]
	IL-13	S+			[115]
Th17	IL-23	P−	P−		[14, 58](Fidel, submitted)
	IL-17	P+/−	P+/−		[13, 58](Fidel, submitted)
	IL-22		P−		(Fidel, submitted)
Proinflammatory	IL-1α	P−			[34]
	IL-6	P−		P+	[13, 34, 44, 114]
	G-CSF	P−	P−		[13](Fidel, unpublished data)
	TNF-α	P−		P+	[34, 114]
Regulatory	TGF-β	I+	I+		[29, 34, 35, 43]
	IL-10	P−	P−, S+	P+	[29, 34, 43, 44, 114]
Chemokines	MCP-1		P−		[29, 30]
	RANTES		P−		[30]
	IL-8/MIP-2	P−, S+	P−		[30, 51, 71]
	MIP-1α		P−		[30]

P, protection; S, susceptibility; I, immunoregulation; +, reported role; −, reported lack of a role

Table 2

Involvement of S100 alarmins in inflammatory diseases

	Disease	Protective	Pathological	Reference
Tumors	Skin/epidermal carcinomas		√	[90, 91]
	Squamous cell carcinomas	√	√	[107, 108]
	Pterygium (benign conjunctiva growth)		√	[116, 117]
	Liver carcinomas		√	[118]
Infections	Vaginal candidiasis		√	[71]
	Oral candidiasis	√		[95]
	HIV		√	[88, 119]
	Mycobacteriosis		√	[120]
	Streptococcal pneumonia	√		[111]
	Urinary tract infection (*Ureaplasma*)		√	[121]
Autoimmunity	Psoriasis		√	[122]
	Rheumatoid arthritis		√	[97, 98]
	Cystic fibrosis		√	[123, 124]
	Chronic inflammatory bowel disease		√	[82, 105]
	Sjogren's syndrome		√	[125]
	Systemic lupus erythematosus		√	[126]
Others	Atherosclerosis		√	[127, 128]
	Gouty arthritis		√	[129]
	Meibomian gland disease (an eyelid cyct)		√	[130]