An estrogen-independent and IL-1-dependent pathway controls vulvovaginal candidiasis through combined IL-17/IL-22 signaling

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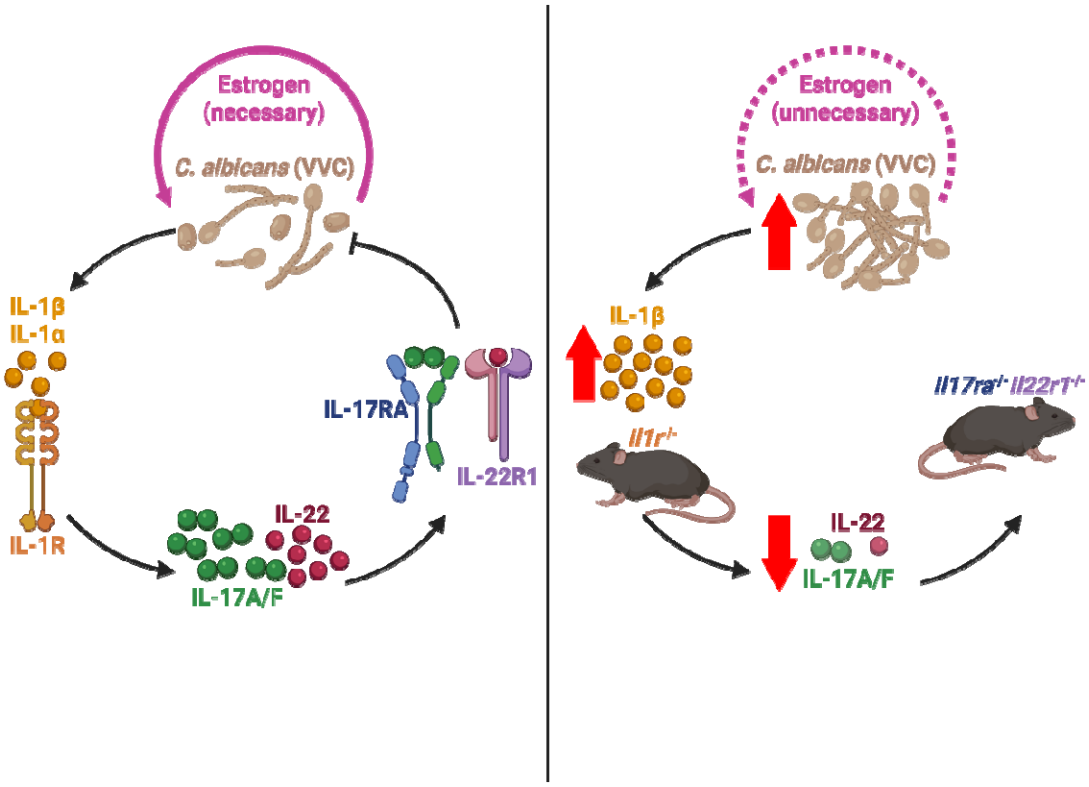
Abstract

Vulvovaginal candidiasis (VVC), caused by the commensal pathobiont *Candida albicans*, affects >75% of women, marring quality of life and incurring significant health costs. Estrogen (E2) activity is tightly linked to VVC susceptibility, and preclinical models employ E2 to establish vaginal colonization. Unlike most forms of candidiasis, VVC is not considered to be a condition of immune compromise. Rather, VVC is characterized by high levels of PMNs and inflammatory cytokines that drive immunopathology but fail to clear the fungus. The role of the Type 17 pathway in this condition is controversial. Th17 signature profiles are upregulated in vaginal tissue during VVC in mice and humans. However, loss of individual Th17 components by gene deletion or anti-cytokine administration does not predispose to disease. Here, we reveal an IL-1/Type 17-driven arm of immunity that operates to control *C. albicans* in the vaginal mucosa independently of estrogen. *Il1r*$^{-/-}$ mice subjected to VVC bore high vaginal loads, accompanied by reduced IL-17A/F and IL-22 expression and suppressed PMN influx. Although mice lacking IL-17, IL-22/IL-22R or IL-23 individually exhibited normal susceptibility to VVC, mice lacking receptors for both cytokines (*Il17raIl22ra1*$^{-/-}$) had high and persistent fungal loads, with increased vaginal tissue damage and elevated IL-1α/β levels. Thus, IL-1R serves as a master regulator of protective Type 17 responses, and moreover IL-1 signaling alone is insufficient to control fungal colonization. Interestingly, *Il1r*$^{-/-}$ and *Il17raIl22ra1*$^{-/-}$ mice showed high fungal colonization in the absence of exogenous estrogen, and this susceptibility persisted even when mice were given progesterone to prevent estrus. Together, these data reveal an estrogen-independent pathway of vaginal antifungal host defense mediated by combinatorial actions of IL-17 and IL-22 and governed by upstream IL-1R signaling.

Graphical Abstract



Introduction

The commensal pathobiont *Candida albicans* colonizes healthy individuals and is a WHO "critical priority" pathogen [1]. The immune correlates of host defense to fungi are complex and highly tissue-specific, and to date, there are no licensed vaccines against any fungal pathogens. Vulvovaginal candidiasis (VVC) and oropharyngeal candidiasis (OPC) are the most common manifestations of *C. albicans* infections [2]. Whereas oral candidiasis arises primarily from immunocompromise or impairments in oral immunity (e.g., head-neck irradiation or xerostomia), VVC occurs in at least 75% of otherwise healthy women, for many, occurs more than 3 times per year (recurrent, RVVC). VVC symptoms include vaginal itching, burning, redness and discharge, imposing a major detriment on quality of life and mental health [3, 4, 5, 6]. Contributing factors to VVC susceptibility are microbiota abundance and composition, vaginal pH, behavioral factors, diabetes, and hormones that establish a permissive environment within the reproductive mucosa [6]. Although often treatable with over-the-counter antifungal medications, drug resistance is an increasing problem and medical costs are significant.

Unlike other forms of candidiasis, VVC is considered a condition of immunopathology [3, 7, 8, 9]. Many individuals are colonized with *C. albicans* asymptomatically, while symptomatic infection is associated with high polymorphonuclear leukocyte (PMN) influx into the vaginal lumen. [10]. Yet, in the vaginal microenvironment, neutrophils do not adequately facilitate fungal clearance [11]. Instead, this robust neutrophil response is associated with elevated pro-inflammatory cytokine release and pathological inflammation [11, 12]. Additionally, genetic studies implicate innate immune sensing of fungal components and physiological factors in contributing to VVC susceptibility, including the mannan receptor, SIGLEC15, Dectin-1/CARD9, and heparan sulfate, among others [3]. VVC is linked to high estrogen activity, with VVC being rare in pre-pubescent and post-menopausal individuals and high in patients receiving hormone replacement therapy or during high estrogenic phases of pregnancy. In keeping with this, exogenous estradiol administration is used to establish persistent vaginal *Candida* colonization in animal models of VVC [13, 14, 15].

Adaptive T cell immune responses are important to control fungal infections including candidiasis, but they play surprisingly disparate roles that vary by anatomical site. Th17 cells and intact IL-17 receptor signaling maintain immunity to *C. albicans* in the mouth and skin, as mice

and humans lacking the IL-17 receptor (IL-17RA, IL-17RC), IL-22 or related Th17 cell components are highly susceptible to oropharyngeal and cutaneous candidiasis [16, 17, 18, 19, 20]. However, the role of the Type 17 pathway in VVC is far less clear. Strong Th17 gene signatures are elicited in both human and murine vaginal tissue during *C. albicans* infections, which prompted inferences that the Th17 pathway is involved in some capacity in this condition [21, 22, 23]. Supporting this, VVC is a statistically significant, albeit modest, adverse event associated with anti-IL-17 biologic therapy [24]. However, in contrast to OPC, women infected with HIV/AIDS or harboring individual Th17/IL-17R gene mutations do not exhibit increased incidence of VVC [3, 25]. Moreover, deletion of individual genes within the Th17 pathway in mice (*Il17a*, *Il17ra*, *Il22*, *Act1)* does not cause elevated vaginal fungal loads or signs of immunopathology in standard VVC murine models [5, 22, 26]. Collectively, these observations have led to an accepted paradigm that VVC is distinct among manifestations of candidiasis in not requiring the Th17 immune axis.

Nonetheless, there is good evidence that adaptive immune effectors contribute to control of VVC, including roles for Type 17 cytokines. Infections with avirulent *C. albicans* strains protect mice from subsequent infections, as does immunization with recombinant *C. albicans* cell wall proteins [3, 27]. Halofuginone, an aminoacyl-tRNA synthetase inhibitor that restrains Th17 cells [28, 29], worsened vaginal candidiasis in mice [21]. An experimental *Candida* vaccine showed benefit for VVC in mice and in a Phase Ib/IIa clinical trial, where RVVC patients showed delayed time to recurrence and generated *Candida*-specific Th1 and Th17 adaptive responses [30, 31, 32, 33]. Hence, despite continuing work in this area, there is no consensus regarding the extent to which the Th17 pathway participates in VVC, and generally the immune landscape in the vaginal tract is a neglected area of inquiry.

Here, we present data that sheds light on how Type 17 cytokines serve to control VVC, perhaps reconciling some of these observations. Using a murine VVC model, we find that IL-22R signaling alone or the upstream Type 17 activator IL-23 are dispensable for controlling VVC. However, IL-17R and IL-22R signaling in combination are required to limit disease. IL-1 signaling is also required to control fungal loads and is nonredundant for activation of IL-17/22 activation in the vaginal mucosa. Moreover, IL-1R signaling in the absence of Type 17 signaling is insufficient to control VVC. Normally estrogen conditioning is required to maintain vaginal fungal colonization, but VVC susceptibility persists in IL-17R/IL-22R-deficient mice even in the absence of estrogen treatment. Together, these findings indicate that an IL-1-driven Type 17

immune pathway that appears to be independent of estrogen is a significant and previously unrecognized contributor to *Candida* immunity in the vaginal mucosa.

Results

IL-1 receptor signaling mediates immunity to VVC and activates Type 17 cytokines

Multiple studies show that IL-1 cytokines (IL-1α, IL-1β) are upregulated in both vaginal lavage fluid (VLF) and vaginal tissue during VVC in humans and mice [22, 34]. To test the role of IL-1 receptor (IL-1R) signaling in VVC, we subjected mice lacking the receptor for IL-1α and IL-1β (*Il1r*$^{-/-}$) to the standard murine model of VVC. To that end, mice were administered 17-β-estradiol (E2) three days prior to and four days after vaginal infection with 5x10^{6} CFU of *C. albicans* (strain SC5314) (**Fig 1a)**. VLF was collected on days 3 and 7 post-infection, and fungal loads were assessed by CFU enumeration. *Il1r*$^{-/-}$ mice had significantly quantitatively higher vaginal fungal colonization compared to WT controls (**Fig 1b**), indicating a protective role for IL-1 signaling in VVC. IL-1R-dependent reduction appears to be reliant specifically on IL-1β, as *Il1b*$^{-/-}$ mice also had higher CFU on days 3 and 7 post-infection (**Fig. 1c**).

PMN infiltration to the vaginal lumen in *Il1r*$^{-/-}$ mice was determined by cytological evaluation (Pap smear) (**Fig S1**). PMN levels were modestly reduced on day 3 but were similar to WT by day 7 **(Fig 1d).** This was evident in histological sections of vaginal tissue (**Fig 1e**). Interestingly, in IL-1β-deficient mice, PMNs were elevated at both time points (**Fig 1d, e**), which suggests a disconnect between IL-1R-mediated control of fungal loads versus control of PMN levels, possibly due to sustained responsiveness to other IL-1 isoforms.

To understand how the IL-1R pathway drives antifungal host defense, vaginal mucosal tissue was subjected to bulk RNASeq. Strikingly, *Il1r*$^{-/-}$ mice exhibited impaired expression of Type 17 cytokines (*Il17a, Il17f, Il22*) together with signature downstream gene targets associated with the Type 17 pathway, such as *Defb3, Saa1, S100a8/a9* [35, 36] (**Fig 1f**). IL-1 is known to be involved in differentiation of Type 17 cells [37], and pointed to the possibility that this axis is involved in VVC immunity.

Cooperative signals from IL-17 and IL-22 but not IL-23 control VVC

In addition to IL-1, IL-23 is a key upstream activator of Type 17 cell cytokine production. IL-23 is required for immunity to oral and systemic candidiasis [35, 38, 39, 40, 41]. Given the impaired vaginal expression of Type 17 cytokine transcripts in *Il1r*$^{-/-}$ mice, including *Il12b* (IL-12p40, the shared subunit of IL-12 and IL-23), we asked whether IL-23 was also required for *C. albicans* colonization in the vaginal tract. In contrast to *Il1r*$^{-/-}$ mice and consistent with prior

studies [26], mice lacking IL-23 did not exhibit elevated fungal loads in E2-induced VVC (**Fig 2a**). Surprisingly, *Il17a* transcript levels were not statistically lower in the vaginal mucosa of infected *Il23*$^{-/-}$ mice, though *Il17f* levels were. Interestingly, *Il1b* was elevated, which may explain why *Il17a* could be found in an IL-23-deficient environment (**Fig 2b**). Collectively, these data indicate that IL-1 rather than IL-23 appears to be the master regulator of the Type 17 response in vaginal candidiasis.

We noted in the transcriptomic data that infected *Il1r*$^{-/-}$ mice showed alterations in vaginal expression of several cytokines that use the shared IL-22RA1 receptor subunit, including *Il22, Il20* and *Il24* (see **Fig 1f**) [42]. We previously demonstrated that loss of IL-22 alone does not increase susceptibility to VVC [22], but it was conceivable that other IL-22-family cytokines might serve redundant functions. Therefore, to capture the cumulative impact of the IL-22R-dependent ligands, we evaluated vaginal fungal burdens in *Il22ra1*$^{-/-}$ mice. As shown, mice lacking IL-22RA showed the same susceptibility to VVC as WT counterparts (**Fig 2b**), arguing against additive or redundant impacts of other IL-22R-ligands during VVC.

Expression of Type 17 signature cytokines (*Il17a, Il17f, Il22*) are among the most significantly induced genes in VVC [22] and were considerably reduced in *Il1r*$^{-/-}$ mice (**Fig 1**). IL-17A/F and IL-22 exhibit cooperative activities in various contexts including OPC [35, 43, 44, 45, 46, 47, 48]. Accordingly, while loss of these cytokines individually did not render mice susceptible to VVC, we postulated that their combined activities might contribute to vaginal host defense. To test this, we assessed VVC in mice lacking both IL-17RA (lacking responsiveness to IL-17A, IL-17F, IL-17C and IL-25 [49]) and IL-22RA1 (lacking responses to IL-22, IL-20 and IL-24 [50]). Strikingly, *Il17raIl22ra1*$^{-/-}$ mice showed more than 50-fold greater fungal loads compared to WT controls, examined at day 3 (20,438 vs. 341 CFU/mL and day 7 (32,792 vs 611 CFU/mL (**Fig 2d**). Thus, combined activities of IL-17R and IL-22R serve to control *C. albicans* colonization in the vaginal tract.

IL-17 and IL-22 signaling limits VVC pathology

VVC is considered a disease of immunopathology, and thus fungal colonization per se does not necessarily indicate a diseased state. Accordingly, we next examined immunopathological hallmarks of VVC in the context of IL-17R/IL-22R deficiency. Vaginal tissue damage was assessed by evaluating VLF levels of lactate dehydrogenase (LDH), which is

released during hyphal-mediated epithelial destruction through the fungal toxin candidalysin [4, 51, 52]. LDH levels were higher in *Il17raIl22ra1*$^{-/-}$ mice at both early and late time points, correlating with the high fungal burdens in this condition (**Fig 3a**). However, unlike *Il1r*$^{-/-}$ mice, PMN numbers in VLF were surprisingly unchanged in *Il17raIl22ra1*$^{-/-}$ mice compared to WT, despite the high fungal loads that might be expected to provoke a neutrophil response (**Fig 3b**). This observation aligns with prior findings that PMNs are insufficient to control fungal loads in VVC [11, 53]. These data hinted that since PMNs were impaired early in *Il1r*$^{-/-}$ mice but not in *Il17raIl22ra1*$^{-/-}$ mice, PMN levels in VLF are governed by an IL-1-dependent but Type 17-independent pathway. Even so, histological evaluation of the vaginal mucosa indicated considerable invading hyphae, tissue sloughing and clusters of PMNs along the surface mucosa, indicative of ongoing inflammation (**Fig 3c**).

We also examined VLF levels of 40 cytokines and chemokines associated with inflammation and Type 17 pathways (**Fig 3d, Table S1**). IL-17A and IL-17F were substantially elevated in *Il17raIl22ra1*$^{-/-}$ mice, which was expected based on prior studies in OPC and other settings that have similarly observed high IL-17A/F levels in *Il17ra*$^{-/-}$ mice [35, 54]. IL-1β, CCL3, CCL4, eotaxin, and VEGF were also increased in infected *Il17raIl22ra1*$^{-/-}$ mice, indicative of elevated tissue inflammation and likely driven by elevated fungal loads. In contrast, IL-22, CXCL1, and TNF levels were lower in *Il17raIl22ra1*$^{-/-}$ mice (**Fig 3d**). Collectively, these results indicate that the Type 17 pathway not only serves to limit fungal loads in VVC but concomitantly restricts expression of inflammatory cytokines and chemokines that contribute to inflammatory pathology.

The IL-1-Type 17 axis restricts fungal burden independently of estrus cycle

Because VVC correlates with high estrogen activity [55, 56], the standard mouse model of this disease employs the most potent form of estrogen (estradiol, E2) to synchronize cycling in recipient mice and thereby facilitate persistent *C. albicans* colonization. Although the precise mechanisms are unclear, E2 plays numerous potential roles in enabling colonization, including complement evasion, altered cytokine and PRR expression, indirect impairment of neutrophil function and NETosis and stimulation of hyphal growth [13, 57, 58, 59, 60]. Most studies of VVC using gene-targeted mice only assess disease in the presence of estrogen, so comparatively little is

known about estrogen-independent pathways that contribute to vaginal infection.

Accordingly, we asked whether susceptibility to VVC was evident in the absence of E2 in any of the Type 17-deficient mice used in this study. In keeping with the well-known kinetics of this VVC model [15], WT controls given the vehicle (sesame oil) control (Veh) usually fully clear *C. albicans* from the vaginal tract by day 7 (**Fig 4a-d**). *Il23*$^{-/-}$ and *Il22ra1*$^{-/-}$ mice similarly cleared *C. albicans* by day 7 (**Fig 4a, b**) and is in line with our reported findings that *Il17ra*$^{-/-}$ and *Il22*$^{-/-}$ individual knockout mice also do not show elevated susceptibility to VVC in the absence of E2 [22]. However, here we observed that non-estrogenized *Il1r*$^{-/-}$ and *Il17raIl22ra1*$^{-/-}$ mice both harbored substantially higher fungal burdens compared to WT (**Fig 4c, d**). Non-estrogenized *Il1r*$^{-/-}$ and *Il17raIl22ra1*$^{-/-}$ mice also showed delayed clearance kinetics compared to WT controls (**Fig 4c, d**). While PMN numbers trended higher in in *Il17raIl22ra1*$^{-/-}$ mice on day 7, they did not reach statistical significance (**Fig 4e**), again suggesting that IL-17R/IL-22R signaling is dispensable for neutrophil migration into the vaginal lumen. Unlike in the context of E2-treated VVC, *Il17raIl22ra1*$^{-/-}$ mice given SO vehicle do not display increased LDH activity of VLF compared to WT mice (**Fig 4f**). Of the 40 cytokines and chemokines tested, only IFNγ and IL-17F were increased in the VLF from these mice (**Fig 4g, Table S2**). Therefore, the Type 17 pathway is important in controlling fungal loads but does not markedly impact neutrophil recruitment in the absence of exogenous estrogen administration.

E2 administration synchronizes the murine estrus cycle to the state most receptive to fungal infection. Although *Il17raIl22ra1*$^{-/-}$ mice are fertile with normal litter sizes (data not shown), a possible explanation for the increased fungal loads in the *Il17raIl22ra1*$^{-/-}$ mice could be abnormal elevation of endogenous estrogen or dysregulation of the estrus cycle. Therefore, we tracked the estrus cycle in WT or *Il17raIl22ra1*$^{-/-}$ mice daily by vaginal cytology [61]. *Il17raIl22ra1*$^{-/-}$ mice spent similar amounts of time in estradiol high phases (proestrus/estrus) and estradiol-low phases (metestrus/diestrus) as WT animals (**Fig 5a, b**). Also similar were levels of estradiol, cortisol and testosterone during the proestrus/estrus phases (**Fig 5c**). Unexpectedly, and despite the apparently normal estrus cycle dynamics, *Il17raIl22ra1*$^{-/-}$ mice showed somewhat elevated levels of progesterone (P4) at this time point (**Fig 5c**), although the basis for this is unclear. Regardless, this finding may be inconsequential, since elevated P4 would be expected to drive higher fungal clearance, yet the opposite is observed in this knockout background.

To determine if the Type 17-driven susceptibility to VVC was hormone-driven, we administered progesterone (P4) in order to put mice into diestrus/metestrus phase at the time of infection (**Fig 5d**), confirmed by cytology. Progesterone does not enhance *C. albicans* vaginal colonization [13], but rather actively promotes fungal clearance [62, 63, 64]. Nonetheless, *Il17raIl22ra1*$^{-/-}$ mice given P4 still had higher fungal loads compared to WT (**Fig 5e**), despite being in a less permissive hormonal environment for *C. albicans* colonization. This was supported by P4-treated *Il17raIl22ra1*$^{-/-}$ mice showing increased LDH activity indicating tissue damage infection compared to P4-treated WT mice (**Fig 5f**). Collectively, these data support the existence of a significant Type 17-dependent component to antifungal protection in the vaginal tract that is likely independent of estrogen/estrus cycle.

Discussion

Vulvovaginal candidiasis (VVC) is one of the most common fungal infection of humans, as an estimated 75% of childbearing aged women develop this condition at least once in their lifetime, and 5-8% suffer from recurrent infection. VVC imposes detriments on finances, mental health and overall wellbeing, emphasizing the need to better understand the underlying pathogenesis of this condition for development of targeted therapeutic interventions [3, 6, 65].

The driving role of IL-17 in oral and dermal candidiasis has been recognized for over 15 years [66], but the contribution of the Type 17 pathway to VVC immunity has been less clear. Studies of individual knockout mice in these pathways surprisingly, however, did not reveal contributions of IL-17A/F or IL-22 signaling [22, 26]. Similarly, rare individuals with inborn mutations in Th17-related cytokines are highly prone to mucocutaneous oral and dermal candidiasis, yet not to VVC [67, 68, 69]. VVC is also not apparent in patients taking anti-IL-23 biologics [24], consistent with findings in IL-23-deficient mice (this study, [26]). Anti-IL-22 Abs are not approved for clinical use, so its contributions to clinical VVC are unknown. *AIRE* gene mutations that cause the genetic autoimmune syndrome APECED are sometimes accompanied by both, anti-IL-17A/F and anti-IL-22 Abs, but VVC is not a hallmark of this disease state. Moreover, recent evidence indicates these auto-antibodies are likely not causative for the candidiasis observed [70].

Despite this, we were prompted to revisit the possibility that combinatorial activities of IL-17 and IL-22 in VVC susceptibility for several reasons. There is a striking induction of IL-17 and IL-22 in vaginal tissue during VVC [21, 22]. A meta-analysis of over 17,000 patients taking anti-IL-17 biologics for autoimmune diseases reported a statistically significant increase in susceptibility to VVC [24]. OPC is far more severe in *Il17raIl22ra1*$^{-/-}$ mice compared to mice lacking either receptor alone [44], suggesting that antifungal properties of these cytokines are potentially synergistic. CARD9-deficient mice are susceptible to VVC, associated with reduced Type 17 cytokine responses [71]. Indeed, the higher vaginal loads seen in *Il17raIl22ra1*$^{-/-}$ mice reconcile these dichotomies; indicating that, although loss of either IL-17 or IL-22 signaling alone can be tolerated in the vaginal mucosa, loss of both causes high and persistent colonization.

IL-1 is a central player in both innate and adaptive immune responses to OPC and

systemic candidiasis, with IL-1 cytokine family or IL-1R deficiency enhancing disease susceptibility [72, 73, 74, 75]. Supporting a contribution for this pathway in VVC was the marked impairment of IL-17/IL-22 profiles in susceptible *Il1r*$^{-/-}$ mice. IL-1 acts upstream of Type 17 responses, including in vaginal immunity to herpes simplex virus and chlamydial infections [38, 39, 72, 73, 76, 77]. Increased *C. albicans* colonization was shown after IL-1 signaling inhibition with an IL-1R antagonist or antibody neutralization [78]. Surprisingly, there is not strong evidence linking IL-1 inhibition in humans to clinical VVC, which might be due to underreporting or to differences in how the Th17 axis is controlled in mice versus humans [79, 80, 81].

Our data reveal an unexpected divergence of IL-1R versus Type 17 signals with respect to control of neutrophils. Whereas *Il1r*$^{-/-}$ mice showed modestly impaired PMN numbers, *Il17raIl22ra1*$^{-/-}$ mice trended to increased PMNs, concomitant with high fungal loads. Increased vaginal fungal burdens and reduced neutrophil influx in *Il1r*$^{-/-}$ mice were similarly reported in murine OPC, where keratinocyte-derived IL-1α contributed to neutrophil recruitment [74]. Thus, IL-1 signaling appears to control two distinct arms of immunity in VVC – one pathway activates PMNs which are insufficient to contain disease, and the other activates Type 17 cytokines which we show here are absolutely essential for control of disease. While IL-1R signaling is driven by both IL-1α and IL-1β, our data indicate that IL-1β dominates for control of vaginal fungal burden. Thus, future work to delineate isoform- and compartment-specific roles of IL-1 isoforms underlying immune activation, inflammation, or burden control is warranted [82].

The source of cytokines was not examined in this study. IL-1 cytokines are broadly expressed and likely derive from many cell types. Vaginal epithelial cells, in particular, produce high levels of IL-1 cytokines [4, 5, 83, 84]. Immunization with a subunit vaccine corresponding to the immunodominant adhesin Als3 generates adaptive T and B cell responses that protect mice and humans from VVC [33]. Unlike humans, mice are immunologically naïve to *C. albicans* [46, 72, 85, 86], so given that these effects are observed as early as day 3 before antigen-specific adaptive immunity is established, it is likely that producers of IL-17A/F and IL-22 are innate-acting lymphocytes [87, 88, 89, 90, 91, 92]. In this regard, γδ-T cells produce IL-17 and IL-22 downstream of IL-1/IL-23 signaling [93, 94, 95]. In light of the susceptibility of *Il1r*$^{-/-}$ but not *Il23*$^{-/-}$ mice to VVC, it appears that IL-1R signaling plays a dominant role in shaping the Type 17 cytokine response within the vaginal tract. Much like the Type 17 pathway, the contributions of γδ-T cells to host

defense during VVC is controversial [88, 89, 96]. Given the present findings, defining the source(s) of IL-17/22 in this context is worth addressing.

Several fungal correlates contribute to VVC and influence host cytokine responses. Candidalysin (encoded by *Ece1*) is a secreted pore-forming fungal toxin that is required to induce the Type 17 response in OPC via epithelial tissue damage [72, 73] and is similarly required to promote tissue damage in VVC [4, 52]. Pra1 facilitates zinc metabolism in *C. albicans*, and is associated with the elevated cytokines that characterize VVC pathology; moreover, zinc administration in humans ameliorates disease [97]. There is a strong influence of vaginal microbiota in this context [65, 98], which could be altered in *Il17raIl22ra1*$^{-/-}$ mice. Still, coordination between microbe and host in VVC is still not fully understood, and the Type 17 pathway is likely to be influential.

VVC differs from other manifestations of candidiasis in being strongly hormone-dependent. In humans, hormone replacement therapies, high-estrogen oral contraceptives, and pregnancy all increase the risk of VVC [6, 65]. In mice, E2 supplementation is used to synchronize the estrus cycle, permitting maintenance of fungal loads in the vaginal tract [13] and also in systemic candidiasis [99]. Consequently, most studies of knockout mice have not included non-estrogenized controls, and thus susceptibility driven by estrogen may have masked contributions from other arms of immunity in this setting. Surprisingly, the mechanism for E2-driven susceptibility is not well understood. Estrogen is thought to act on the host specifically through the *Esr1* receptor to enable VVC [63], potentially by creating an immunosuppressive environment [100]. Estrogen also impacts *C. albicans* directly, promoting its virulence and reducing immune recognition [57, 59].

A surprise from this work was that *Il1r*$^{-/-}$ and *Il17raIl22ra1*$^{-/-}$ mice exhibited substantially elevated vaginal fungal loads even in the absence of exogenous E2 administration. Increased VVC susceptibility was seen in *Il17raIl22ra1*$^{-/-}$ mice despite the endogenously elevated levels of progesterone (P4) as well as in the presence of exogenously administered P4, a hormone known to promote clearance of *C. albicans* from the vaginal tract [13, 64]. P4-driven clearance of VVC was linked to TNF and neutrophils [62, 63, 64], but our data argue for an additional contribution from IL-17/22 signaling. It was not straightforward to test the impact of estrogen directly, as clinically-used estrogen inhibitors such as tamoxifen have direct antifungal activity [101]. However, estrogen levels and estrus cycle dynamics were normal in *Il17raIl22ra1*$^{-/-}$ mice. The alterations in

progesterone would not be predicted to increase *C. albicans* susceptibility based on prior studies [13], nor did P4 do so in *Il17raIl22ra1*$^{-/-}$ mice. Collectively, our data point to an impactful estrogen/estrus-independent host defense pathway in VVC that is mediated by the combinatorial actions of both IL-17 and IL-22.

Methods

Candida albicans and VVC

One colony of *Candida albicans* strain SC5314 (ATCC) was grown overnight in Yeast Peptone Dextrose (YPD) broth containing 50 µg/mL chloramphenicol at 30°C. 5×10^6 CFU *C. albicans* yeast in PBS was administered by intravaginal inoculation, as described [22]. If indicated, on days -3 and 4 relative to infection, mice were subcutaneously administered 0.1 mg of β-estradiol 17-valerate (E2; MilliporeSigma, E1631) in sesame oil (SO), SO vehicle, or 2 mg progesterone (MilliporeSigma, P0130) in SO. At days 3 and 7 post-infection, vaginal lavage fluid (VLF) in PBS (100 µL) was diluted onto YPD agar with 50 µg/mL chloramphenicol and colonies enumerated after growth for 24 h at 37°C, 48 h at 30°C, or 72 h at room temperature.

Mice

Animal protocols were approved by the University of Pittsburgh and the University of Tennessee Health Science Center Institutional Animal Care and Use Committees. Wild-type (WT) mice were from the Jackson Laboratory, Taconic Farms, or generated in-house from breeding. *Il1r*$^{-/-}$ (strain #003245) mice were from The Jackson Laboratory and *Il1b*$^{-/-}$ mice were provided by Nathan Archer, Johns Hopkins University [102]. *Il23a*$^{-/-}$ mice were from Genentech and *Il17ra*$^{-/-}$ mice were from Amgen, provided under MTA. *Il22ra1Il17ra*$^{-/-}$ mice were described [44]. Mice were housed in SPF conditions and used at 6-12 weeks of age. All mice were on the C57BL/6 background.

Microscopy

PMN recruitment was assessed in VLF smears by Papanicolaou (PAP) staining. PMNs were enumerated in 5 nonadjacent fields on an EVOS FL Auto Imaging system (Invitrogen) acquired at 20X or 40X and counts normalized to a 20X field. Investigators were blinded to sample

identity. Each mouse is represented as the average PMN count from 5 images. Excised vaginal tissue was fixed in 10% neutral buffered formalin, paraffin-embedded, and slides were co-stained with Periodic acid–Schiff (PAS), hematoxylin, and eosin and imaged on an EVOS FL Auto Imaging system (Invitrogen). Size bars included in images.

Estrus phase tracking and hormone measurement

VLF from uninfected WT and *Il17raIl22ra1*$^{-/-}$ mice were collected daily at approximately the same time daily for 12 consecutive days. Vaginal slides were subjected to Papanicolaou staining for estrus cycle assessment [61]. Blood at proestrus/estrus or estrus was collected by cardiac puncture. Sera was isolated using serum separator tubes (BD Microtainer, 365967). Hormones were measured using Luminex xMAP technology by Eve Technologies Corporation (Calgary, Canada) with the Steroid/Thyroid 6-Plex Discovery Assay® Multi Species Array panel.

RNASeq, qPCR

RNA from frozen vaginal tissue was isolated using RNeasy Mini Kits (Qiagen) and cDNA generated with the iScript cDNA Synthesis Kit (Bio-Rad). Real-time qPCR was performed using 2X Universal SYBR Green Fast qPCR Mix (ABclonal) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Quantitect primer assays were used for transcript quantification (Qiagen). Sequencing data is registered with the Bioproject database and will be made available upon publication.

ELISA, Luminex

Cytokines and chemokines were measured in clarified VLF using Luminex xMAP technology by Eve Technologies Corporation (Calgary, Canada) with the mouse Cytokine/Chemokine 32-Plex Discovery Assay Array (MD32) and Cytokine Th17 12-Plex Discovery Assay Array (MDTH17-12) panels. For samples too dilute for calculation, values were plotted at the specified limit of detection for each analyte.

LDH activity

LDH clarified in VLF was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780). OD_{492} was determined using a BioTek Synergy 2 microplate reader. Samples

were compared to a standard curve of purified LDH (Sigma, L7525).

Statistics

Data were analyzed using Prism (GraphPad Software Inc.) by Student's *t*-tests, Mann-Whitney, Mantel-Cox and Kruskal-Wallis tests with corrections for multiple comparisons as indicated in the figure legends. Data are pooled from at least two independent experiments unless noted. Throughout, *P < 0.05, **P < 0.01, and ***P < 0.001; ns, not significant.

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Abbreviations

CFU, colony forming units; E2, 17-β-estradiol; OPC, oropharyngeal candidiasis; LDH, lactate dehydrogenase; P4, progesterone; Pap, Papanicolaou; SO, sesame oil; VVC, vulvovaginal candidiasis; Veh, vehicle; VLF, vaginal lavage fluid

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Figure Legends

Fig 1. IL-1R signals control vaginal immunity to VVC a. VVC model **b, c.** Mice administered E2 on day -3 and day 4 were infected vaginally with *C. albicans*. Fungal burdens in WT (C57BL/6, n=8-9), *Il1r*$^{-/-}$ (n=8) or *Il1b*$^{-/-}$ (n=9) mice on day 3 and 7. Data are geometric mean + geometric SD and significance determined by two-sided Mann-Whitney U test. **d.** Representative vaginal histology day 3 p.i. Green arrows denote *C. albicans* hyphae. Black arrows indicate PMNs **e.** PMN counts in VLF of indicated mice on day 3 and 7. Data are mean ± SEM, analyzed by two-sided Student's t-test. **F.** Differentially expressed genes comparing whole vaginal tissue of WT versus *Il1r*$^{-/-}$ mice at day 3 (adjusted $p < 0.05$, Student's t-test and Benjamini and Hochberg's analysis). Type 17 cytokines and their downstream effectors in blue.

Figure 2. Combined loss of IL-17R and IL-22R signaling increases VVC susceptibility. a. Fungal loads in VLF of E2-treated *Il23a*$^{-/-}$ mice (n=5-9) mice on days 3 and 7 post-infection. **b.** Cytokine mRNA levels in whole vaginal tissue of *Il23a*$^{-/-}$ mice by qPCR, relative to *Gapdh* and normalized to sham. **c, d.** Fungal loads in VLF from *Il22ra*$^{-/-}$ (n=6-11) and *Il17raIl22ra1*$^{-/-}$ mice (n=15-16). Data in **a, c,** and **d** are geometric mean ± geometric SD and data in **b** are mean ± SEM; two-tailed unpaired Mann-Whitney test (**a, c,** and **d**) and two-tailed unpaired Student's t-test (**b**).

Figure 3. IL-17/22-driven signaling dampens inflammation and tissue damage. Fungal loads in VLF of E2-treated mice on days 3 and 7 post-infection. **a.** LDH activity in VLF on days 3 and 7. **b.** PMNs in VLF determined by cytology. **c,** Representative PAS and H&E staining of vaginal tissue on day 3 p.i.. Green arrows denote *C. albicans* hyphae. Black arrows indicate PMNs **d.** Indicated cytokine/chemokine levels were determined by Luminex. Data in **a, b,** and **d** are mean ± SEM; two-tailed unpaired Student's t-test (**a, d**) and two-tailed unpaired Mann-Whitney test (**b**).

Figure 4. IL-17/22 receptor deficiency drives VVC susceptibility in the absence of E2 conditioning.

a-d. Indicated mice were administered sesame oil (SO) vehicle (Veh). Left: fungal loads in VLF assessed on days 3 and 7 (*Il23a*$^{-/-}$ n=8-9; *Il22ra1*$^{-/-}$ n=10-11; *Il1r*$^{-/-}$ n= 9; *Il17raIl22ra1*$^{-/-}$ n=14-21). Right: % of mice with detectable fungal load over the indicated time course. **e.** PMN counts on day 3. **f.** LDH activity in VLF on day 3 and 7. **g.** Cytokine levels in VLF on day 3. Data in **a-d,** and **h** show geometric mean ± geometric SD and data in **f** and **g** show mean ± SEM; two-tailed unpaired Mann-Whitney test (**a-d: left, e**), Clearance data analyzed by Mantel-Cox and two-tailed unpaired Student's t-test (**f, g**).

Figure 5. Sex hormone dynamics in *Il17raIl22ra1*$^{-/-}$ mice. a. Hormone predominance and cell populations in VLF during the murine estrus cycle, image adapted from Ref. [61] **b.** Estrus cycle tracking by PAP staining of VLF over 12 days. Each color/symbol represents an individual mouse. D: diestrus, M: metestrus, E: estrus, P: proestrus/estrus. **c.** Serum hormone levels at proestrus or estrus (n=4). **d.** Representative VLF imaging after E2 or P4 treatment at day -1. **e.** Fungal loads in VLF on days 3 and 7 post-infection after sesame oil (Vehicle) (n=7), estrogen (E2) (n=6-7), or progesterone (P4) (n=11-13) given on days -3 and 4 relative to infection. **f.** LDH activity in VLF on days 3 and 7. Data in **c** and **f** are mean ± SEM; two-tailed unpaired Student's t-test and data in **e** are geometric mean ± geometric SD with Kruskal-Wallis test with Dunn's multiple comparisons.

Supplementary Materials

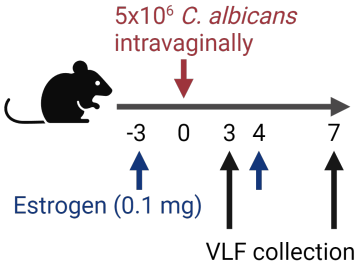
Table S1. Cytokine changes in VLF in E2-treated mice

Table S2. Cytokines in VLF in Vehicle (SO)-treated mice

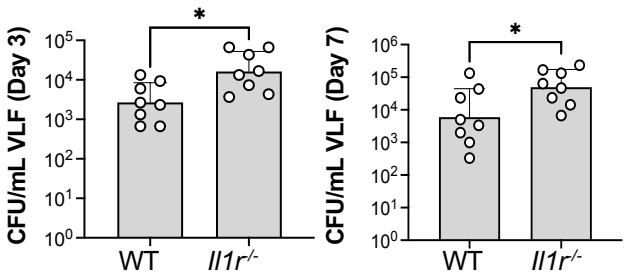
Fig S1. Sample cytology of WT and *Il17raIl22ra1*$^{-/-}$ mice determined by Pap staining. Size bar indicates 200 µM.

Fig S2. Sample cytology of estrus cycle phases in WT and *Il17raIl22ra1*$^{-/-}$ mice determined by Pap staining. Size bar indicates 200 µM.

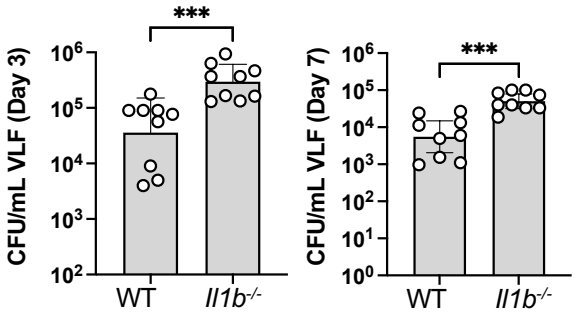
A.


5x10⁶ C. albicans
intravaginally
-3
0
3
4
7
Estrogen (0.1 mg)
VLF collection

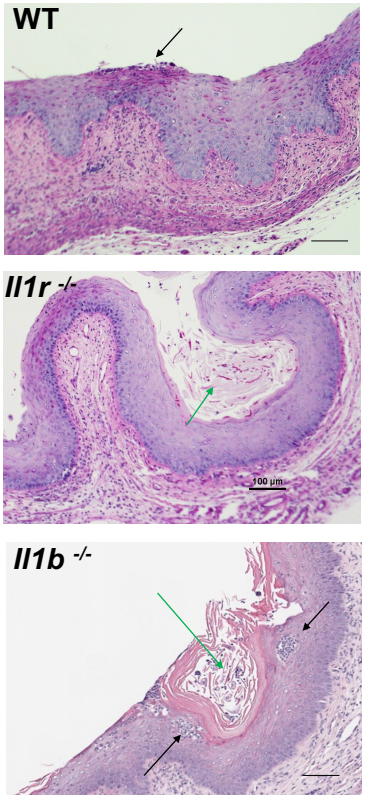
B.


CFU/mL VLF (Day 3)
CFU/mL VLF (Day 7)
*
WT
Il1r-/-

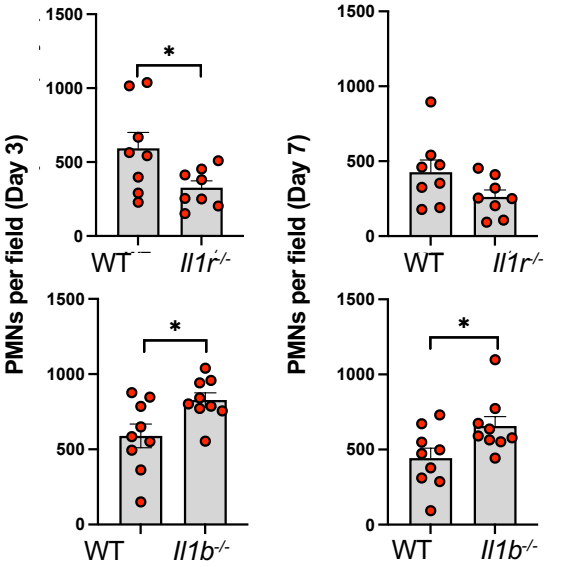
C.


CFU/mL VLF (Day 3)
CFU/mL VLF (Day 7)
***
WT
Il1b-/-

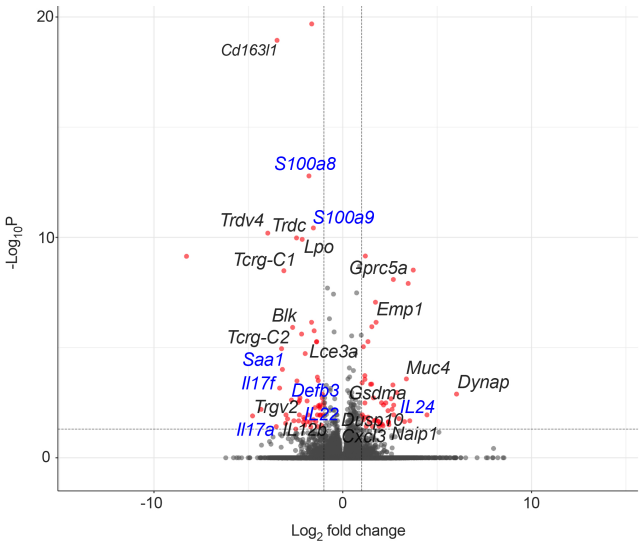
D.

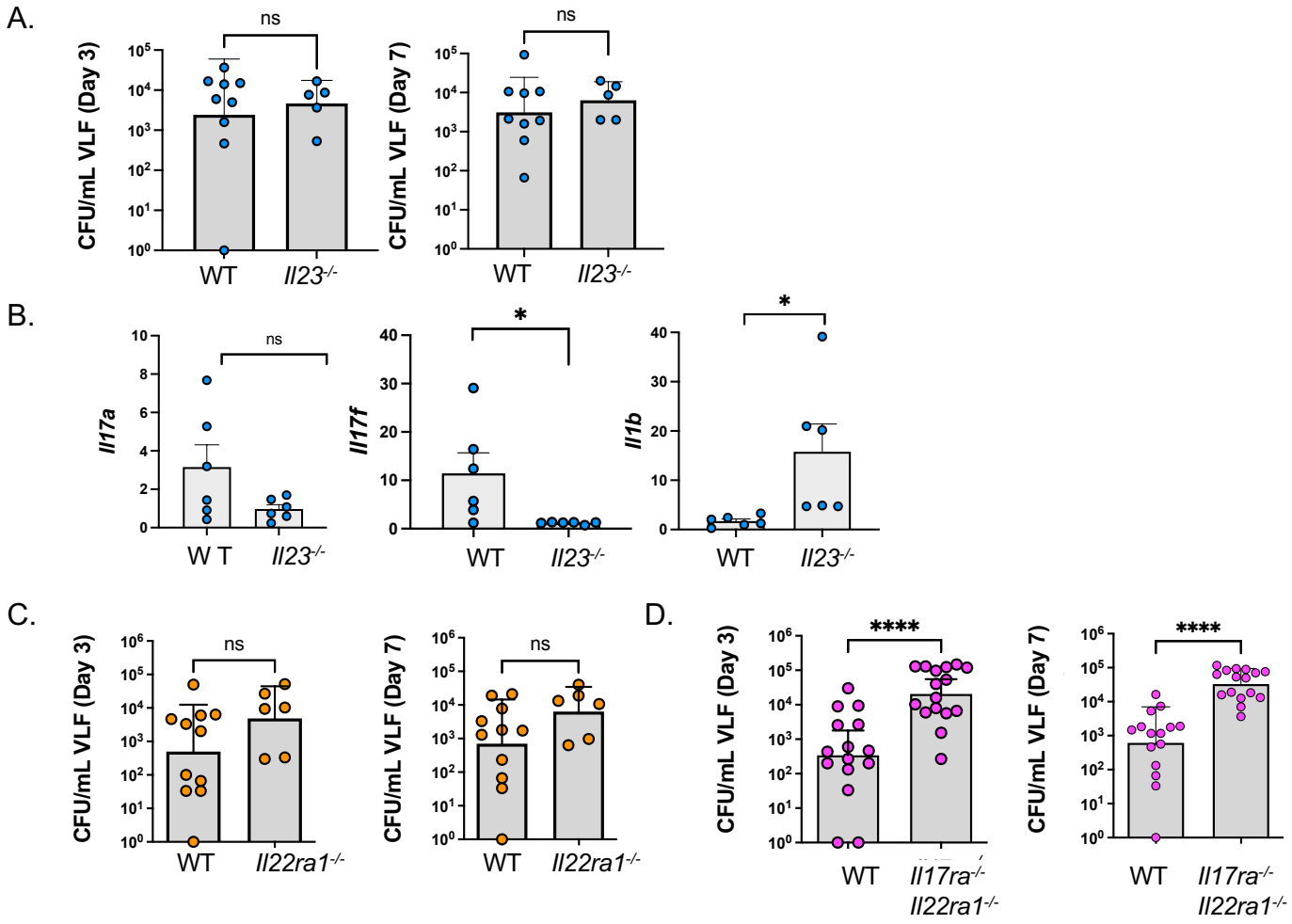

WT
Il1r -/-
Il1b -/-
100 µm

E.


PMNs per field (Day 3)
PMNs per field (Day 7)
*
WT
Il1r-/-
Il1b-/-

F.


Cd163l1
S100a8
S100a9
Trdv4
Trdc
Lpo
Tcrg-C1
Gprc5a
Emp1
Blk
Tcrg-C2
Lce3a
Saa1
Muc4
Dynap
Il17f
Defb3
Gsdma
IL24
Trgv2
IL22
Dusp10
Il17a
Il12b
Cxcl3
Naip1
-Log10P
Log2 fold change


A.
CFU/mL VLF (Day 3)
CFU/mL VLF (Day 7)
ns
WT
Il23-/-
B.
Il17a
Il17f
Il1b
ns
*
WT
Il23-/-
C.
CFU/mL VLF (Day 3)
CFU/mL VLF (Day 7)
ns
WT
Il22ra1-/-
D.
CFU/mL VLF (Day 3)
CFU/mL VLF (Day 7)
****
WT
Il17ra-/-
Il22ra1-/-

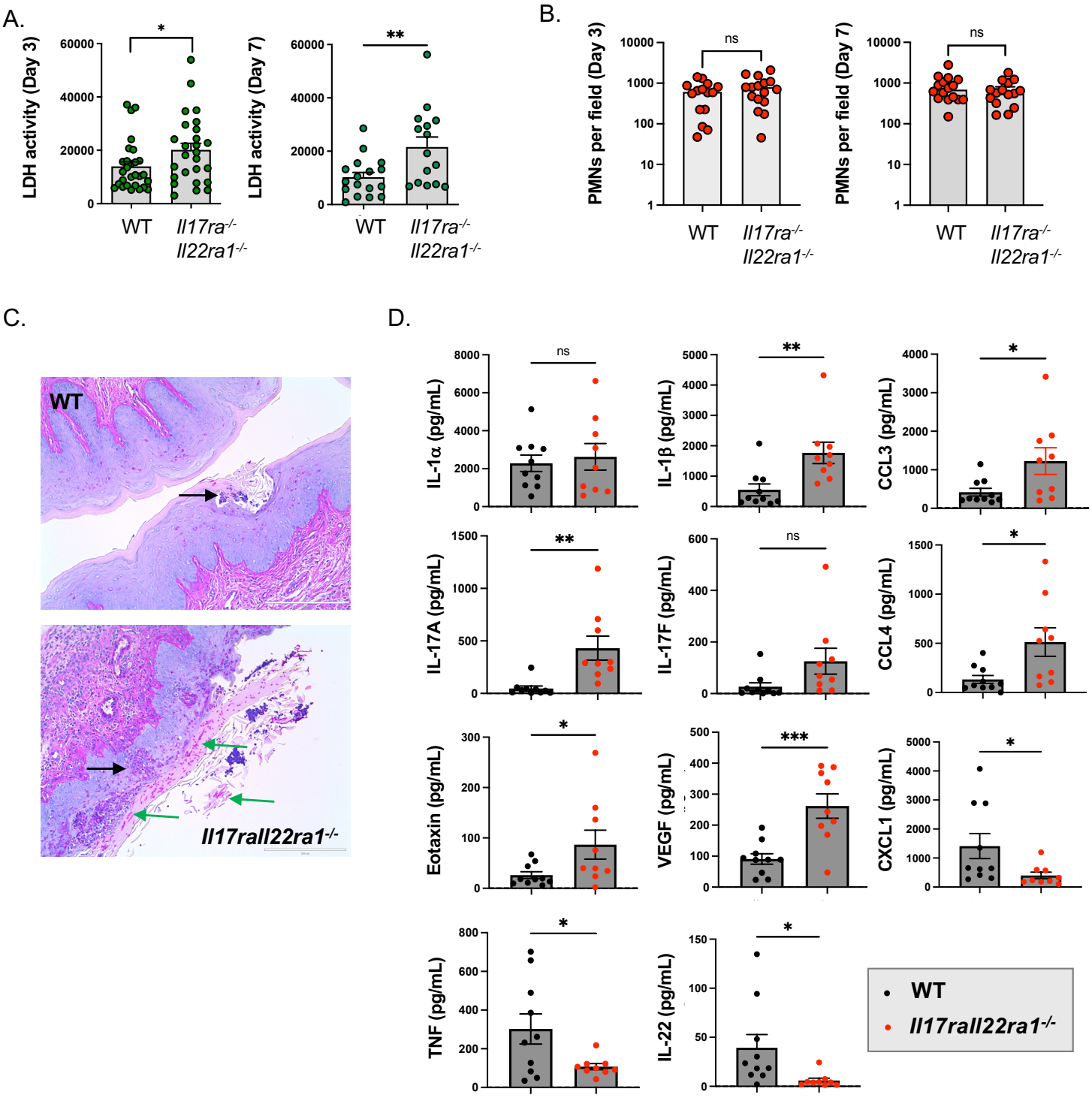
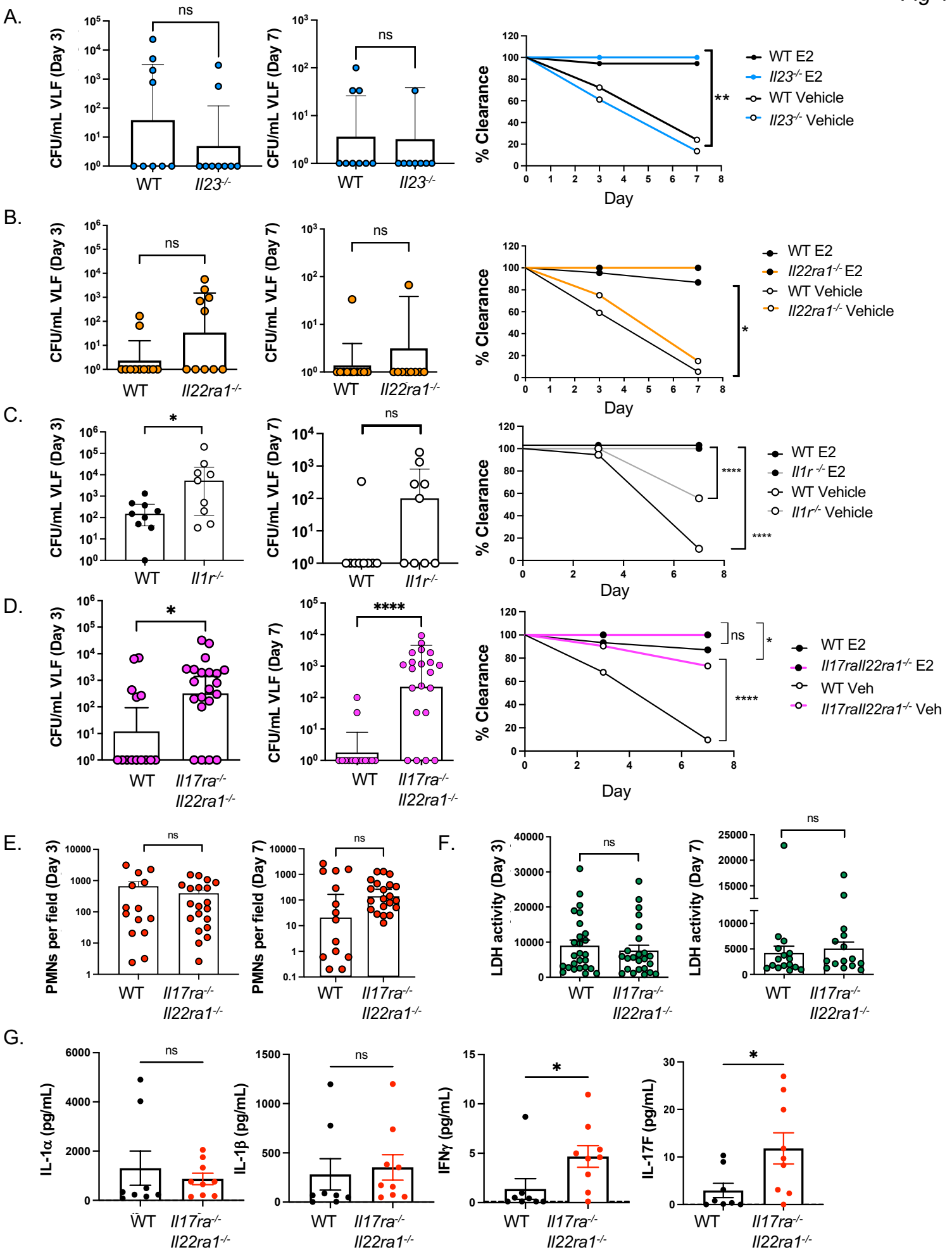
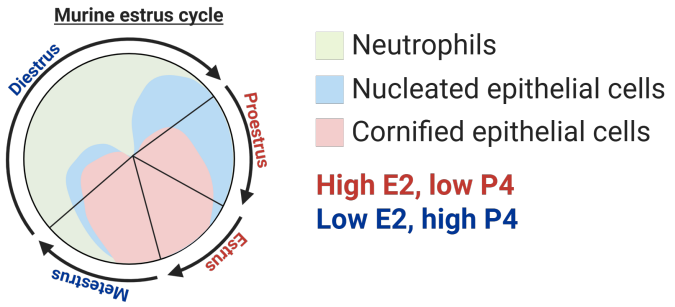

A.
LDH activity (Day 3)
60000
40000
20000
0
*
WT
Il17ra-/-
Il22ra1-/-
LDH activity (Day 7)
**
B.
PMNs per field (Day 3)
10000
1000
100
10
1
ns
PMNs per field (Day 7)
C.
WT
Il17raIl22ra1-/-
D.
IL-1α (pg/mL)
IL-1β (pg/mL)
CCL3 (pg/mL)
IL-17A (pg/mL)
IL-17F (pg/mL)
CCL4 (pg/mL)
Eotaxin (pg/mL)
VEGF (pg/mL)
***
CXCL1 (pg/mL)
TNF (pg/mL)
IL-22 (pg/mL)
WT
Il17raIl22ra1-/-

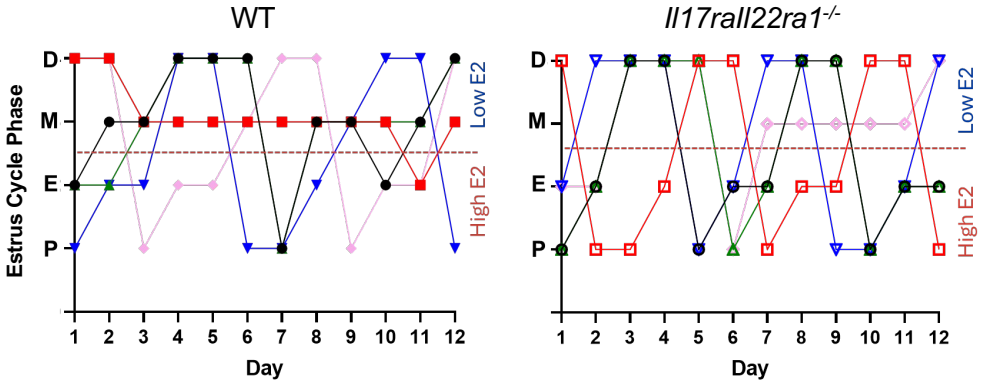
Fig 4


A.
CFU/mL VLF (Day 3)
ns
WT
Il23-/-
CFU/mL VLF (Day 7)
ns
% Clearance
Day
**
WT E2
Il23-/- E2
WT Vehicle
Il23-/- Vehicle
B.
CFU/mL VLF (Day 3)
ns
WT
Il22ra1-/-
CFU/mL VLF (Day 7)
ns
% Clearance
Day
*
WT E2
Il22ra1-/- E2
WT Vehicle
Il22ra1-/- Vehicle
C.
CFU/mL VLF (Day 3)
*
WT
Il1r-/-
CFU/mL VLF (Day 7)
ns
% Clearance
Day
****
****
WT E2
Il1r-/- E2
WT Vehicle
Il1r-/- Vehicle
D.
CFU/mL VLF (Day 3)
*
WT
Il17ra-/-
Il22ra1-/-
CFU/mL VLF (Day 7)
****
% Clearance
Day
ns
*
****
WT E2
Il17raIl22ra1-/- E2
WT Veh
Il17raIl22ra1-/- Veh
E.
PMNs per field (Day 3)
ns
PMNs per field (Day 7)
ns
F.
LDH activity (Day 3)
ns
LDH activity (Day 7)
ns
G.
IL-1α (pg/mL)
ns
IL-1β (pg/mL)
ns
IFNγ (pg/mL)
*
IL-17F (pg/mL)
*

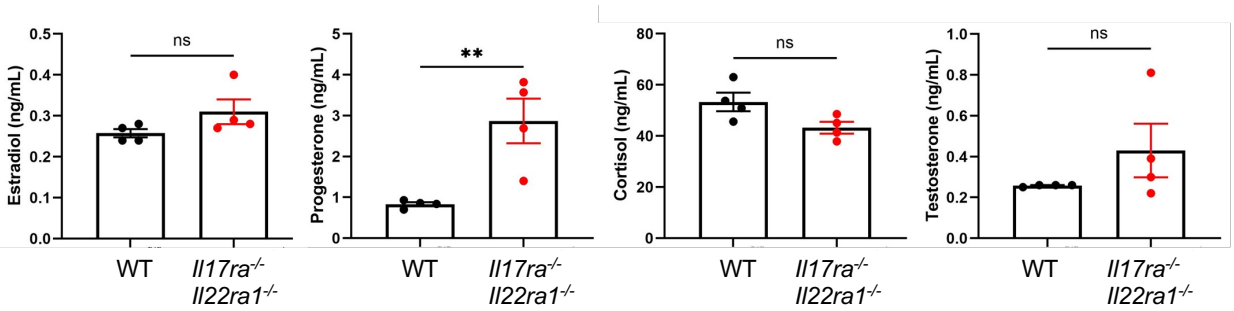
A.


Murine estrus cycle
Diestrus
Proestrus
Estrus
Metestrus
Neutrophils
Nucleated epithelial cells
Cornified epithelial cells
High E2, low P4
Low E2, high P4

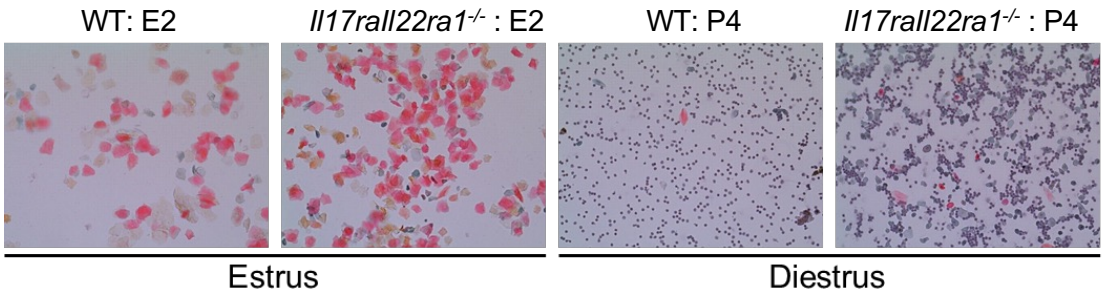
B.


WT
Il17ralI22ra1-/-
Estrus Cycle Phase
D
M
E
P
Low E2
High E2
Day
1 2 3 4 5 6 7 8 9 10 11 12

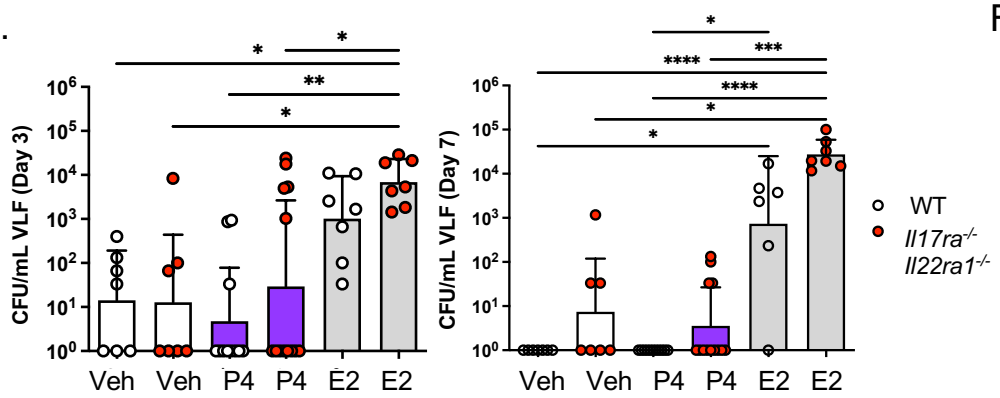
C.


Estradiol (ng/mL)
ns
Progesterone (ng/mL)
**
Cortisol (ng/mL)
ns
Testosterone (ng/mL)
ns
WT
Il17ra-/- Il22ra1-/-

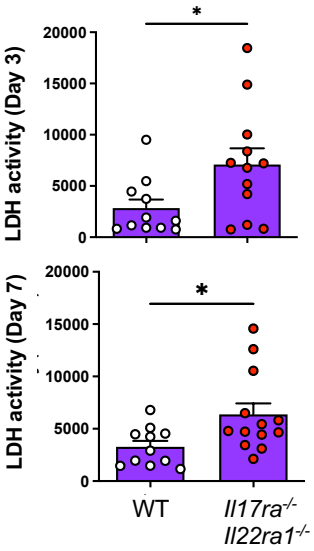
D.


WT: E2
Il17ralI22ra1-/- : E2
WT: P4
Il17ralI22ra1-/- : P4
Estrus
Diestrus

E.


CFU/mL VLF (Day 3)
CFU/mL VLF (Day 7)
Veh Veh P4 P4 E2 E2
WT
Il17ra-/- Il22ra1-/-

F.


LDH activity (Day 3)
LDH activity (Day 7)
WT
Il17ra-/- Il22ra1-/-