Il4ra-independent vaginal eosinophil accumulation following helminth infection exacerbates epithelial ulcerative pathology of HSV-2 infection

Graphical abstract

Highlights
- Helminth infection promotes pathology to vaginal HSV-2 infection
- Promoted pathology is IL-5, IL-33 mediated but Il4ra independent
- The enhanced vaginal epithelial ulceration is eosinophil driven

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In brief
Chetty et al. demonstrate that helminth infection systemically enhances vaginal pathology in subsequent HSV-2 infection. This is a result of an IL-5-promoted type 2 immunity expanding eosinophils in the vagina. These eosinophils cause an enhanced vaginal epithelial ulceration in co-infected mice.
**Summary**

How helminths influence the pathogenesis of sexually transmitted viral infections is not comprehensively understood. Here, we show that an acute helminth infection (*Nippostrongylus brasiliensis* [Nb]) induced a type 2 immune profile in the female genital tract (FGT). This leads to heightened epithelial ulceration and pathology in subsequent herpes simplex virus (HSV)-2 infection. This was IL-5-dependent but IL-4 receptor alpha (*Il4ra*) independent, associated with increased FGT eosinophils, raised vaginal IL-33, and enhanced epithelial necrosis. Vaginal eosinophil accumulation was promoted by IL-33 induction following targeted vaginal epithelium damage from a papain challenge. Inhibition of IL-33 protected against Nb-exacerbated HSV-2 pathology. Eosinophil depletion reduced IL-33 release and HSV-2 ulceration in Nb-infected mice. These findings demonstrate that Nb-initiated FGT eosinophil recruitment promotes an eosinophil, IL-33, and IL-5 inflammatory circuit that enhances vaginal epithelial necrosis and pathology following HSV-2 infection. These findings identify a mechanistic framework as to how helminth infections can exacerbate viral-induced vaginal pathology.

**Introduction**

Parasitic nematode infections and sexually transmitted viral infections (STVIs) occur at high rates in the same geographical locations, especially in low-middle income countries (LMICs) (Looker et al., 2015; WHO, 2002). Nematode infections do not normally colonize or transit the female genital tract (FGT), yet these infections have been associated with changes in both female fecundity (Blackwell et al., 2015) and FGT immunity (Chetty et al., 2020; Gravitt et al., 2016). For example, *Trichuris-trichiura*-infected women can display a distinct FGT type 2 cytokine profile associated with increased risk of human papillomavirus (HPV) infection (Gravitt et al., 2016). Nematode infections therefore appear to profoundly influence FGT biology and...
a critical consequence of this effect may be increased risk of infection and/or pathology from STVI.s.

Nematode infections are known to alter host immunity in uncolonized tissue, these systemic effects can have important consequences for unrelated conditions at these sites. Effects can be complex and appear to be largely dependent on the context and biology of the helminth infection. For example, Schistosoma-
mansonii- and Nippostrongylus brasiliensis (Nb)-induced IL-4 can promote CD8⁺ T cell-driven control of murine gammaher-
pesvirus (Rolut et al., 2018), while Heligmosomoides polygyrus
infection and S. mansoni egg challenge can impair host control of
this virus (Reese et al., 2014). Natural and vaccine-mediated
immunity to a pathogen can also be impaired; Nb infection
reduces host control of non-typhoidal salmonella infection and
induction of protective vaccination (Bobat et al., 2014). Addition-
ally, canonical cellular responses to helminth infection, such as
eosinophil recruitment, have been strongly associated with
improved host control during Mycobacterium tuberculosis infec-
tion (O’Shea et al., 2018). Helminth infections therefore have
diverse and critical influences on a host’s ability to control unre-
lated infections. How helminths infections alter vaginal immunity
and susceptibility to infections is currently not fully understood.

Genital herpes simplex virus (HSV)-2 is a common STVI world-
wide, with high prevalence in helmint endemics regions and is
associated with poorer male and female reproductive health
(Freeman et al., 2006; Looker et al., 2017, 2015; Phipps et al.,
2016). Initial host immunity to HSV-2 is classically driven by
innate type 1 interferon (IFN)–promoted natural killer (NK) cell
activity and subsequent IFN-γ, CD4⁺, and CD8⁺ T cell control (Lee
et al., 2017; Milligan and Bernstein, 1997; Thapa et al., 2007).
Type 1 responses are sensitive to downregulation by pre-existing or concurrent type 2 Th2 immune responses (Chang and
Aune, 2007; Szabo et al., 1997; Wei et al., 2010). To date, direct
demonstration of the consequences of helmint-induced type 2
immunity in the FGT, on immune control of HSV-2 infection, has
not been described. Artificial induction of type 2 immunity in
the FGT can increase pathogenesis of HSV-2 infection, for example, raised IL-33 levels were shown to enhance HSV-2 pathology and impair type 1 responses (Oh et al., 2016). Demonstrating whether nematode infections drive altered immunity to HSV-2 therefore remains an important and largely unanswered biological question that could have significant implications for female reproduc-
tive health. To gain insight into how helmint infections can alter
STVI infections, this study uses a pre-clinical model to dem-
onstrate that current and prior Nb infections initiate profound
Il4ra-independent expansion of eosinophils in the FGT. This
eosinophil expansion promotes increased vaginal epithelial ul-
ceration following HSV-2 infection. These findings identify a
nematode-induced systemic effect on immunity that promotes
enhanced pathogenesis from a subsequent HSV-2 infection.

RESULTS

Nb infection results in a type 2 immune signature in
the FGT
To test whether helmint infections can alter the underlying
immune function of the FGT and associated lymph nodes, immune
homeostasis in the FGT of hormone-synchronized mice was as-
Sessed at day 9 (immediately post-worm expulsion) and day 21
(12 days post-resolution) following infection (Figure 1A). In FGT
of Nb-infected mice, raised levels of the epithelial alarmin IL-33
and canonical type 2 cytokines, IL-4 and IL-5, were found
when compared with uninfected controls (Figure 1B). Raised
IL-33 level is likely to be a consequence of epithelial stress,
immunofluorescence (IF) staining of vaginal tissue for IL-33 and
β-catenin (Bcat) confirmed vaginal epithelial cells to be the pre-
dominant source of IL-33 following Nb infection (Figure S1).
Further histological analysis of FGT tissue demonstrated
increased myeloid cell infiltration (Figure 1C) in Nb-infected
mice when compared with uninfected mice. Sirius red staining
indicated a significant element of this infiltrate were eosinophils
(Figure 1D). Flow cytometric analysis of FGT myeloid cell popu-
lations according to the applied gating strategy (Figure S2) confirmed this observation. Nb-infected mice showed a signifi-
cant increase in the proportions and numbers of eosinophils
(CD11b⁺SiglecF⁺SSCh⁺), a modest induction of Ly6C⁺inflamma-
tory monocytes (CD11b⁺Ly6C⁺Ly6G⁻), and a trend for
increased neutrophils (CD11b⁺Ly6G⁺) in the FGT, in comparison
with uninfected mice (Figure 1E). Comparative analysis of
myeloid cells in the associated iliac lymph nodes (iLNs) also re-
vealed increased numbers of eosinophils, Ly6C⁺ monocytes,
and neutrophils in mice 9 days post-infection (dpi) compared
with uninfected controls (Figure S3A). The expanded FGT eosin-
ophil population was maintained at 21 dpi (Figure 1E), indicating
a long-term effect of Nb on FGT immunity.

Little data exist on eosinophil phenotypes in the FGT. To
address this, we compared the expression of established
markers of eosinophil function in the lung and FGT (Figure 1F).
As expected, in the lung, we identified Siglec-F⁺ and Siglec-
Fint eosinophil populations that are representative of recruited
and resident eosinophils (Mesnil et al., 2016). Relative expres-
sion of CD11b⁺, CD62L⁺, and CD49d on eosinophils in the lung
and FGT was used to identify molecular characteristics of these
populations (Grayson et al., 1998; Percopo et al., 2017; Veen
et al., 1998; Walker et al., 1993). We found Nb-infected FGT eosin-
ophils to be Siglec-F⁻ and Siglec-F⁺ populations that are representative of recruited
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populations (Grayson et al., 1998; Percopo et al., 2017; Veen
et al., 1998; Walker et al., 1993). We found Nb-infected FGT eosin-
ophils to be Siglec-F⁻CD11b⁻CD62L⁻CD49d⁻, a pheno-
type distinct from Siglec-F⁺ and int populations found at the
site of infection (lung) (Figure 1F). Nb-induced FGT eosinophils
expressed raised levels of the integrins CD11b and L-selectin
(CD62L) relative to Siglec-F hi and int populations found at the
site of infection and Nb 9 dpi mice (Figure S3B). Together, this suggests that FGT eosinophils are predominantly
a recruited population following Nb infection.

To identify changes in FGT epithelial integrity following Nb
infection, IF staining for Bcat and cleaved caspase-3 (c-Casp-
3) was carried out. This revealed equivalent epithelial integrity
in vaginal tissue of uninfected and Nb-infected mice (Figure S3B).
Together these findings show that Nb infection induces expan-
sion of myeloid cell populations in the FGT, and although this
did not result in distinct histological changes to epithelium,
raised levels of IL-33 in vaginal lavages and in the epithelium (Fig-
ure S1) suggests the presence of epithelial stress.

HSV-2 FGT pathology is exacerbated with prior Nb
infection
To test whether Nb-associated immune changes in the FGT had
a consequence on an unrelated infection, we infected mice

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intravaginally with HSV-2, 7 days after Nb infection (Figure 2A). Nb + HSV-2 co-infection resulted in a step shift elevation in genital pathology, with raised vaginal inflammation from day 3 post-HSV-2 infection and increased genital ulceration by day 6, when compared with HSV-2-only-infected mice (Figure 2B). No significant differences in viral shedding were observed between co-infected and virus-only mice, at days 3 and 6 post-HSV-2 infection (Figure 2C), which suggests that the increased pathology following a prior Nb infection was not a result of changes to viral replication. Histological analysis of vaginal tissue at day 6 post-HSV-2 infection revealed increased vaginal epithelial ulceration in co-infected mice when compared with HSV-2-only mice (Figure 2D). IF analysis of vaginal tissue at day 3 post-HSV-2 infection identified a trend for increased epithelial cell necrosis (i.e., loss of membranous Bcat, dispersed DNA, but negative for apoptosis marker c-Casp-3) at the site of ulcer formation in co-infected mice when compared with HSV-2-only mice (Figures 2E and S4). This supports virus-induced epithelial necrosis, rather than apoptosis, underlying epithelial ulceration and that prior Nb infection enhances the onset of necrosis.

Further analysis of vaginal epithelial cells at day 3 post-HSV-2 infection identified reduced expression of MHCII on Nb + HSV-2 vaginal epithelial cells and a trend for less MHCII (Figure 2F). Suppressed MHC presentation in virally infected epithelial cells is associated with evasion of host cytotoxic lymphocyte responses (Neumann et al., 2003; Orr et al., 2005). Host interferon responses can counteract virus-induced downregulation of MHC expression (Härle et al., 2001; Mikloska and Cunningham, 2001; Parr and Parr, 1999). We found reduced vaginal epithelial MHC expression in co-infected mice associated with reduced...
Figure 2. Prior Nb exposure results in earlier and exacerbated HSV-2 pathology

(A) 7 days post-Nb infection, mice were infected intravaginally with $5 \times 10^5$ plaque-forming units (PFUs) HSV-2.

(B) Viral progression was determined by daily pathology scoring.
detection of antiviral IFN-γ (Figure 2G) in vaginal lavages compared with HSV-2-only mice at day 2 post-viral infection. Levels of IFN-γ-induced transcription factor STAT1 were also reduced in the FGT of co-infected mice compared with virus-only controls (Figure 2H). Together, these findings indicate that prior Nb infection may impair epithelial anti-viral responses; however, this effect may be insufficient to alter viral load. We also detected raised IL-33 levels in lavages of co-infected mice compared with HSV-2-only-infected mice (Figure 2I). IF analysis of HSV-2- and Nb + HSV-2-infected vaginal tissue identified a predominant detection of IL-33 in epithelial cells (Figure 2J). We suggest that the raised IL-33 detected in vaginal lavages is a consequence of increased epithelial necrosis and subsequent release of IL-33 in co-infected mice.

**Exacerbated viral pathology is associated with Nb-induced type 2 immunity in the FGT**

Histological analysis of FGT at the onset of more severe pathology in Nb + HSV-2 mice (day 3 post-virus infection), identified increased cellular infiltration at the sites of epithelial ulceration in co-infected mice compared with HSV-2 alone (Figure S5A). Flow cytometric analysis of the FGT at this time point supported these observations with significantly increased proportions and numbers of eosinophils, as well as inflammatory monocytes and neutrophils in the FGT of co-infected mice compared with virus-only controls. (Figures 3A and S5B). Importantly, increased FGT eosinophils were a feature of co-infected mice and not HSV-2 infection alone. Numbers of neutrophils, Ly6Ch monocytes, macrophages (CD11b+F4/80+), and eosinophils were also raised in the iLN of Nb + HSV-2 mice (Figure S5C). Sirius red staining confirmed increased infiltration of eosinophils into the genital submucosa compared with virus-only-infected mice at day 3 post-HSV-2 infection (Figures 3B and 3Ci). Eosinophil infiltration was also observed at the vaginal epithelial layer in co-infected tissues but not HSV-2-only-infected tissue (Figure 3Ci). Increased detection of the eosinophil granule protein, major basic protein (MBP) was found in co-infected FGT compared with HSV-2 alone, at day 2 post-virus infection (Figure 3D). This supported a role for eosinophils in causing epithelial necrosis as MBP has been demonstrated to be an important cause of epithelial necrosis (Filley et al., 1982).

To further identify if helminth-induced genital eosinophils could promote pathology following HSV-2 infection, expression of established markers of eosinophil function were quantified (Figures 3E and S5D). This analysis revealed increased expression of Ly6C, a proinflammation marker (Percoo et al., 2017), and integrin CD49d, a marker of eosinophil recruitment (Walsh et al., 1996) and survival (Meerschaert et al., 1999) in Nb + HSV-2 genital eosinophils compared with Nb 9dpi (Figure 3E). Together with increased MBP, this suggests raised persistence and inflammatory action of Nb-induced FGT eosinophils during vaginal viral infection that may promote epithelial pathology.

During Nb infection, epithelial IL-33 has been shown to be a significant contributor to the induction of mucosal type 2 immunity, activating ILC2s, which in turn release IL-5 to promote eosinophil recruitment (Hung et al., 2013; Nussbaum et al., 2013). Here, we identified that Nb infection promoted this effect in the FGT; increased levels of lavage IL-5, along with raised proportions and numbers of Lin+IL-7Rα+ICOS+S'T2+ ILC2s, and IL-5-producing Lin+IL-7Rα+ICOS+ cells were found in the FGT and iLN of Nb + HSV-2 compared with HSV-2-only-infected mice (Figures 3F–3H and S5C). Significantly increased proportions and numbers of IL-5-producing inflammatory monocytes were also found in co-infected mice (Figure 3I). Together, these findings identify that host lymphoid and myeloid IL-5 responses in the FGT provide an environment conducive to enabling eosinophil population expansion in Nb + HSV-2 mice.

To test whether IL-5 contributed to Nb-exacerbated viral pathology, we depleted the cytokine with anti-IL-5 (x-IL-5), prior to and during early HSV-2 infection (Figure S6A). x-IL-5-treated co-infected mice displayed significantly reduced pathology and ulcerated epithelium (Figures 3J and S6B) compared with isotype control. Flow cytometry analysis of iLN and Sirius red staining of vaginal tissue at day 6 post-HSV-2 infection confirmed eosinophil depletion following x-IL-5 treatment (Figures 3J and S6C). Exacerbated viral pathology in helminth-exposed mice was therefore associated with type 2 immune imprinting in uncolonized FGT, which was characterized by an eosinophil influx associated with expanded ILC2 and monocyte sources of IL-5.

**Nb-induced raised vaginal HSV-2 pathology is independent of Il4ra signaling**

Increased genital pathology in Nb + HSV-2-infected mice correlated with raised canonical type 2 IL-4 and IL-5 responses in the FGT and an impaired IFN-γ response. To identify whether, in addition to IL-5, IL-4/IL-13 also contributed to enhanced pathology, either directly or via a classical type 2 antagonism of type 1
immunity, i.e., IL-4 and STAT6 impairment of IFN-γ production (Reese et al., 2014). Il4ra knockout (Il4ra−/−), mice were infected with Nb + HSV-2 or HSV-2 alone. Significantly reduced pathology and viral shedding was observed in HSV-2-infected Il4ra−/− mice when compared with wild-type (WT) counterparts. However, unexpectedly, we did not observe significant differences in pathology or viral PFUs, between WT and Il4ra−/− co-infected mice (Figures 4A and 4B). Therefore, Nb-promoted HSV-2 pathology was Il4ra independent.

Associated with the onset of genital pathology in both WT and Il4ra−/− Nb + HSV-2 co-infected mice was increased IL-5 and reduced IFN-γ levels, in FGT tissue and lavages, respectively, at day 2 post-HSV-2 infection (Figure 4C). Furthermore, histological analysis of vaginal tissue at day 6 post-HSV-2 revealed increased vaginal epithelial ulceration in WT and Il4ra−/− co-infected mice compared with HSV-2-only controls (Figure 4D). Sirius red staining exposed increased infiltration of eosinophils in the vaginal stroma and epithelium of co-infected WT and Il4ra−/− mice (Figure 4E). Flow cytometry analysis of eosinophils confirmed that heightened HSV-2 pathology following Nb infection was accompanied by raised FGT eosinophils in both WT and Il4ra−/− co-infected mice at day 3 post-viral infection (Figure 4F). Of note was the detection of raised eosinophil populations in Il4ra−/− co-infected mice. We suggest that this may be a consequence of persistence of Nb infection in these mice. Together, these findings and those in Figure 3 identify that enhanced pathology in co-infected mice is independent of Il4ra and instead mediated by the activation of an IL-5 type 2 immune axis.

**Il4ra-independent, IL-33-mediated induction of FGT eosinophils**

To test whether epithelial-damage-associated IL-33 release could contribute to eosinophil recruitment in the FGT, we challenged WT and Il4ra−/− mice intravaginally with the serine protease papain (Figure 5A), which promotes IL-33 release predominantly by vaginal epithelial cells (Oh et al., 2017) and has been shown to drive an IL-33-dependent abrogation of host control of HSV-2 (Oh et al., 2016). We found that papain treatment resulted in equivalent numbers of FGT eosinophils in both WT and Il4ra−/− mice compared with untreated controls (Figure 5B). Moreover, induction of FGT ILC2s following intravaginal papain treatment, was also maintained in the absence of Il4ra signaling (Figure 5C).

To identify if IL-33 contributed to raised pathology in co-infection, we treated Nb + HSV-2 mice intravaginally with the IL-33 inhibitor heligmosomoides polygyrus alarmin release inhibitor (HpARI) (Figure 5D) (Osbourn et al., 2017). Both WT and Il4ra−/− HpARI-treated co-infected mice displayed reduced pathology compared with untreated counterparts (Figure 5E), as well as reduced epithelial ulceration at day 6 post-HSV-2 infection (Figure 5F). Flow cytometry analysis at this time point showed a trend for reduced numbers of ILC2s and significantly less IL-5-producing ILC2s in the FGT of HpARI-treated Nb + HSV-2 mice compared with untreated counterparts (Figures S7B and S7C). Together, these findings support IL-33 driving an Il4ra-independent induction of the IL-5 type 2 immune axis, which contributes to exacerbated HSV-2 pathology in co-infected mice.

**Eosinophil depletion abrogates helmint-exacerbated HSV-2 pathology**

To confirm if Nb-induced FGT eosinophils contributes to elevated HSV-2 pathology, we depleted eosinophils using α-Siglec-F antibody prior to virus infection (Figure 6A). α-Siglec-F treatment significantly reduced eosinophils in the FGT, ILN, spleen and lung compared with treatment with isotype control (Figure S7D). Following HSV-2 infection, α-Siglec-F-treated co-infected mice displayed rescued pathology equivalent to HSV-2-only mice and significantly less than isotype-treated co-infected mice (Figure 6B). No significant differences in viral shedding at day 3 and day 6 post-HSV-2 infection were found (Figure 6C). These findings were also supported by co-infection of ΔdblGata1−/− mice, which lack eosinophils, also resulting in significant reduction in vaginal pathology (Figure S7E). Histological analysis of vaginal tissue at day 6 post-HSV-2 infection demonstrated reduced ulcerated epithelium in α-Siglec-F-treated Nb + HSV-2 mice equivalent to that seen in virus-only-infected mice (Figure 6D). If analysis of vaginal tissue at this time point showed equivalent detection of c-Casp-3-positive cells in the intact epithelium of all groups, notable loss of vaginal epithelium (Bcat) was observed in isotype-treated Nb + HSV-2 mice compared with HSV-2-only and α-Siglec-F-treated co-infected mice (Figure 6E). Flow cytometry analysis and Sirius red staining...
of the FGT at days 3 and 6, respectively, confirmed a significant reduction in FGT eosinophils in α-Siglec-F-treated co-infected mice (Figures 6F and 6G). These findings support increased epithelial ulceration and reduced tissue integrity in co-infected mice, as being mediated by FGT eosinophil accumulation. Along with these findings, α-Siglec-F treatment also reduced numbers of the FGT at days 3 and 6, respectively, confirmed a significant reduction in FGT eosinophils in α-Siglec-F-treated co-infected mice (Figures 6F and 6G). These findings support increased epithelial ulceration and reduced tissue integrity in co-infected mice, as being mediated by FGT eosinophil accumulation. Along with these findings, α-Siglec-F treatment also reduced numbers...
of ILC2s in FGT (Figure 6H). This reduction in FGT ILC2s associated with significantly reduced levels of vaginal IL-33 in α-Siglec-F-treated co-infected mice (Figure 6I). Together, these data demonstrate that the exacerbation of HSV-2 pathology following prior Nb infection is dependent on an Nb-induced FGT eosinophil population. Additionally, we show that prior Nb infection results in a step increase in pathology to a subsequent vaginal HSV-2 infection. Depletion of eosinophils protected against this increased HSV-2-induced genital pathology. Furthermore, increased HSV-2 ulceration was dependent on IL-5 and IL-33 and correlated with an Il4ra-independent induction of ILC2s, which would further promote eosinophil action in the FGT. These findings may provide a mechanistic framework that explains the association between raised type 2 cytokine profiles in cervical fluids of nematode-infected women and their increased risk of viral infection (Gravitt et al., 2016).

DISCUSSION

In this study, we identify that systemic immunity to nematode infection results in a type 2 immune profile in uncolonized female genital tissue, a salient feature of this is an enhanced and persistent FGT eosinophil population. Additionally, we show that prior Nb infection results in a step increase in pathology to a subsequent vaginal HSV-2 infection. Depletion of eosinophils protected against this increased HSV-2-induced genital pathology. Furthermore, increased HSV-2 ulceration was dependent on IL-5 and IL-33 and correlated with an Il4ra-independent induction of ILC2s, which would further promote eosinophil action in the FGT. These findings may provide a mechanistic framework that explains the association between raised type 2 cytokine profiles in cervical fluids of nematode-infected women and their increased risk of viral infection (Gravitt et al., 2016).
Weller, 2012; Chitkara and Krishna, 2006; Ekin et al., 2016; Simet et al., 2017). But our findings do agree with roles of helminthutor to pathologies resulting from helminth migration including

(F) Numbers of FGT eosinophils at day 3 post-virus infection in isotype control and

(E) IF analysis of day 6 post-HSV-2 vaginal tissue. White boxes indicate magnified sections.

(C) Viral shedding was measured by plaque assay of days 3 and 6 vaginal washes.

(G) Representative magnified sections (n = 3) of Sirius-red-stained vaginal tissue at day 6 post-HSV-2. Images were taken at 1,000 magnification. White boxes indicate magnified sections.

Figure 6. Depletion of eosinophils rescues HSV-2 pathology in co-infected mice
(A) Co-infected mice were treated with 20 μg α-Siglec-F or isotype control antibody at days 5, 7, and 9 post-Nb infection.

(B) Viral progression in HSV-2-only, α-Siglec-F-, and isotype-treated mice was determined by daily pathology scoring (*Nb + HSV-2 isotype control versus Nb + HSV-2-α-Siglec-F; †HSV-2 only versus Nb + HSV-2 isotype control).

(C) Viral shedding was measured by plaque assay of days 3 and 6 vaginal washes.

(D) Representative H&E-stained sections (n = 3) of vaginal tissue at day 6 post-HSV-2 infection. Images were taken at ×50 magnification. Ulcerated vaginal epithelium is indicated by black dotted lines and qualified as percentage (%) of ulcerated epithelium. Yellow boxes indicate magnified sections in (G).

(F) Numbers of FGT eosinophils at day 3 post-virus infection in isotype control and α-Siglec-F-treated Nb + HSV-2 mice compared with Nb 9dpi and HSV-2 only controls.

(H) Numbers of FGT IL22s at day 3 post-HSV-2 infection.

(I) At day 2 post-HSV-2 infection, lavage or FGT levels of IL-33 and IFN-γ were measured by ELISA. Data are representative of two independent experiments with 3 mice per group (mean ± SEM). Statistical significance was calculated by two-way ANOVA with Bonferroni correction for multiple comparisons and Mann-Whitney t-test: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
and eosinophil accumulation equivalent to that seen in WT mice identifies redundancy in Il4ra signaling in our model. Rapid induction of both ILC2 and eosinophils, suggests T cell independence and lack of Il4ra largely precludes a role for IL-13. Our observations and experiments therefore strongly support an IL-33, ILC2/monocyte, IL-5 axis driving vaginal eosinophils.

The current understanding of eosinophil function in the FGT is limited, yet the evidence indicates complex functions, depending on the location of the eosinophil response. Related to our findings, antibiotic-mediated vaginal dysbiosis has been shown to increase HSV-2 vaginal pathology, via epithelial IL-33 impairment of anti-viral immunity; however, this study does not address any role for eosinophils in promoting pathology (Oh et al., 2016). Conversely, eosinophils in the uterus have been shown to promote endometrial repair and reduce tissue destruction following Chlamydia trachomatis murine infection (Vicetti Miguel et al., 2017). Our results clearly demonstrate that nematode-induced genital eosinophil infiltration mediates vaginal tissue disruption, predisposing an exacerbation of HSV-2-induced ulceration in the vagina.

In the co-infection model presented here, helminth infection is naturally cleared by an immune-competent host. In Il4ra−/− mice, Nb infections persist (Horsnell et al., 2013; Urban et al., 1998), which suggests that our findings will also have relevance to chronic helminth infections. Chronic enteric helminth infection has been shown to systemically alter immunity in the lung mucosa, protecting against pulmonary virus infection (McFarlane et al., 2017). Irrespective, further investigation is needed to understand the systemic effects of chronic helminth infections on genital susceptibility to viral infections.

In conclusion, we have shown that an acute, self-resolving nematode infection systemically induced canonical type 2 immunity in uncolonized genital tissue. Moreover, helminth-induced genital eosinophils were associated with vaginal tissue disruption, elevated IL-33 responses, and expansion of genital ILC2s and IL-5 levels during subsequent HSV-2 infection. This directly demonstrates systemic influences of gastrointestinal nematode infections on genital responses to viral infection and provides important experimental support for the relevance of reported clinical associations between soil-transmitted helminth infection and vaginal viral infection (Gravitt et al., 2016). These findings represent a conceptual advance in our understanding of how a non-FGT
infection can systemically alter pathogenesis to an important STVI.

**STAR METHODS**

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Supplemental Information can be found online at https://doi.org/10.1016/j.chom.2021.02.004.

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**AUTHOR CONTRIBUTIONS**

A.C. and M.G.D. performed experiments and the parasite lifecycle. P.M.V., M.M.-A., and A.F. performed experiments and analyzed data. W.G.C.H., A.C., M.R., B.R., M.J.O., and A.F.C. designed the experiments, analyzed the data, prepared the figures, and wrote the paper. F.B. supervised mouse breeding and provided Il4ra+/- mice. W.G.C.H. and L.E.L. acquired funding and supervised the work. H.J.M., M.J.O., B.R., and B.G.D provided reagents. All authors discussed the results and commented on the paper.

**DECLARATION OF INTERESTS**

The authors declare no conflict of interest.

**REFERENCES**


**STAR METHODS**

**KEY RESOURCES TABLE**

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr William G. C. Horsnell (wghorsnell@gmail.com).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This study did not generate any unique datasets or code.

**EXPERIMENTAL MODELS AND SUBJECT DETAILS**

**Cells**
African green monkey kidney (Vero) cells were obtained from ATCC (ATCC® CCL-81™, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich®) supplemented with 10% FCS, 100 U/ml penicillin, 100mg/ml streptomycin and 2mM glutamine, at 37°C, 5% CO₂.
Virus
Human herpesvirus 2 strain G (HSV-2, ATCC® VR-734™) was propagated in Vero cells (Blaho et al., 2005; Marshak et al., 2014). Confluent Vero cells were washed with serum-free media and the HSV-2 inoculum was added, at a multiplicity of infection (MOI) of 0.1. At 2-3 days post-infection, cells and supernatant was collected and viral titres were determined by plaque assay. Briefly, confluent Vero cells were incubated with serial dilutions of viral stock, at 37°C, 5% CO₂, for 2 hours (hrs) to allow for absorption. The inoculum was then replaced with supplemented DMEM and cells were incubated for 2 days, fixed with methanol + 2% H₂O₂ and stained with Giemsa stain (Sigma-Aldrich®) to determine plaque forming units (PFU). Viral aliquots were stored at -80°C until use.

Animals
Mice were bred and housed in specific pathogen-free conditions at the Research Animal Facility, University of Cape Town, South Africa. Food and water were provided ad libitum. All studies carried out are in accordance with ethical protocols 014/027 or 018/002, approved by the Faculty of Health Science Animal Ethics Committee from the University of Cape Town. Mice were randomly sorted into experimental groups. Female wildtype BALB/c, Il4ra⁻/⁻ and ΔdblGata1⁻/⁻ (BALB/c background) mice, aged 6-8 weeks, were injected subcutaneously with 2 mg Depo Provera® (Pfizer) in sterile phosphate buffered saline (PBS), 7 days prior to infection, to synchronize estrous cycles and facilitate consistent intravaginal viral infection (Marshak et al., 2014).

Parasite maintenance and infection
N. brasiliensis (Nb) was maintained in male Wistar rats (ethics protocol 014/042 or 018/037). Briefly, rats were injected subcutaneously with 5000 x infectious Nb L3. Feces were collected during peak helminth egg production (day 6-8 post infection). Fecal cultures were prepared by placing a feces/charcoal mix on wet raised filter paper. Hatched L3 larvae migrate to the edge of filter paper and are collected by gently washing off with water. Collected L3 larvae were counted under a dissecting microscope and resuspended in an appropriate volume for infection. Mice were infected with 500 x Nb L3 larvae subcutaneously delivered in 200 μl of water, 7 days prior to viral infection.

METHODS DETAILS

Intravaginal infection with HSV-2
Mice were anesthetized and inoculated intravaginally with 5 x 10⁵ PFU HSV-2. Virus-associated illness severity was determined by pathology scoring: 0 - No pathology observed; 1 - Slight genital/perianal erythema; 2 - Genital/perianal swelling and erythema; 3 - Genital lesions and/or visible weight loss; 4 - Hind limb paralysis and/or purulent lesions; 5 – Premoribund (Marshak et al., 2014). Vaginal lavages were performed by 10x flushing the vaginal vault with 50 μl sterile PBS. This was repeated three times. Viral shedding was quantified by plaque assay as described previously.

Intravaginal papain treatment
Female WT or Il4ra⁻/⁻ mice were treated subcutaneously with 2 mg Depo Provera® to equilibrate hormone levels. One week later, mice were treated with 20 μg of serine protease Papain intravaginally under deep anesthesia, for three consecutive days.

Intravaginal HpARI treatment
Hormone-synchronized female WT and Il4ra⁻/⁻ mice were infected subcutaneously with 500x L3 Nb, one week prior to intravaginal infection with 5 x 10⁵ PFU HSV-2. To inhibit vaginal IL-33, mice were treated intravaginally with 5 μg Heligmosomoides polygyrus Alarmin Release Inhibitor (HpARI) (Osborn et al., 2017) consecutively from 3 days prior until 3 days post HSV-2 infection.

Antibody depletions
To deplete eosinophils and IL-5, mice were treated with 20 μg/mouse anti-Siglec-F (α-Siglec-F, monoclonal Rat IgG2A clone: # 238047, R&D systems) or anti-IL-5 (α-IL-5, monoclonal Rat IgG1 clone: TRFK5, eBioscience™ antibody intraperitoneally on day 5, 7 and 9 post Nb infection. Control mice were treated with rat IgG2A or rat IgG1 isotype antibody, respectively.

Histology
Vaginal tissue was excised and fixed in phosphate-buffered formalin solution (Sigma-Aldrich®) overnight (Horsnell et al., 2007). Following paraffin embedding, tissue was cut into 5 μm cross-sections (2 sections per vaginal at varying depths) and stained with hematoxylin and eosin (H&E) to visualize vaginal epithelial integrity and inflammation. Sections were also stained with hematoxylin and Sirius red, to identify eosinophils. All sections were viewed with Zeiss Axioskop Microscope (Zeiss) and images were taken with a color AxioCam HRc and AxioVision 4.7 supporting software. ‘Ulcerated epithelium’ was defined as the complete loss of the stratified squamous cells from a given length of vaginal epithelium. ‘Total epithelial length’ was defined as the total length of the tissue-lumen interface. Total and ulcerated epithelium length was measured using ImageJ Software (NIH) and percentage of ulcerated epithelium was calculated as follows:

% ulcerated epithelium = ("Measured length of ulcerated epithelium" / "Total measured epithelial length") x 100.
Immunofluorescent (IF) staining and confocal imaging: Briefly, paraffin embedded sections (4 μm) were subjected to deparaffinization in Neo-clear (Sigma-Aldrich®), followed by rehydration and antigen retrieval in Tris-EDTA buffer (pH 9). Slides were blocked (5% normal goat serum, 5% BSA, 0.3% Tx-100, 0.05% Tween 20 in PBS) and then incubated with primary antibodies (anti-β-catenin, Mouse monoclonal Ab, BD Biosciences, 610154; anti-cleaved caspase 3, Rabbit polyclonal Ab, Cell signaling, 9661) overnight at 4°C (humid). Following three washes, slides were then incubated with appropriate secondary antibodies (goat anti-Rabbit IgG Alexa Fluor 488, Invitrogen, A11034; goat anti-Mouse IgG Alexa Fluor 647, Invitrogen, A32728) for 1 hr at room temperature (dark, humid). Following three washes, slides were incubated with anti-smooth muscle actin (SMA)-Cy3 directly labelled antibody (mouse mAb, Sigma-Aldrich® C6198) and Hoechst 33342 for 30 min at RT (dark, humid).

For IL-33 staining, antigen retrieval was performed by boiling slides in citrate buffer (pH 6), followed by blocking (5% BSA, 0.3% Tx-100, 0.05% Tween 20 in PBS). The primary antibodies (anti-IL33, Goat polyclonal, AF3626, R&D Systems; anti-β-catenin) were incubated in 2.5 % BSA, 0.3% Tx-100, 0.05% Tween 20 in PBS. Secondary antibody (Donkey anti-Goat IgG Alexa Fluor Plus 488, Invitrogen, A32814; Donkey anti-Mouse IgG Alexa Fluor Plus 647, Invitrogen, A32787) incubation was performed in the same buffer for 1 h at RT adding Hoechst 33342.

After four washes, slides were mounted in Fluoromount G medium (Thermo Fisher Scientific) and imaged using Zeiss Airyscan confocal microscope, with a 10x and 20x objective. Images were analyzed using Zen black edition software (Zeiss). Maximal intensity projections are shown (Zwiggelaar et al., 2020).

Flow cytometry
Female genital tissue (FGT, excluding ovaries) was removed from individual mice, finely cut, and digested in supplemented DMEM containing 1% HEPES and 20 μg/ml LiberaseTM TL (Roche), for 1 hr at 37°C with gentle shaking. Digested tissue was passed through a 70 μm cell strainer and dispersed cells resuspended in fresh supplemented DMEM. Iliac lymph nodes (iLN) were excised and passed through a 40 μm cell strainer. To isolate vaginal epithelial cells, excised vaginal tissue was incubated in 0.25% Trypsin/EDTA, for 1 hr at 37°C with gentle shaking.

Cells were stained with staining buffer (PBS + 0.5% BSA and 2mM Ethylenediaminetetraacetic acid (EDTA)) containing 2% heat-inactivated rat serum, 1 μg anti-mouse CD16/32 antibody (clone: 93, BioLegend) and fluorochrome-conjugated antibodies against cell-surface markers for 20 min (min) at 4°C (dark): CD45 Alexa Fluor® 700 (clone: 30-F11, BioLegend) to define hematopoietic cells; CD11b Brilliant violet (BV) 421™ (clone: M1/70, BioLegend), F4/80 BV605™ (clone: BM8, BioLegend), Ly6C FITC (clone: HK1.4, BioLegend), Ly6G APC Cy7 (clone: 1A8, BioLegend) and Siglec-F PE (clone: S17007L, BioLegend) to identify myeloid cells; lineage cocktail PE (CD3e, clone: 145-2C11; Ly-6G/Ly-6C, clone: RB6-8C5; CD11b, clone: M1/70; CD45R/B220, clone: RA3-6B2; TER-119, clone: Ter-119, BioLegend), IL-7Rz (CD127) PE Cy7 (clone: A7R34, BioLegend), ICOS APC (clone: C998.4A, BioLegend) and ST2 (IL-33Rz) BV421™ (clone: DIH9, BioLegend) to define ILC2s; and EPCAM (CD326) APC (clone: G8.8, BioLegend), CD90.2 BV605™ (clone: 30-H12, BioLegend), MHCII (HLA-DR) PE (clone: 34-2-12, BioLegend) and MHCIi (I-A/I-E) FITC (clone: M5/114.15.2, BioLegend) to identify vaginal epithelial cells. 7-aminoactinomycin D (7-AAD) staining was used to identify ‘Live’ cells.

For intracellular cytokine staining, cells were incubated in complete DMEM, containing 50 ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich®) and 1 μg/ml lonomycin (Sigma-Aldrich®) for 3 hrs at 37°C, in the presence of 10 μg/ml brefeldin A (BFA; Sigma-Aldrich®). Following ex vivo stimulation, cells were stained for viability (LIVE/DEAD® Fixable Aqua) and surface markers, fixed in 2% Formalin/PBS, and washed in permeabilization buffer (eBioscience™), before incubation with anti-mouse/human IL-5 BV421™ (clone: TRFK5, BioLegend) for 30 min at 4°C (dark).

Samples were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences) and data were analyzed by FlowJo® V10 (Treestar, Ashland, OR). Appropriately stained compensation beads and unstained controls were run to compensate for spectral overlap between fluorochrome emissions.

Enzyme-linked immunosorbent assay (ELISA) and luminex
Isolated FGT was snap frozen in liquid nitrogen and stored at -80°C until use. For cytokine analysis, tissue was homogenized in RIPA lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich®), and homogenates were centrifuged at 10,000 rpm for 10 min to isolate the supernatant. Protein concentrations were quantified by bicinchoninic acid (BCA) assay (Pierce®), and all samples were standardized for cytokine analysis by ELISA or Luminex (Thawer et al., 2016).

Quantification of cytokines IL-4 and IFN-γ was performed using ELISA MAX™ Standard kits (BioLegend) following the manufacturer’s instructions. IL-33 quantification was performed using Mouse IL-33 DuoSet ELISA kit (R&D systems) as per manufacturer’s instructions. The plates were developed with TMB microwell peroxidase substrate system (Thermo Fisher Scientific), and the reaction was stopped with 1M H3PO4. The plates were read at an absorbance of 450nm (Lm1) and 570 nm (Lm2; background) using a VersaMax microplate reader (Molecular Devices Corporation, CA, U.S.A.).

FGT homogenate and vaginal lavage cytokine levels were measured by Luminex xMap technology using the Invitrogen ProcartaPlex™ 36-plex mouse kit (Cat# EPX360-2692-901, Lot # 189084327), as per manufacturers. The Luminex plate was
read using Bio-Rad Bio-Plex® 200 system and analysis software. Luminex was performed once, with samples from two independent experiments. Analyte levels below the lower detection limits were given an arbitrary value of half the Lower Limit of Quantification (LLOQ), defined by the manufacturer.

**Western blotting**

For detection of proteins of interest in the FGT, 25 μg of tissue homogenates were analyzed by reduced SDS-PAGE and Western blotting according to conventional protocols (Ujma et al., 2019). Primary antibodies used were rabbit polyclonal anti-STAT1 (abcam; ab47425), rabbit polyclonal anti-Major Basic Protein (MBP; abcam; ab187523) and rabbit polyclonal anti-GAPDH loading control (abcam; ab9485). For signal detection, secondary goat anti-rabbit IgG-HRP (abcam; ab205718) was used. All Western blots were visualized using the LumiGlo® chemiluminescent substrate (KPL, Milford, MA, USA), with the Biospectrum™ 500 Imaging System (UltraViolet Products, UVP, Mile End South, SA, Australia). Densitometry analysis were performed using ImageJ software, to determine relative changes in protein expression.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical details (statistical tests used, value of n (number of animals), definition of center, and dispersion and precision measures) can be found in the figure legends. Data are represented as group mean and standard error of the mean (mean±sem). Statistical analysis was performed either by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test or by non-parametric Mann-Whitney test, with a 95% confidence interval. A *p value* ≤ 0.05 was considered significant and are indicated by an asterisk (*). Statistical analyses were performed using GraphPad Prism V6.