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THE EMBO JOURNAL

Vaginal bacterium *Prevotella timonensis* turns protective Langerhans cells into HIV-1 reservoirs for virus dissemination

Nienke H van Teijlingen[1,2,†], Leanne C Helgers[1,2,†], Ramin Sarrami-Forooshani[1,3], Esther M Zijlstra-Willems[1,2], John L van Hamme[1,2], Celia Segui-Perez[4], Marleen Y van Smoorenburg[1,2], Hanneke Borgdorff[5], Janneke HHM van de Wijgert[6], Elisabeth van Leeuwen[7], Joris AM van der Post[7], Karin Strijbis[4,‡], Carla MS Ribeiro[1,2,‡] & Teunis BH Geijtenbeek[1,2,*]

Abstract

Dysbiosis of vaginal microbiota is associated with increased HIV-1 acquisition, but the underlying cellular mechanisms remain unclear. Vaginal Langerhans cells (LCs) protect against mucosal HIV-1 infection via autophagy-mediated degradation of HIV-1. As LCs are in continuous contact with bacterial members of the vaginal microbiome, we investigated the impact of commensal and dysbiosis-associated vaginal (an)aerobic bacterial species on the antiviral function of LCs. Most of the tested bacteria did not affect the HIV-1 restrictive function of LCs. However, *Prevotella timonensis* induced a vast uptake of HIV-1 by vaginal LCs. Internalized virus remained infectious for days and uptake was unaffected by antiretroviral drugs. *P. timonensis*-exposed LCs efficiently transmitted HIV-1 to target cells both *in vitro* and *ex vivo*. Additionally, *P. timonensis* exposure enhanced uptake and transmission of the HIV-1 variants that establish infection after sexual transmission, the so-called Transmitted Founder variants. Our findings, therefore, suggest that *P. timonensis* might set the stage for enhanced HIV-1 susceptibility during vaginal dysbiosis and advocate targeted treatment of *P. timonensis* during bacterial vaginosis to limit HIV-1 infection.

Keywords HIV-1; Langerhans cells (LCs); *Prevotella timonensis*; transmission; vaginal microbiome

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction

DOI 10.15252/embj.2022110629 | Received 10 January 2022 | Revised 8 July 2022 | Accepted 18 July 2022 | Published online 15 August 2022

The EMBO Journal (2022) 41: e110629

Introduction

HIV-1 infection is a major global health problem as 38 million people live with HIV and annually 1.8 million people become newly infected (UNAIDS, 2020). A large proportion of these infections (26%) occurs in young sub-Saharan African women (UNAIDS, 2020). Reducing high HIV-1 infection rates among young women in Sub-Saharan Africa is an important challenge in HIV-1 prevention. The major route of HIV-1 transmission to women is via the cervicovaginal mucosa during sexual intercourse (UNAIDS, 2020). Large prospective epidemiological studies have shown that the composition of the microbiome populating the female genital tract strongly influences susceptibility to HIV-1 infection (Atashili *et al*, 2008; Low *et al*, 2011; Gosmann *et al*, 2017). A healthy vaginal microbiome is dominated by *Lactobacillus* species (*spp*.). However, vaginal dysbiosis is caused by an increase in relative abundance and concentration of other bacteria at the expense of *Lactobacillus spp*. and is associated with increased HIV-1 susceptibility (Sha *et al*, 2005; Atashili *et al*, 2008; Low *et al*, 2011; Borgdorff *et al*, 2014; Gosmann *et al*, 2017).

During the most common type of vaginal dysbiosis, referred to as bacterial vaginosis by clinicians, anaerobic bacteria dominate the vaginal microbiome. These anaerobes are almost always present in

1 Department of Experimental Immunology, Amsterdam UMC Location University of Amsterdam, Amsterdam, The Netherlands
2 Amsterdam institute for Infection and Immunity, Amsterdam, The Netherlands
3 ATMP Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran
4 Division Infectious Diseases and Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands
5 Amsterdam Institute for Global Health and Development (AIGHD), Amsterdam, The Netherlands
6 Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands
7 Department of Obstetrics and Gynecology, Amsterdam UMC Location University of Amsterdam, Amsterdam, The Netherlands
*Corresponding author. Tel: +3120-5668590; E-mail: t.b.geijtenbeek@amsterdamumc.nl
†These authors contributed equally to this work
‡These authors contributed equally to this work

diverse bacterial communities and include *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera spp*., and different *Prevotella spp*. (Fredricks *et al*, 2005; Aldunate *et al*, 2015). In particular, *Prevotella spp*. including *P. amnii*, *P. bivia*, and *P. timonensis* show a strong positive association with increased risk for HIV-1 infection (Atashili *et al*, 2008; Gosmann *et al*, 2017). However, it is unclear whether and how different microbiota such as *Prevotella spp*. affect HIV-1 susceptibility.

Human Langerhans cells (LCs) are a specific subset of mucosal dendritic cells (DCs) that survey the vaginal mucosa and function as antigen-presenting cells (Steinman & Banchereau, 2007; Kubo *et al*, 2009). LCs serve as the first line of defense against HIV-1 upon sexual transmission (Hladik *et al*, 2007; Hladik & McElrath, 2008; Haase, 2010). Under non-inflammatory conditions, LCs are in an immature state and restrict HIV-1 infection via capture and subsequent degradation of the virus, thereby preventing infection and dissemination of HIV-1 (Valladeau *et al*, 2000; De Witte *et al*, 2007; De Jong *et al*, 2008; van den Berg *et al*, 2014; Ribeiro *et al*, 2016). However, when LCs become activated, their antiviral function is impaired and activated LCs become infected by the virus and efficiently transmit HIV-1 to $CD4^+$ T cells (De Witte *et al*, 2007; De Jong *et al*, 2008). Vaginal LCs are in continuous contact with the microbiota that populate the female genital tract (Van De Wijgert *et al*, 2014). Yet, it is unclear whether these bacteria, in particular those associated with vaginal dysbiosis, directly affect the antiviral function of LCs.

Here, we have investigated the impact of commensal and dysbiosis-associated vaginal bacteria on the antiviral function of LCs. We identified *P. timonensis* as a bacterium that greatly affected the antiviral function of LCs. *P. timonensis* strongly induced uptake of HIV-1 by vaginal LCs, and sequestered virus remained infectious for several days. *P. timonensis* not only enhanced susceptibility to laboratory HIV-1 strains but also enhanced uptake and transmission of Transmitted Founder (T/F) HIV-1 variants, further underscoring the relevance for HIV-1 susceptibility. Our findings suggest that *P. timonensis* could contribute to increased HIV-1 susceptibility by enhancing HIV-1 uptake and rendering the virus insensitive to anti-HIV-1 prophylaxis.

Results

P. timonensis enhances HIV-1 uptake in vaginal LCs

Epidermal as well as vaginal LCs were exposed to bacteria associated with a healthy vaginal mucosal microbiome *Lactobacillus crispatus* and *L. iners* or those associated with vaginal dysbiosis, *G. vaginalis*, *A. vaginae*, *M. elsdenii*, and *P. timonensis*. After stimulation, LCs were exposed to HIV-1 for 5 days and HIV-1 content was analyzed by detection of capsid protein p24 by flow cytometry. Notably, *P. timonensis* enhanced the amount of HIV-1-positive vaginal as well as epidermal LCs (Fig 1A and B). However, none of the other tested bacteria increased the percentage of HIV-1-positive epidermal or vaginal LCs (Fig 1A and B). Since *P. timonensis* increased HIV-1 acquisition, we exposed epidermal LCs to different UV-killed *Prevotella spp*. or related *Bacteroides spp*. Strikingly, only *P. timonensis* significantly increased the amount of HIV-1-positive LCs in contrast to the other *Prevotella spp*. (Fig 1C). To investigate whether *P. timonensis* also affected LC susceptibility to HIV-1 in physiologically relevant conditions, we exposed *ex vivo* vaginal explants to HIV-1 in the presence of selected vaginal commensal and dysbiosis-associated bacteria. Stimulation with *P. timonensis*, but not with the other bacteria, resulted in a significant increase of HIV-1-positive LCs among those who emigrated from *ex vivo* vaginal explants (Fig 1D).

Next, we investigated whether the increase in HIV-1-positive LCs induced by *P. timonensis* was due to enhanced HIV-1 uptake or replication. Replication inhibitors zidovudine (AZT), tenofovir (TFV), indinavir (IDV), or lamivudine (3TC) did not reduce the amount of HIV-1-positive epidermal or vaginal LCs after *P. timonensis* exposure (Fig 2A–D), although the inhibitors were proven to be effective (Fig EV1). Moreover, *P. timonensis* increased the number of vaginal LCs positive for p24 after exposure to HIV-1-GFP reporter virus (NL4.3eGFP-Bal), whereas no increase in eGFP expression, a measure for productive infection, was observed (Fig 2E and F). Activated $CD4^+$ T cells were used as a positive control for eGFP expression (Fig EV2). Additionally, neither blocking of HIV-1 entry (co-) receptors CD4 and CCR5, nor inhibition of viral fusion with T20 reduced the amount of HIV-1-positive epidermal LCs after *P. timonensis* and HIV-1 exposure (Fig 2G). These data strongly suggest that *P. timonensis*, but none of the other bacteria tested, increases HIV-1 uptake by epidermal and vaginal LCs independent of HIV-1 entry receptors and infection.

M. elsdenii and *P. timonensis* exposure induces minor LCs activation

While immature LCs resist HIV-1 infection, LC activation abrogates this HIV-restrictive function resulting in LC infection and subsequent transmission of HIV-1 (De Witte *et al*, 2007; De Jong *et al*, 2008). Therefore, we investigated whether vaginal microbiota exposure leads to LC activation. Both isolated immature vaginal and epidermal LCs were exposed to *L. crispatus*, *M. elsdenii*, and *P. timonensis*, and after 24 h, the activation phenotype of LCs was determined by flow cytometry. As a control, migrated LCs were isolated after culture of the epidermis as these migrated LCs have a mature phenotype expressing increased levels of maturation markers CD80 and CD86, and chemokine receptor CCR7 (Fig 3A–E). The same trend for both vaginal and epidermal LCs was observed. *M. elsdenii* significantly increased expression levels of CD80, while *L. crispatus* exposure did not affect the expression of any maturation markers. *P. timonensis* stimulation of immature epidermal and vaginal LCs increased the expression of CD80, CD86, and CCR7, but expression levels remained lower than mature LCs (Fig 3A–E). Additionally, expression of langerin was slightly, but not significantly, reduced upon exposure of immature epidermal LCs to *M. elsdenii* and *P. timonensis*, whereas langerin expression was strongly decreased on LCs activated through migration (Figs 3B and EV3). Next, we investigated whether vaginal microbiota influence migration of epidermal LCs from *ex vivo* explants. *Ex vivo* explants were exposed to *L. crispatus*, *M. elsdenii*, *P. timonensis* or positive control polycytidylic acid (poly(I:C)), and after 3 days, the migratory fraction was analyzed in number and phenotype. The number of migrated LCs was significantly lower in response to *M. elsdenii*, whereas *P. timonensis* showed a trend in the decrease of migration (Fig 3F). *L. crispatus* did not affect migration and poly(I:C) induced

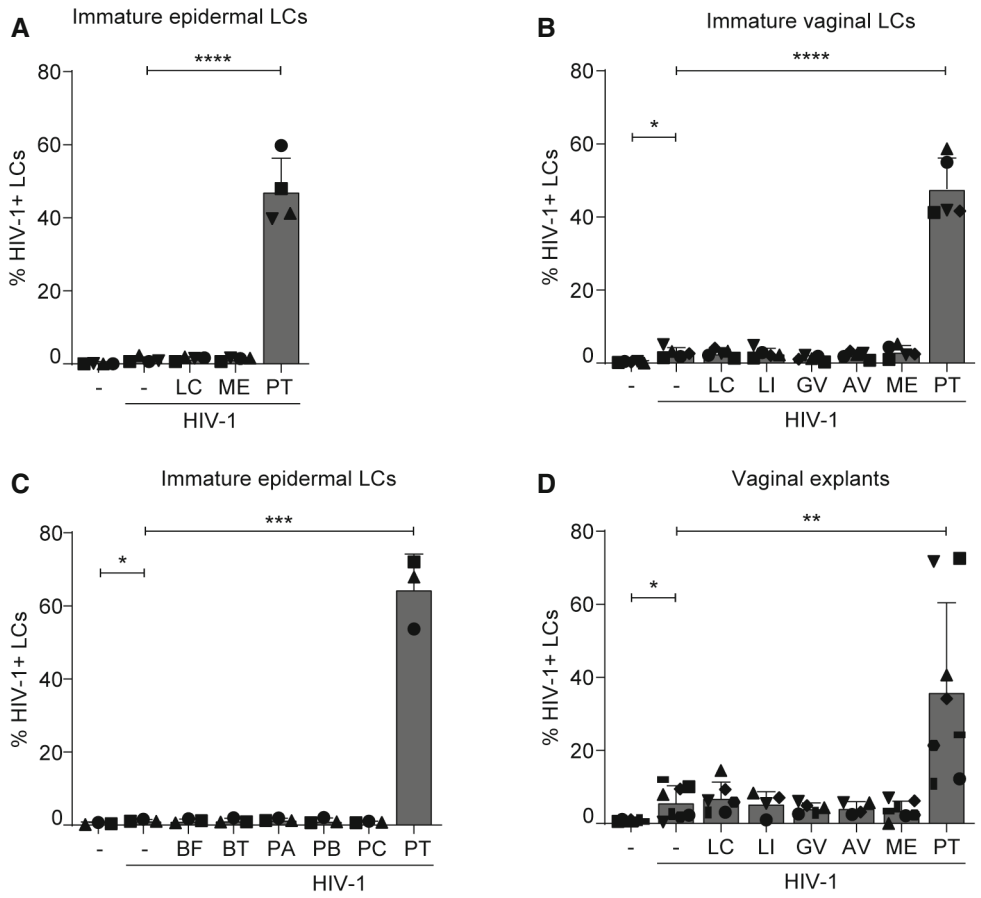

Figure 1. *P. timonensis* increases HIV-1 content in primary LCs.

A, B Isolated epidermal LCs (A, $n = 4$) or isolated vaginal LCs (B, $n = 5$) were stimulated O/N with a variety of vaginal microbiota and subsequently exposed to HIV-1 (SF162; MOI 0.5). Epidermal LCs were stimulated with *L. crispatus* (LC), *M. elsdenii* (ME), and *P. timonensis* (PT), whereas vaginal LCs were additionally stimulated with *L. iners* (LI), *G. vaginalis* (GV), and *A. vaginae* (AV). After 5 days, HIV-1 p24 in LCs was determined by an intracellular staining for CD1a and p24 by flow cytometry.

C Isolated epidermal LCs ($n = 3$) were stimulated O/N with *B. fragilis* (BF), *B. thetaiotaomicron* (BT), *P. amnii* (PA), *P. bivia* (PB), *P. copri* (PC), and *P. timonensis* (PT), followed by HIV-1 (SF162; MOI 0.5) infection for 5 days and HIV-1 p24 determination by flow cytometry.

D Vaginal epithelium explants ($n = 8$) were exposed to vaginal microbiota as described for isolated vaginal LCs. HIV-1 p24 in emigrated LCs was determined by flow cytometry.

Data information: Symbols represent independent donors. Data are mean ± SD. Two-tailed *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

migration. *M. elsdenii* induced expression of CD80 and CD86 albeit at lower levels than poly(I:C), whereas *P. timonensis* only showed increased expression of CD86 (Fig 3G and H). These data suggest that in contrast to *L. crispatus*, *M. elsdenii* and *P. timonensis* induced LC maturation whereas *P. timonensis*.

HIV-1 escapes langerin-mediated degradation and is internalized in *P. timonensis*-exposed LCs

Next, we performed confocal microscopy to investigate intracellular uptake of HIV-1 in *P. timonensis*-exposed vaginal LCs. Large intracellular vesicles containing HIV-1 virions were observed in *P. timonensis*-stimulated LCs, whereas unstimulated LCs contained low numbers of small HIV-1-positive vesicles (Fig 4A). Removal of extracellular-bound HIV-1 by Trypsin treatment did not abrogate *P. timonensis*-induced HIV-1 uptake in epidermal LCs compared with unstimulated LCs as measured in cell lysates by ELISA (Fig 4B). The comparison of trypsin-treated with untreated epidermal LCs shows that a major part of the virus was insensitive to trypsin treatment, suggesting viral internalization by LCs (Fig 4C). Furthermore, we measured viral fusion using the HIV-1-BlaM-vpr-based fusion assay. *P. timonensis* strongly induced fusion of HIV-1 to vaginal LCs, which was comparable to levels observed with the positive control VSV-G-pseudotyped HIV-1 (Fig 4D and E). Moreover, the amount of HIV-1-positive epidermal LCs after *P. timonensis* exposure was not reduced by antibodies against langerin or the C-type lectin-inhibitor mannan (Fig 4F). Langerin captures and targets HIV-1 into autophagy vesicles for viral degradation and autophagy-enhancing drugs decrease HIV-1 infection of mucosal LCs (Ribeiro *et al*, 2016; Cloherty *et al*, 2021). However, treatment with autophagy inducer rapamycin only marginally decreased the amount of HIV-1-positive epidermal LCs after *P. timonensis*

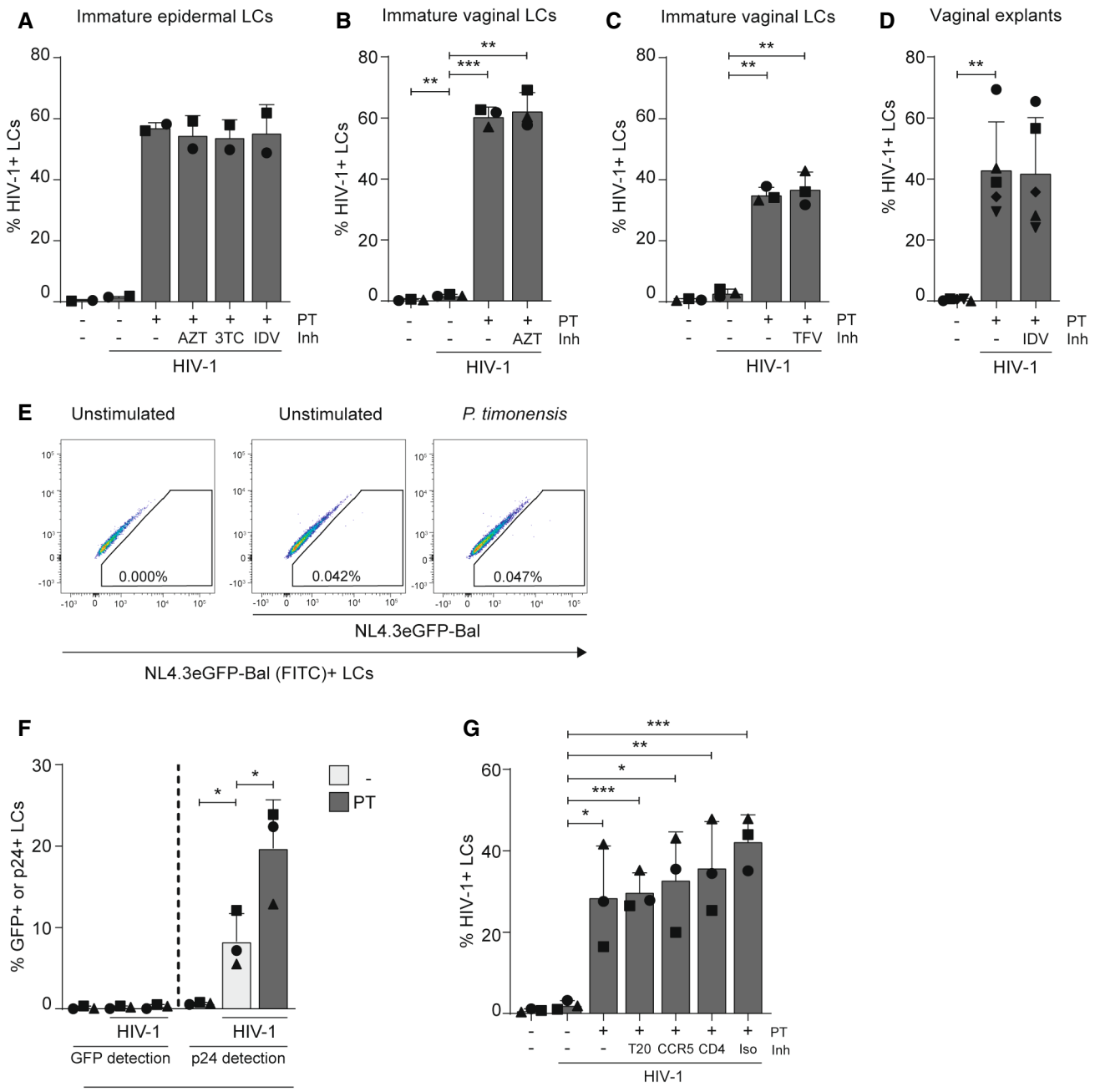

Figure 2. *P. timonensis* increases HIV-1 uptake but not productive infection.

A–D Isolated epidermal LCs (A, $n = 2$), isolated vaginal LCs (B and C, both $n = 3$), or vaginal explants (D, $n = 5$) were stimulated O/N with *P. timonensis* (PT) and exposed to HIV-1 (SF162; MOI 0.5) for 5 days in the presence or absence of HIV-1 replication inhibitors (Inh) zidovudine (AZT), tenofovir (TFV), lamivudine (3TC), or indinavir (IDV). HIV-1 levels were determined by intracellular staining for HIV-1 p24 using flow cytometry.

E, F Representative plots (E) and pooled data (F, $n = 3$) of HIV-1 (NL4.3eGFP-Bal) infection of O/N *P. timonensis*-stimulated (PT) isolated vaginal LCs as determined by both GFP-detection (*de novo* replication) and HIV-1 p24 (detection of *de novo* replication and uptake) by flow cytometry.

G Isolated epidermal LCs ($n = 3$) were stimulated O/N with *P. timonensis* (PT). Next, LCs were treated with T20, CCR5 inhibitor Maraviroc (CCR5), neutralizing antibodies against CD4 (CD4) and isotype control (Iso), subsequently followed by HIV-1 exposure for 5 days (SF162; MOI 0.5). Intracellular HIV-1 p24 levels were determined using flow cytometry.

Data information: Symbols represent independent donors (mean of duplicates). Data are mean ± SD. Two-tailed *t*-test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

exposure (Fig 4G). Together, these data demonstrate that *P. timonensis*-mediated HIV-1 uptake by immature LCs is independent of langerin and bypasses autophagy-mediated degradation. Our data, therefore, strongly indicate that *P. timonensis* enhances internalization of HIV-1 into intracellular vesicles where the virus remains infectious for several days.

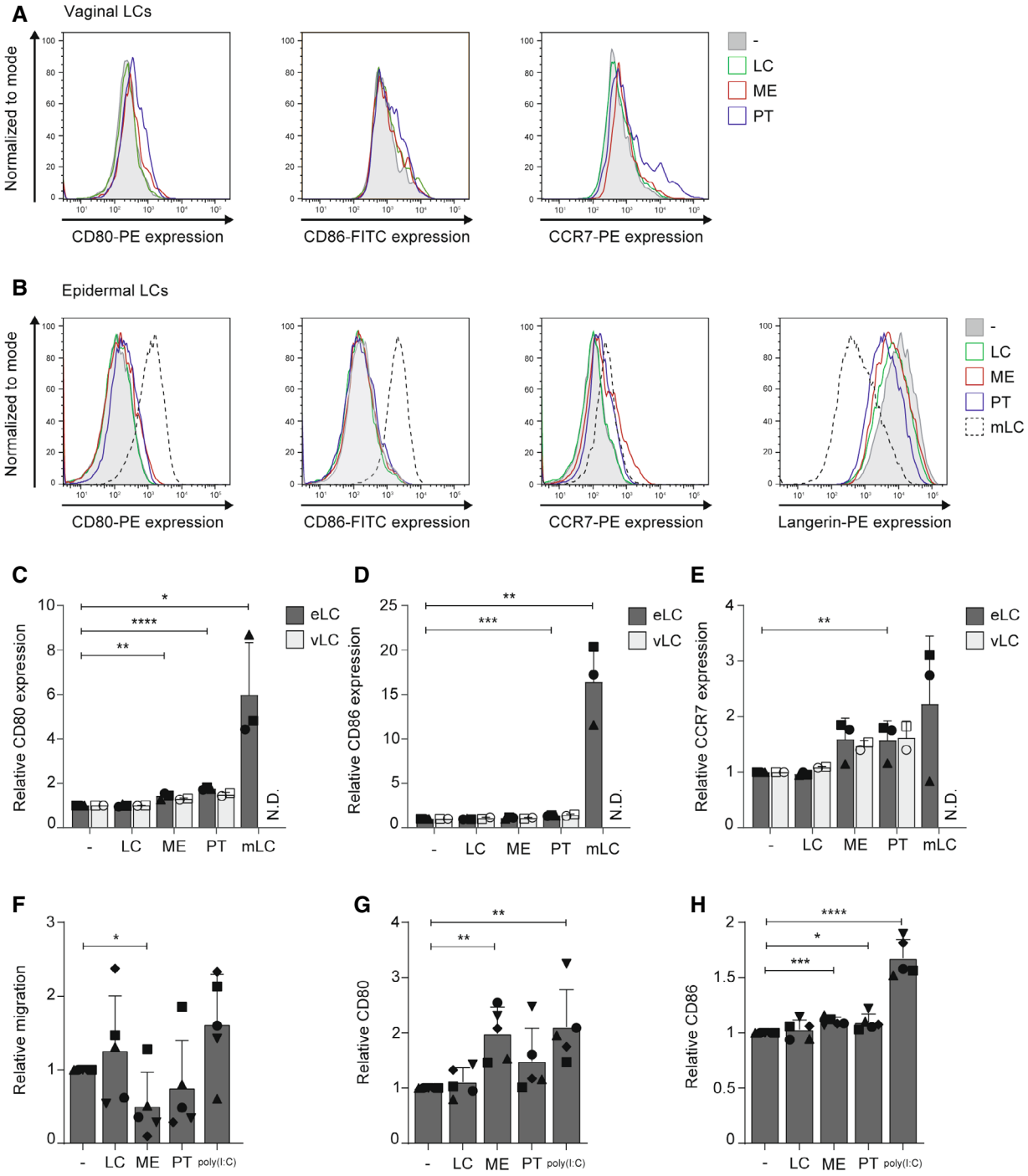

Figure 3. *P. timonensis* induces minor maturation in immature and migratory LCs.

Immature vaginal (vLC) and epidermal LCs (eLC) were stimulated O/N with *L. crispatus* (LC), *M. elsdenii* (ME), or *P. timonensis* (PT), and activation phenotype was determined by flow cytometry. Same donor emigrated epidermal LCs (mLC) served as a matured and activated control. Same donor data of emigrated vaginal LCs could not be obtained due to limited amount of tissue (N.D.).

A–H Representative plots of vaginal (A) or epidermal (B) donors and combined experiments (MFI expression relative to untreated condition) of CD80 (C, $n = 3$), CD86 (D, $n = 3$), and CCR7 (E, $n = 3$) surface expression are shown. *Ex vivo* skin explants were stimulated O/N with *L. crispatus* (LC), *M. elsdenii* (ME), *P. timonensis* (PT) or poly(I:C) and at day 3 post-inoculation, emigrated LCs were collected and washed and activation phenotype was determined by flow cytometry. Cells were analyzed for CD1a expression, and the absolute number of CD1a-positive cells, that is LCs, migrated from the epidermis was determined using counting beads; the graph shows the migration relative to the untreated condition (F, $n = 5$). CD1a-positive cells, that is LCs, were analyzed using flow cytometry, and the graphs show combined experiments (MFI expression relative to untreated condition) of CD80 (G, $n = 5$) and CD86 (H, $n = 5$) surface expression.

Data information: Symbols represent independent donors (mean of duplicates). Data are mean ± SD. Two-tailed *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

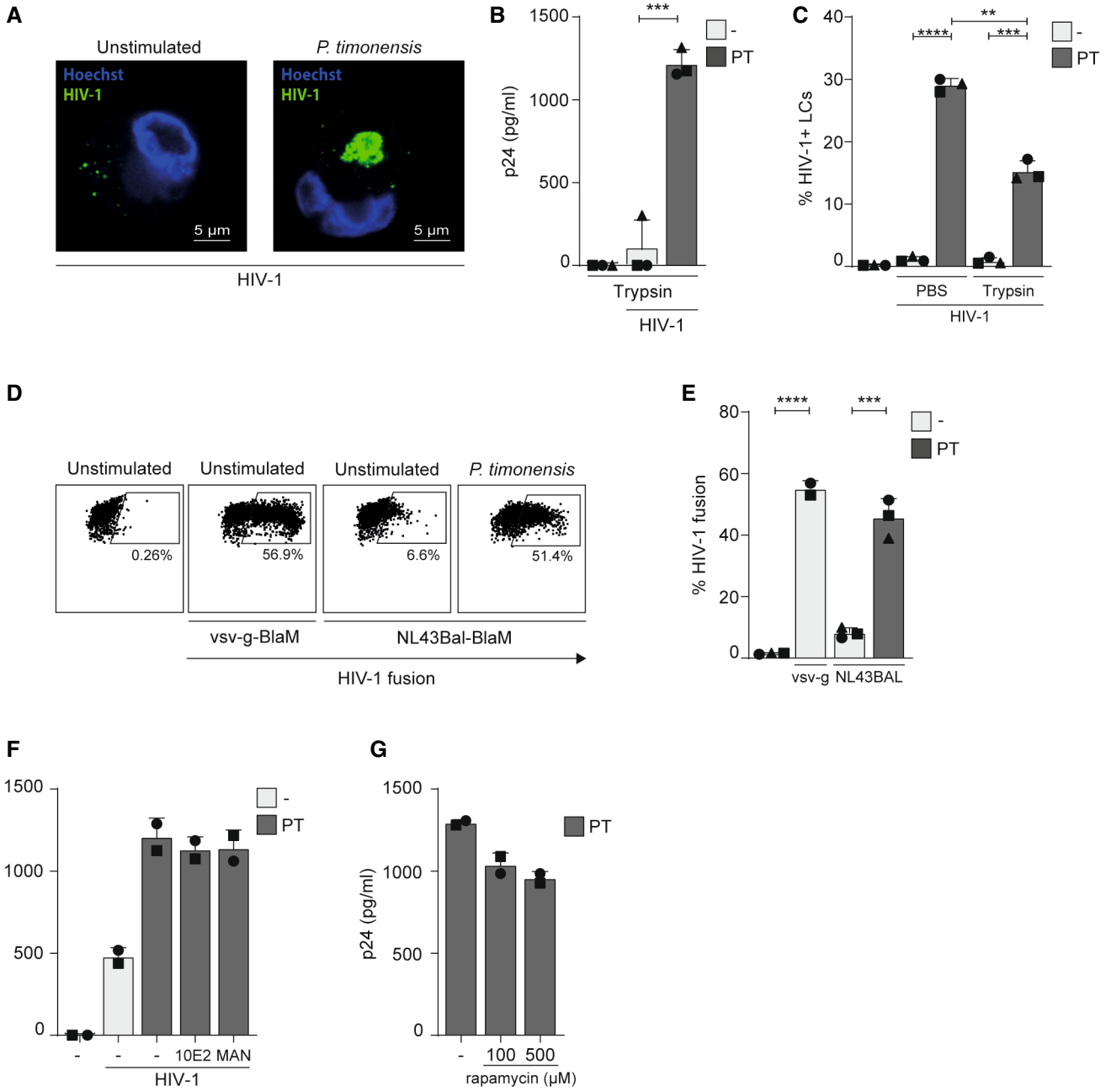

Figure 4. HIV-1 is internalized and protected from the langerin-mediated degradation pathway in *P. timonensis*-exposed LCs.

Isolated vaginal LCs were stimulated O/N with *P. timonensis* (PT) followed by HIV-1 (SF162; MOI 0.5) exposure.

A Intracellular HIV-1 detection was determined by confocal microscopy (Hoechst in blue, HIV-1 p24 in green); scale bar represents 5 µm (representative donor).

B Isolated epidermal LCs were stimulated O/N with *P. timonensis* (PT) followed by a 4-h exposure to HIV-1. HIV-1 p24 was determined by ELISA after trypsin treatment and lysis of LCs ($n = 3$).

C Isolated epidermal LCs were stimulated O/N with *P. timonensis* (PT) followed by HIV-1 (SF162; MOI 0.5) exposure for 3 days. After 3 days, LCs were treated with PBS or trypsin and HIV-1 p24 was determined by flow cytometry ($n = 3$).

D, E Isolated vaginal LCs were stimulated O/N with *P. timonensis* (PT) followed by infection with VSV-g-BlaM-Vpr fusion (positive control) and NL4.3Bal-BlaM-Vpr fusion. The figure shows representative plots (D) and pooled data (E, $n = 3$) of viral fusion as determined by β-lactamase-Vpr (BlaM-Vpr) activity.

F Isolated epidermal LCs were stimulated O/N with *P. timonensis* (PT) followed by HIV-1 exposure (SF162; MOI 0.5) in the presence or absence of anti-langerin (10E2) or mannan (MAN). HIV-1 p24 content was determined by ELISA after trypsin treatment and subsequent lysis of LCs ($n = 2$).

G Isolated epidermal LCs were stimulated O/N with *P. timonensis* (PT) followed by treatment with Rapamycin (µM) and exposure to HIV-1 (SF162). After 3 days, HIV-1 p24 was measured by ELISA after trypsin treatment and lysis of LCs ($n = 2$).

Data information: Symbols represent independent donors (mean of duplicates). Data are mean ± SD. Two-tailed *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

P. timonensis increases HIV-1 transmission by LCs to target cells

To investigate whether internalized HIV-1 remains infectious, *P. timonensis*-exposed epidermal LCs were exposed to HIV-1 and cultured for 2 days. Next, LCs were treated with trypsin to remove extracellular-bound virus and lysed by repetitive freeze–thaw cycles in order to release internalized HIV-1. The cell lysates were added to HIV-1-susceptible U87-CD4-CCR5 cells, and infection was determined. Notably, the lysate of *P. timonensis*-exposed and HIV-1-treated LCs caused a stronger infection of U87-CD4-CCR5 cells than the lysate from LCs treated with HIV-1 only (Fig 5A). We next investigated whether *P. timonensis*-induced HIV-1 uptake by LCs results in HIV-1 transmission to target cells using an epidermal *ex vivo* transmission model. *Ex vivo* epidermal explants were exposed to vaginal bacteria *L. crispatus*, *M. elsdenii*, or *P. timonensis* and subsequently infected with HIV-1 in the presence or absence of antiretroviral drugs to investigate whether transmission is dependent on HIV-1 replication in LCs. After 3 days, LCs that emigrated from the tissue were harvested, washed extensively, and co-cultured with HIV-1-permissive U87-CD4-CCR5 cells to determine virus transmission by flow cytometry. *P. timonensis*-exposed LCs were more efficient in transmitting HIV-1 than untreated LCs or LCs exposed to the other bacteria (Fig 5B and C). Notably, pre-treatment of explants with tenofovir, T20 or maraviroc before HIV-1 exposure did not abrogate HIV-1 transmission by *P. timonensis*-exposed LCs, although a trend in a decrease in HIV-1 transmission was observed with tenofovir (Fig 5C and D). Next, we investigated the effect of *P. timonensis* on HIV-1 transmission by vaginal LCs. Strikingly, *P. timonensis* induced strong HIV-1 transmission by vaginal LCs compared with unstimulated vaginal LCs (Fig 5E and F). These data suggest that *P. timonensis* enhances HIV-1 uptake by epidermal LCs *ex vivo* and by vaginal LCs *in vitro*, leading to efficient HIV-1 transmission which is largely insensitive to antiretroviral drugs.

P. timonensis enhances HIV-1 uptake and transmission of Transmitted Founder HIV-1 strains

Transmitted Founder (T/F) HIV-1 variants are the variants that establish infection after sexual transmission (Derdeyn *et al*, 2004; Salazar-Gonzalez *et al*, 2008; Hertoghs *et al*, 2019). These variants have specific properties that allow them to infect immature LCs (Hertoghs *et al*, 2019). Here, we investigated whether T/F HIV-1 interaction with LCs is affected by *P. timonensis* or closely related species. In contrast to other *Prevotella spp*. and *Bacteroides spp*., *P. timonensis* exposure of epidermal LCs increased the amount of HIV-1 CH058-positive LCs (Fig 6A and B). This increase was not affected by treatment with AZT or tenofovir. To investigate T/F transmission, *ex vivo* epidermal explants were exposed to *P. timonensis* followed by infection with laboratory-adapted strain SF162 or T/F variant CH058 in the presence or absence of tenofovir. After 3 days, emigrated LCs were harvested, washed, and co-cultured with U87-CD4-CCR5 cells and transmission was determined by flow cytometry. LCs that migrated from *P. timonensis*-exposed skin explants transmitted both SF162 and CH058 more efficiently than LCs that migrated from untreated skin explants (Fig 6C). Notably, CH058 transmission was more efficient compared with laboratory strain SF162 and this effect was magnified after *P. timonensis* stimulation (Fig 6C). Tenofovir treatment did not abrogate HIV-1 transmission. These data strongly suggest that *ex vivo* transmission of T/F strains to HIV-1 target cells is strongly increased in the presence of *P. timonensis* and is largely replication-independent. These results show that of all the bacteria tested, only *P. timonensis* specifically induces strong HIV-1 uptake resulting in efficient transmission of T/F HIV-1 by LCs.

Discussion

The microbiome affects the immune system in different ways, for example, by training and development of tolerance but also by altering the susceptibility to viral pathogens (Zheng *et al*, 2020). Previous studies have shown that imbalances in the microbiome can underlie increased HIV-1 susceptibility in women (Atashili *et al*, 2008; Gosmann *et al*, 2017). Here, we have uncovered a novel effect of the vaginal dysbiosis-associated bacterium *P. timonensis* on HIV-1 susceptibility. *P. timonensis* strongly enhanced HIV-1 uptake by vaginal and epidermal LCs and induced increased HIV-1 transmission to target cells by *P. timonensis*-exposed epidermal and vaginal LCs. The effect was specific for *P. timonensis*, as other tested vaginal commensals or dysbiosis-associated bacteria not affect LC function. Even in the presence of antiviral prophylaxis, *P. timonensis* increased HIV-1 uptake and transmission. In addition, *P. timonensis* strongly enhanced transmission of T/F HIV-1 variants in *ex vivo* epidermal explants, providing further proof for the role of the identified mechanism in HIV-1 susceptibility. Taken together, this study provides compelling evidence that increased HIV-1 susceptibility via vaginal mucosa DC subsets is directly influenced by interaction with *P. timonensis*. Our data emphasize that improving the prevention and treatment of bacterial vaginosis, and specifically *P. timonensis*, could attenuate HIV-1 susceptibility in vulnerable populations.

Mucosal LCs are resistant to HIV-1 infection and have been suggested to function as a barrier to HIV-1 (Valladeau *et al*, 2000; De Witte *et al*, 2007; De Jong *et al*, 2008; van den Berg *et al*, 2014; Ribeiro *et al*, 2016). In this study, we show that immature vaginal LCs, similar to immature epidermal LCs, restrict HIV-1 infection. As mucosal LCs are in continuous contact with the vaginal microbiome, we investigated whether vaginal bacteria affect LC function. Of the bacteria that we tested, we observed that *P. timonensis* strongly enhanced HIV-1 uptake by epidermal and vaginal LCs *in vitro* and *ex vivo*, in contrast to the other common vaginal bacteria including other *Prevotella spp*. We did not observe any differences between epidermal and vaginal LCs suggesting that HIV-1 uptake and transmission is similarly induced by *P. timonensis* in both LC subtypes. As *P. timonensis* is also detected in anal and oral swaps these observations are relevant for LC-mediated HIV-1 transmission via different types of mucosal surfaces (Fredricks *et al*, 2022).

During vaginal dysbiosis, *P. timonensis* is known to almost always co-occur with other *Prevotella spp*. and other strict anaerobes (Atashili *et al*, 2008; Passmore *et al*, 2016; Gosmann *et al*, 2017; McClelland *et al*, 2018). Yet, it is possible that other species that were not part of our study have similar effects. Additional studies including a wider range of *Prevotella spp*. and other dysbiosis-associated vaginal bacteria are needed to further unravel the impact of the vaginal microbiome on HIV-1 acquisition.

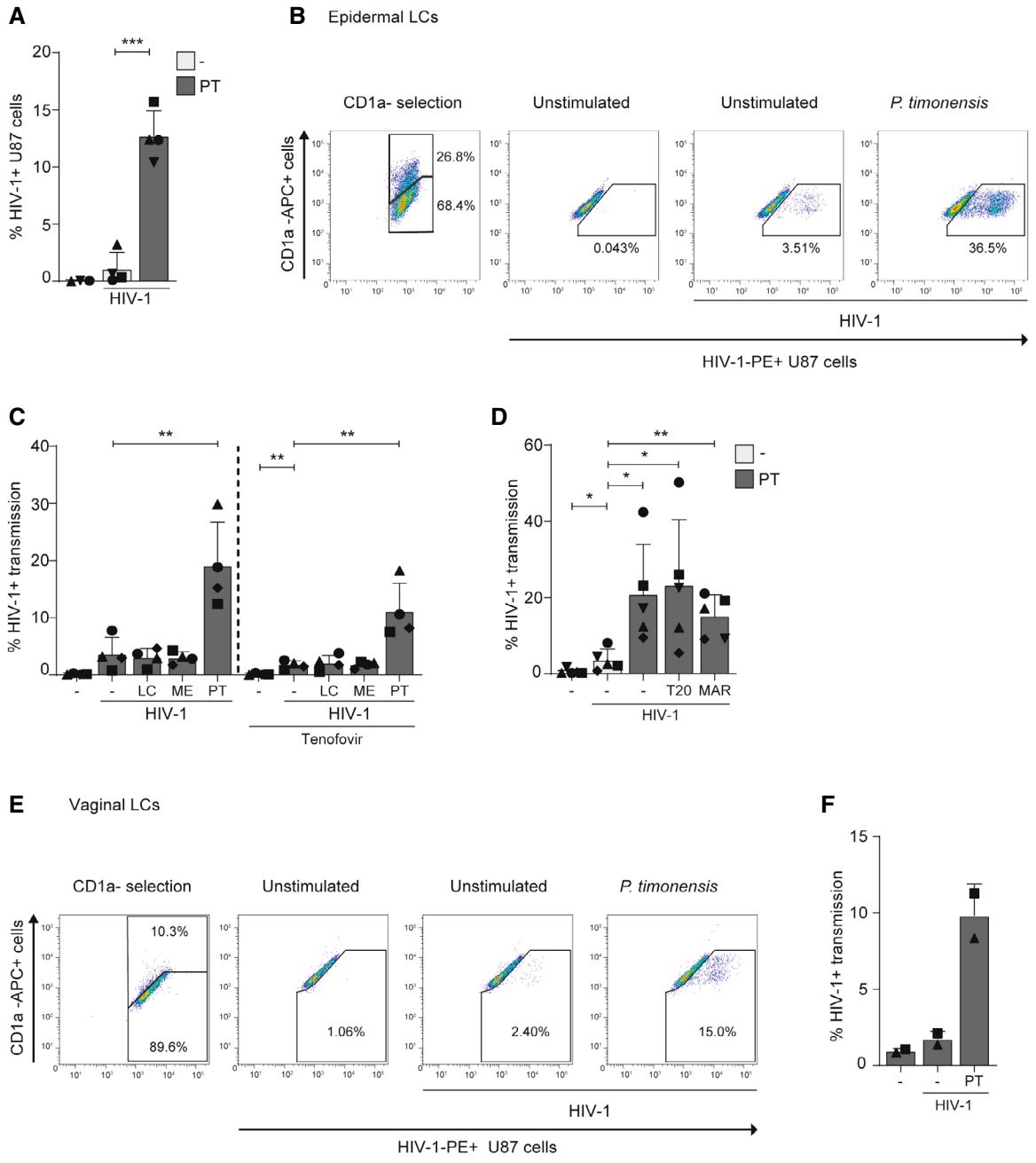

Figure 5. *P. timonensis* induces transmission of HIV-1 by LCs in a replication-independent manner.

A Isolated epidermal LCs were stimulated O/N with *P. timonensis* (PT) followed by HIV-1 (SF162; MOI 0.5) exposure. LCs were subjected to repetitive freeze–thaw cycles, and cell lysates were added to U87.CD4.CCR5 cell line. HIV-1 infection of U87.CD4.CCR5 was determined by an intracellular staining for p24 by flow cytometry ($n = 3$).

B, C *Ex vivo* skin explants were stimulated O/N with *L. crispatus* (LC), *M. elsdenii* (ME), or *P. timonensis* (PT) and inoculated with HIV-1 (JRCSF; MOI 0.5) in the presence or absence of tenofovir. At day 2 post-inoculation, emigrated LCs were collected, washed, and co-cultured with U87.CD4.CCR5 cells for 3 days. Cells were analyzed for CD1a expression and p24 content by flow cytometry and here presented as representative plots (B) and pooled data of HIV-1-positive cells (%) of the CD1a-negative U87.CD4.CCR5 cells (C, $n = 4$).

D *Ex vivo* skin explants were stimulated with *P. timonensis* (PT), pre-treated with T20 or maraviroc (MAR), and exposed to HIV-1 (JRCSF; MOI 0.5) for 2 days, after which the migratory fraction was co-cultured with U87.CD4.CCR5 cells. HIV-1-positive cells (%) of the CD1a-negative fraction, that is, U87.CD4.CCR5 cells, were determined using flow cytometry ($n = 5$).

E, F Isolated vaginal LCs were stimulated O/N with *P. timonensis* (PT) and infected with HIV-1 (SF162; MOI 0.5). After 3 days, LCs were washed and co-cultured with U87.CD4.CCR5. Infection was analyzed by flow cytometry and here shown as representative plots (E) and pooled data of HIV-1-positive cells (%) of the CD1a-negative U87.CD4.CCR5 cells (F, $n = 2$).

Data information: Symbols represent independent donors (mean of duplicates). Data are mean ± SD. Two-tailed *t*-test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

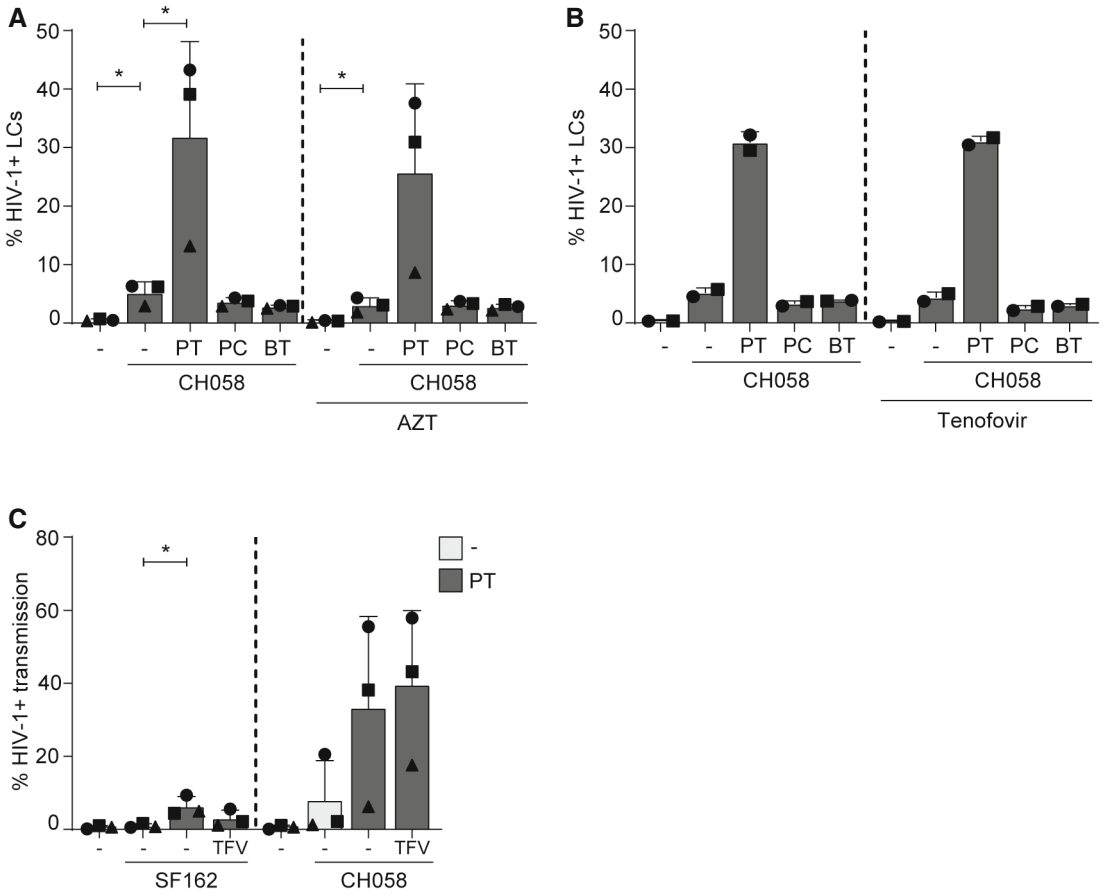

Figure 6. *P. timonensis* specifically induces uptake and transmission of Transmitted Founder HIV-1.

A, B Isolated epidermal LCs were stimulated by different *Prevotella spp.* and *Bacteroides spp.* followed by T/F variant (CH058; MOI 0.25) exposure in the presence or absence of AZT (A, $n = 3$) or tenofovir (B, $n = 2$). A lower MOI was used due to higher susceptibility of LCs for CH058 than for laboratory strains.

C HIV-1 uptake was determined by flow cytometry. *Ex vivo* skin explants ($n = 3$) were stimulated O/N with *P. timonensis* (PT) followed by pre-treatment with tenofovir (TFV). After pre-treatment, skin explants were inoculated with lab-adapted strain (SF162; MOI 0.25) or HIV-1 T/F strain (CHO58; MOI 0.25). After 2 days, the migratory fraction was collected and co-cultured with U87.CD4.CCR5 cells. Cells were analyzed for CD1a expression and p24 content in U87.CD4.CCR5 cells by flow cytometry. A lower MOI was used due to higher susceptibility of LCs for CH058 than for laboratory strain SF162.

Data information: Symbols represent independent donors (mean of duplicates). Data are mean ± SD. Two-tailed *t*-test, $*P < 0.05$.

Strikingly, we found that only *P. timonensis* had this unique effect on HIV-1 uptake by LCs. Several research groups have reported that during vaginal dysbiosis *P. timonensis* prevalence can be as abundant as 30–35% of the total vaginal microbiome (Cho *et al*, 2022; France *et al*, 2022; Fredricks *et al*, 2022). Additionally, data from 110 people of reproductive age revealed that the vagina contains a total of 10^{10}–10^{11} bacteria (Chen *et al*, 2021) and supports the conclusion that hundreds of million *P. timonensis* bacteria can be present in the vagina during vaginal dysbiosis. Hence, the ratio of *P. timonensis* bacteria to LCs increases during vaginal dysbiosis most likely modulating the function of vaginal LCs possibly resulting in enhanced HIV-1 uptake and transmission.

The increased viral cargo in *P. timonensis*-exposed LCs was due to HIV-1 uptake and not to HIV-1 replication as antiretroviral drugs did not abrogate the observed effects. Moreover, we did not observe any induction of HIV-1 replication in *P. timonensis*-treated LCs infected with reporter eGFP-expressing HIV-1 even though the reporter virus was efficiently taken up by *P. timonensis*-treated LCs. Our data demonstrate that *P. timonensis* strongly increases HIV-1 uptake by LCs rather than that HIV-1 establishes a productive infection in *P. timonensis*-exposed LCs. This hypothesis is supported by the fact that *P. timonensis*-induced HIV-1 internalization is independent of CD4, CCR5, and viral fusion, and in line with the intracellular HIV-1-positive vesicles observed in *P. timonensis*-exposed LCs.

Normally, HIV-1 is internalized by langerin resulting in viral fusion via CD4 and CCR5. In turn, viral fusion triggers autophagy leading to degradation of fused HIV-1 as well as HIV-1 stored in Birbeck granules (Valladeau *et al*, 2000; De Witte *et al*, 2007; De Jong *et al*, 2008; van den Berg *et al*, 2014; Ribeiro *et al*, 2016). Strikingly, fusion was significantly increased in *P. timonensis*-exposed LCs, whereas we did not observe viral integration nor replication. These data strongly suggest that the autophagy-dependent restriction of HIV-1 infection in LCs is not affected by *P. timonensis*. Indeed, *P. timonensis*-induced HIV-1 uptake was independent of langerin and mostly unaffected by increasing

autophagy. Moreover, neither CD4 nor CCR5 were involved in viral uptake by *P. timonensis*-exposed LCs and viral fusion inhibitors failed to reduce *P. timonensis*-mediated uptake. Taken together, these results indicate that *P. timonensis* does not abrogate the autophagy-restricted pathway but induced HIV-1 uptake via another pathway that allows escape of HIV-1 from langerin-mediated autophagic degradation. As a result, internalized HIV-1 remained infectious for several days within *P. timonensis*-exposed LCs and was efficiently transmitted to HIV-1 susceptible cells upon co-culture independent of viral fusion and replication in LCs. Our observations show striking resemblances with the formation of multivesicular bodies (MVBs) in DCs during HIV-1 infection. These MVBs have shown to contain large intracellular pools of captured infectious HIV-1, which can be efficiently transmitted to $CD4^+$ T cells (Garcia *et al*, 2005). Further studies are required to identify the intracellular vesicles harboring HIV-1 in *P. timonensis*-exposed LCs.

Increased HIV-1 uptake and subsequent transmission by *P. timonensis*-exposed LCs was independent of viral replication as it was not inhibited by antiretroviral drugs such as tenofovir, T20, and maraviroc. Previous research has shown that the effectiveness of prophylactic tenofovir gel treatment is reduced among women with vaginal dysbiosis (Klatt *et al*, 2017). This is thought to be due to the increase in anaerobic bacteria that metabolize these antiretroviral drugs, thereby lowering their bioavailability in the vaginal mucosal tissue (Klatt *et al*, 2017). However, the enhanced sequestering of HIV-1 in *P. timonensis*-exposed LCs, which is not abrogated by antiretroviral drugs, poses an additional mechanism that could explain the reduced effectiveness of vaginal prophylactic tenofovir treatment during vaginal dysbiosis (Atashili *et al*, 2008; Passmore *et al*, 2016; Gosmann *et al*, 2017; McClelland *et al*, 2018).

During sexual transmission, multiple HIV-1 variants are present; however, only a single variant, the T/F variant, establishes infection in the new host (Derdeyn *et al*, 2004; Salazar-Gonzalez *et al*, 2009). Recently, we have shown that T/F strains productively infect immature vaginal as well as epidermal LCs (Hertoghs *et al*, 2019). Here, we show that *P. timonensis* enhanced infection of and transmission by immature LCs of T/F variant CH058. CH058 uptake was not inhibited by AZT nor tenofovir, suggesting that *P. timonensis* also enhances uptake of T/F variants independent of viral replication. These data further support a role for *P. timonensis* in enhancing HIV-1 susceptibility *in vivo*.

In conclusion, we have discovered a novel mechanism via which a dysbiosis-associated bacterium increases HIV-1 susceptibility in women. The effect of *P. timonensis* on mucosal LCs might be one of the underlying causes of increased susceptibility for HIV-1 in women with vaginal dysbiosis and the ineffectiveness of prophylactic treatment in these women. Unfortunately, current treatments for vaginal dysbiosis are suboptimal, and many women are not treated because they are asymptomatic (van de Wijgert & Jespers, 2017). Identification of individuals that carry *P. timonensis* could provide specific treatment targets for HIV-1 prevention. Our study underscores the urgent need to improve the prevention and treatment of bacterial vaginosis containing specific bacteria, like *P. timonensis*, and these new insights could help to develop and re-evaluate treatment protocols to improve HIV-1 prevention among women with vaginal dysbiosis.

Materials and Methods

Ex vivo explants culture

Vaginal and skin tissue was freshly processed for each experiment. To obtain epithelial sheets, vaginal and skin tissue was treated with dispase II (3 U/ml for vaginal tissue and 1 U/ml for skin; Roche Diagnostics), after which the epithelium/epidermis was separated from the rest of the tissue. *Ex vivo* vaginal epithelial explants were extensively washed, cut to size, and placed in a Transwell system (6.5 mm Transwell, 5.0 μm pore polycarbonate membrane inserts; Corning) complete medium (IMDM; Thermo Fisher Science) supplemented with 10% FCS (Invitrogen), L-glutamine (2 mM; Lonza), penicillin (10 U/ml; Invitrogen), and streptomycin (10 mg/ml; Invitrogen), containing the according stimuli. *Ex vivo* skin explants were prepared in a Costar® 24-well plate (Corning) in complete medium containing the according stimuli as described before (Hertoghs *et al*, 2019).

Cell isolation

Isolated immature vaginal LCs and immature epidermal skin LCs were purified as described by de Jong *et al* (2010) and De Witte *et al* (2007). Briefly, vaginal and skin epithelial sheets were incubated in PBS containing DNAse I (20 units/ml, Roche Applied Science) and trypsin (0.05%, Beckton Dickinson) for 10 (vaginal) to 30 (skin) minutes at 37°C to obtain a single cell suspension. Trypsin digestion was inactivated with FCS. Cells were washed in complete medium, layered on a Ficoll (Axis-shield) gradient, and LCs were purified using CD1a-labeled immunomagnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec). Langerin and CD1a expression was quantified by flow cytometry, and immature LCs were routinely 90–98% pure and expressed high levels of langerin and CD1a. Cells were placed in a 96-well plate (Corning) in complete medium containing the according stimuli.

Vaginal bacteria and LC stimulations

Atopobium vaginae (DMSZ-15829), *Bacteroides fragilis* (Utrecht UMC), *Bacteroides thetaiotaomicron* (DSMZ-2079), *Gardnerella vaginalis* (DSMZ-4944), *Lactobacillus crispatus* (DSMZ-20584), *Lactobacillus iners* (DSMZ-13335), *Megasphaera elsdenii* (DSMZ-20460), *Prevotella amnii* (DSMZ-23384), *Prevotella bivia* (DSMZ-20514), *Prevotella copri* (DSMZ-18205), and *Prevotella timonensis* (DSMZ-22865) were cultured as recommended by DSMZ (German Collection of Microorganisms and Cell Cultures GmbH; Braunschweig). After harvesting bacteria during log-phase growth, bacteria were extensively washed in PBS and standardized to an optical density at 600 nm (OD_{600}) of 1. Next, culture purity was determined by plate culture and gram stains. Subsequently, bacterial suspensions were UV-inactivated in a UV crosslinker using five rounds of 100,000 $\mu J/cm^2$. Loss of viability was verified by plating UV-inactivated bacteria. Isolated primary LCs were stimulated with a ratio (MOI) of 1:10. For stimulation of vaginal and skin explants, volumes equivalent to what would have been used for 5×10^5 vaginal LCs were used. Both *ex vivo* tissue explants and isolated LCs were stimulated overnight before HIV-1 infection. Additionally, poly (I:C; 5 μg/ml; Invivogen) stimulation was used.

Viruses, HIV-1 infection, and transmission

SF162, JRCSF, CHO58, CHO106, NL4.3-BaL, NL4.3eGFP-Bal, and NL4.3(ΔEnv) HIV-1 were generated as described previously (van den Berg *et al*, 2014; Hertoghs *et al*, 2019). All produced viruses were titrated using the indicator cells TZM-Bl. Purified immature vaginal and skin LCs were infected with MOI 0.5, and *ex vivo* vaginal and skin explants were exposed to volumes equivalent to what has been used for 5×10^5 isolated LCs. Isolated LCs were collected at day 3–5 of HIV-1 exposure, while the migratory fraction of *ex vivo* tissue explants was collected at day 3 after HIV-1 exposure. Collected cells were either fixed in 4% paraformaldehyde and the intracellular p24 content was assessed by intracellular flow cytometry, lysed for p24 ELISA, or cells were used in transmission assays. For transmission, collected cells were washed extensively in complete medium to remove unbound virus and co-cultured with U87 cells stably expressing CD4 and wild-type CCR5 co-receptor (NIH AIDS Reagent Program, Division of AIDS, NIAID, from H.K. Deng and D.R. Littman), for 3 days at 37°C (Björndal *et al*, 1997). After co-culture, cells were fixed in 4% paraformaldehyde and intracellular p24 was analyzed by flow cytometry.

Flow cytometry

After fixation, cells were permeabilized in PBS supplemented with 0.5% saponin (Sigma) and 1.0% BSA (Sigma) for 10 min. To assess LC infection and transmission to U87 cells, cells were stained with PE-conjugated p24 (KC57, Beckman Coulter) in combination with APC-conjugated CD1a (HI149, BD Biosciences). For some experiments, NL4.3eGFP-Bal was used and HIV-1-1eGFP expression was measured. HIV-1 acquisition was defined as % $p24^+$ CD1a-positive cells (infection of LCs) or % $p24^+$ CD1a-negative cells (transmission of LCs to U87.CD4.CCR5). For surface expression of maturation markers, cells were stained in Tris-buffered saline supplemented with 1.0% BSA with PE-conjugated CD80 (L307.4, BD Pharmingen), FITC-conjugated CD86 (2331, BD Pharmingen), PE-conjugated CCR7 (3D12, BD Pharmingen), PE-conjugated langerin (DCGM4, Beckman Coulter) or CD1a (HI149, BD Biosciences) in different combinations. Cells were quantified by flow cytometry on FACS Canto II (BD Biosciences), and results were analyzed using FlowJo software. Surface marker expression is defined by mean fluorescent intensity (MFI).

Blocking reagents and replication inhibitors

HIV-1 replication inhibitors zidovudine (AZT; 20 μM/ml), enfuvirtide (T20; 10,000 ng/ml) as described before (Eggink *et al*, 2011), lamivudine (3TC; 50 μM/ml), indinavir (IDV; 5 μM/ml), maraviroc, (MAR; 30 μM/ml), and tenofovir (TFV; 50 μM/ml) were added after O/N stimulation with vaginal bacteria and 1 h prior to HIV-1 infection. All replication inhibitors were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Concentrations were optimized for usage in both isolated LCs and *ex vivo* explants. The same accounts for anti-CD4 antibody (20 μg/ml; RPA-T4; Biolegend) and isotype control (20 μg/ml; MOP-C21/P3; E-bioscience), autophagy inducer rapamycin (100 and 500 nM mTOR inhibitor, tlrl-rap, Invivogen), langerin blocking antibody 10e2 (house produced; 40 μg/ml), or langerin blocking reagent mannan (Sigma-Aldrich; 100 μg/ml).

Confocal microscopy

Isolated vaginal LCs were stimulated overnight with vaginal bacteria and subsequently infected with SF162 (MOI 0.5) for 3 days. After attachment onto poly-L-lysine coated slides, LCs were fixed with 4% paraformaldehyde and permeabilized with PBS supplemented with 0.1% saponin (Sigma), 1.0% BSA (Sigma) and 1 mM Hepes (Sigma). Next, LCs were incubated with primary antibodies (5 μg/ml) directed against p24 (KC57, Beckman Coulter), followed by Alexa Fluor 488-conjugated anti-mouse (715-545-150, Jackson) incubation. Nuclei were counterstained with Hoechst (10 μg/ml; Molecular Probes). Images were obtained by Leica TCS SP-8 X confocal microscope, and data analysis was carried out with Leica LAS AF Lite (Leica Microsystems).

HIV-1 uptake assay

After overnight stimulation with medium or bacteria, LCs were incubated over time with SF162 (MOI 0.5). LC samples were then treated with Trypsin–EDTA (0.05%, Invitrogen) to remove membrane-bound HIV-1 virions and washed extensively, and HIV-1 uptake was analyzed by flow cytometry or ELISA. HIV-1 uptake was quantified by determining p24-antigen levels (pg/ml) of cell lysates by means of ELISA (ZeptoMetrix) 4 h after viral incubation. For flow cytometry, isolated skin LCs were fixed 5 days after viral incubation and washed with PBS or Trypsin–EDTA and subsequently intracellular p24 content was assessed using flow cytometry.

Freeze–thaw assay

After overnight stimulation with vaginal microbiota, isolated LCs were infected with SF162 (MOI 0.5). After 3–5 days of infection, LCs were treated with Trypsin–EDTA (0.05%, Invitrogen) to remove non-internalized HIV-1 virions, washed, and subjected to two freeze–thaw cycles (−80°C to RT). Next, LC lysates were added to U87-CD4-CCR5 cells. Release of infectious HIV-1 was determined by analyzing infection levels (% $p24^+$ cells) in U87-CD4-CCR5 cells using intracellular p24 staining and flow cytometry (FACSCanto II, BD Biosciences).

HIV-1 fusion assay

A BlaM-Vpr-based assay was used to quantify fusion of HIV-1 to the vaginal LC membrane as previously described. In short, bacteria-stimulated vaginal LCs were infected with NL4.3-Bal-BlaM-Vpr for 4 h and loaded with substrate CCF2/AM (Life Technologies) in serum-free medium for 1 h, and BLaM-reaction was allowed to develop overnight at 22°C in complete medium supplemented with probenecid (Sigma Pharmaceuticals). LC infection with VSV-G-BlaM-Vpr was used as positive control. The shift from green (520 nm) to blue (450 nm) fluorescence, reflecting the release of BlaM-Vpr into the LC cytoplasm after fusion, was assessed using flow cytometry (LSRFortessa, BD Biosciences).

Data analysis and statistics

FACS data analysis was carried out with FlowJo-V10. Statistical analyses were performed using GraphPad 8.0 software. Two-tailed Student's *t*-test for unpaired observations was performed on data

sets whit $n > 2$. For significance, we employed $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Study approval

Human vaginal tissue was collected from women undergoing vaginal surgery for pelvic organ prolapse in which excessive vaginal tissue was removed of the anterior or posterior vaginal wall. Human skin tissues were obtained from healthy donors undergoing corrective breast or abdominal surgery. This study was approved by the Medical Ethics Review Committee of the Amsterdam University Medical Center (Amsterdam UMC), location Academic Medical Center (AMC), Amsterdam, the Netherlands, reference number: W15_089 # 15.0103 (skin tissue) and reference number: W13_046 # 13.17.0060 (vaginal tissue).

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

Acknowledgements
This work was supported by the AMC PhD Scholarship, Dutch Research Council (NWO-ZonMw) VIDI grant 91718331, Dutch Research Council (NWO-ZonMW) TOP grant 91218017 and the European Research Council (ERC) Advanced Grant 670424.

Author contributions
Nienke H van Teijlingen: Conceptualization; data curation; formal analysis; funding acquisition; validation; investigation; methodology; writing – original draft; project administration. **Leanne C Helgers:** Conceptualization; data curation; formal analysis; validation; investigation; visualization; methodology; writing – original draft; project administration; writing – review and editing. **Ramin Sarrami-Forooshani:** Conceptualization. **Esther M Zijlstra-Willems:** Investigation. **John L van Hamme:** Investigation. **Celia Segui-Perez:** Investigation. **Marleen Y van Smoorenburg:** Investigation. **Hanneke Borgdorff:** Conceptualization. **Janneke HHM van de Wijgert:** Conceptualization; writing – review and editing. **Elisabeth van Leeuwen:** Conceptualization. **Joris AM van der Post:** Conceptualization; supervision. **Karin Strijbis:** Conceptualization; resources; supervision; writing – review and editing. **Carla MS Ribeiro:** Conceptualization; supervision; investigation; writing – review and editing. **Teunis BH Geijtenbeek:** Conceptualization; resources; supervision; funding acquisition; writing – review and editing.

In addition to the CRediT author contributions listed above, the contributions in detail are:

NHT and LCH designed, performed, and interpreted most experiments and prepared the manuscript. RS-F helped to design and interpret experiments. EMZ-W and JLH performed and analyzed some experiments. HB and JHHMvdW assisted with the selection of bacteria to be tested. CSP and KS helped grow the bacteria. HB, JHHMW, EL, JAMP, CS-P, and MYS participated in discussion on the data and writing the manuscript. CMSR and KS helped to design and interpret experiments and helped writing the manuscript. TBHG supervised all aspects of the project.

Disclosure and competing interests statement
The authors declare that they have no conflict of interest.

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