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Productive HIV-1 Infection of Human Cervical Tissue *Ex Vivo* is Associated with the Secretory Phase of the Menstrual Cycle

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Abstract

Cervical tissue explants (CTE) from 22 HIV-1 seronegative women were exposed to R5 HIV-1 *ex vivo*. Eight CTE were productively infected in terms of HIV-1 $p24_{Gag}$ release in culture supernatants whereas 14 were not. Nonetheless, both accumulation of HIV-1_{gag} DNA and of $p24_{Gag}^{+}$ $CD4^{+}$ T cells and macrophages occurred in both productive and, at lower levels, in nonproductive CTE. Nonproductive CTE differed from productive CTE for higher secretion of CCL3 and CCL5. A *post-hoc* analysis revealed that all productive CTE were established from women in their secretory phase of the menstrual cycle, whereas nonproductive CTE derived from women either in their secretory (28%) or proliferative (36%) menstrual cycle phases or with an atrophic endometrium (36%). Thus, our results support the epidemiological observation that sexual HIV-1 transmission from males to women as well as from women to men is more efficient during their secretory phase of the menstrual cycle.

Introduction

Most of the newly human immunodeficiency virus type-1 (HIV-1) infected women acquire infection through vaginal intercourse in which semen-associated virus is deposited in the mucosa of the lower female genital tract before infecting primary HIV-1 target cells and disseminating to the regional lymph nodes [1,2]. The lack of understanding of the basic

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Disclosure

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mechanisms of HIV-1 transmission and dissemination in the female lower genital tract is a limitation to developing efficient protection measures as evidenced from the recent controversial results on the efficacy of vaginal microbicides [3], [4].

In order to reach its primary cell targets in the female lower genital tract, i.e. $CD4^+$ T lymphocytes, dendritic cells (DC) and sub-epithelial macrophages [5–7], the virus must circumvent mucosal barriers [8]. The nature of these barriers is both physical (columnar or multi-stratified epithelial layer, mucus, and acidic pH) and biological, including secreted factors endowed with anti-HIV activity such as CCR5-binding chemokines, secretory leukocyte peptidase inhibitor (SLPI) and β-defensins [9–11]. Noteworthy, these mucosal barrier components as well as the state of activation of HIV-1 target cells are strongly influenced by the menstrual cycle that is under the control of sexual hormones [12–16]. In this regard, different studies performed in non-human primates (NHP) have demonstrated that different phases of the menstrual cycle affect simian immunodeficiency virus (SIV) transmission and infection at the level of the cervico-vaginal mucosa [17,18]. In humans, Heffron et al. [19] have recently demonstrated that systemic administration of hormonal contraceptives can increase significantly the risk of HIV-1 acquisition by women as well as the likelihood of viral transmission from infected women to men.

In vitro studies using a reconstituted mucosal barrier have provided new insights in sexual transmission of HIV-1/SIV [20]. Another fruitful experimental model to study the mechanisms of the early events in HIV interaction with the genital mucosa is represented by human cervical (or cervico-vaginal) tissue explants (CTE), which retain the *in vivo* cyto-architecture and some tissue functions for several days of culture [21,22]. By adopting this latter experimental model we have previously reported that activated tissue-associated $CD4^+$ T cells are major targets and the likely source of CCR5-dependent (R5), but not CXCR4-dependent (X4), HIV-1[22]. While the role of $CD4^+$ T lymphocytes in HIV-1 infection of the cervico-vaginal compartment has been thoroughly investigated, the potential contribution of resident macrophages is much less defined [23,24].

Therefore, we here investigated the potential role of both $CD4^+$ T lymphocytes and macrophages in sustaining R5 HIV-1 replication in CTE obtained from HIV-seronegative women undergoing hysterectomy for benign gynecological conditions. We found that both cell types contribute to propagate HIV-1 infection in this model system. Furthermore, we observed that the menstrual cycle phases of the donors at the time of hysterectomy strongly affected virus production (as measured by $p24_{Gag}$ release) in CTE. In fact, only tissues obtained from women in their secretory phase (dominated by progesterone) at the time of surgery sustained productive virus replication, whereas nonproductive infection of CTE was observed with tissues obtained from women in either their secretory or proliferative phase of the menstrual cycle or with an atrophic endometrium.

Results

R5 HIV-1 efficiently replicates in a subset of CTE established from seronegative women

Fresh human cervical tissues (collected ≤1 h after surgery) were dissected into approximately 2-mm^3 blocks and then cultured on collagen sponge gel rafts for up to 12

days, as described [21,22]. CTE were inoculated with R5 HIV-1_{BaL} and virus replication was evaluated by both $p24_{Gag}$ release into the culture supernatant as well as by the accumulation of HIV DNA in tissue-associated cells [22]. In order to discriminate between the viral inoculum captured nonspecifically by the tissue blocks and *de novo* virus replication, control donor-matched tissue blocks were incubated with R5 HIV-1 in the presence of the RT-inhibitor lamivudine (3TC; 5 μM), as described [22].

Virus replication became evident on day 9 post-HIV-1 inoculation and increased up to day 12 as evaluated by $p24_{Gag}$ release in CTE culture supernatants. In contrast, there was no increase of virus release in 3TC-treated HIV-1 exposed cultures. CTE from different donors showed a clear-cut difference in their capacity to support virus production. We defined as "productive" those CTE in which a progressive accumulation of $p24_{Gag}$ into culture supernatants occurred in comparison to 3TC-treated autologous cultures. Conversely, "nonproductive" CTE were defined as those showing no difference in $p24_{Gag}$ release in the presence or absence of 3TC. Overall, out of 22 CTE (each established from a different donor) 8 were classified as productive, whereas the remaining 14 were defined as nonproductive (Figure 1A).

Higher levels of HIV-1 DNA accumulate in productive than in nonproductive CTE

In order to further investigate whether CTE established from different donors were susceptible or resistant to HIV-1 infection and replication, HIV-1 DNA was quantified by means of RT-PCR after 12 days of infection. Productive CTE showed accumulation of HIV DNA that reached $3.06\pm1.06 \times 10^5$ copies of HIV-1_{gag} DNA per 10^7 cells over 3TC-treated control cultures (that were essentially negative for HIV-1 DNA detection). In comparison to productive CTE, significantly lower levels of HIV DNA ($0.80\pm0.20 \times 10^5$) were detected in nonproductive CTE ($p<0.05$; Figure 1B).

Phenotype and susceptibility to HIV-1 infection of CTE-associated $CD4^+$ T cells and resident macrophages

In order to characterize which potential HIV-1 target cells were present in our CTE, fresh cervical tissues from some donors were digested with collagenase IV and the cell suspensions were stained for different surface antigens. A flow cytometric analysis indicated that the percentage of $CD4^+$ T cells and resident macrophages (defined as lineage negative, $CD3^{neg}$, $CD19^{neg}$, $CD20^{neg}$, $CD56^{neg}$, $CD14^+CD11b^+$ cells) isolated from CTE were not significantly different between productive and nonproductive tissues (data not shown). Most $CD3^+CD4^+$ T cells isolated from either productive or nonproductive CTE were $CCR5^+$ (76.7±3.4% vs. 81.7±12.5%, respectively; n=7; Figure 2A) and showed an "effector memory" phenotype, as defined by the lack of expression of CCR7 and CD45RA (92.1±3% vs. 93,5±0,9 for productive and nonproductive CTE, respectively; n=6; Figure 2B).

Different populations of resident macrophages could be distinguished based on the expression of CD4 and CCR5. A minority of these cells co-expressed CD4 and CCR5 without significant differences between productive and nonproductive CTE (21.1±7.6% and 42.7±14.8% for productive and nonproductive CTE, respectively; Figure 2C), as observed for T cells. Other resident macrophages expressed either CD4 alone or CCR5 alone

(10.7±7.5% vs. 6.9±4.5% and 8.1±2.5% vs. 20.5±7.4% for productive and nonproductive CTE, respectively, Figure 2C). Finally, a macrophage subset was negative for the surface expression of both CD4 and CCR5 (59.6±9.1% 29.8±14.1% vs. for productive and nonproductive CTE, respectively; Figure 2C) thus resembling a phenotype described for intestinal macrophages [6].

Resident macrophages were also evaluated for the expression of mannose receptor (CD206) and of Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN, CD209). No differences were observed in the fractions of $CD206^+$ macrophages between productive and nonproductive CTE (75.3±1.9 vs. 79.1±2,1 respectively; n=4; Figure 2D). In contrast, a higher percentage of $CD209^+$ macrophages was observed in productive vs. nonproductive CTE (53.9±11.6% vs. 27±2.1% respectively; n=9, $p<0.05$; Figure 2D).

Infection of HIV-1 target cells present in the CTE was confirmed by intracellular (i.c.) $p24_{Gag}$ staining of cells isolated from tissue blocks obtained from the 22 different donors 6 days after infection (Figure 3A). A trend of higher frequency of $p24_{Gag}^+$ $CD8^{neg}$ T cells (that were gated in order to include $CD4^+$ T lymphocytes that downregulated CD4 as a consequence of HIV-1 infection [22]) and macrophages was observed in productive vs. nonproductive CTE, although these differences were not statistically significant (14.2+3.2% vs. 8.1+2.3% for productively and nonproductively infected CTE, respectively, for T cells, and 1.7+0.7% vs. 0.6+0.3%, respectively, for macrophages; Figure 3B).

Comparable levels of anti-viral peptides and pro-inflammatory cytokines are secreted by both productively and nonproductively infected and uninfected CTE

Both β-defensins and SLPI have been associated with anti-HIV activities, particularly at the mucosal level [9,10]. Therefore, we investigated whether the secretion of these innate antiviral molecules could explain the different outcomes of CTE infection. However, no differences in the release of these factors were observed between productive and nonproductive CTE, as measured after 3 days (Figure 4, **upper panel)** or 12 days (data not shown) of culture both in the absence or presence of HIV-1.

Since an inflammatory environment also could be involved in the differential permissiveness of CTE to HIV-1 infection and spreading, we determined the levels of secretion of pro-inflammatory cytokines Tumor Necrosis Factor-α (TNF-α), Interferon-γ (IFN-γ) and CCL2 (a chemokine previously reported to be associated with increased virus replication [25,26]), as well as of the anti-inflammatory cytokines Interleukin-4 (IL-4) and IL-10, after 3 and 12 days from virus inoculation. Low levels of TNF-α and IFN-γ were released by both productive and nonproductive CTE irrespective of whether they were exposed or not to HIV-1 (Figure 4, **middle panel**), whereas the concentrations of both IL-4 and IL-10 by all CTE were below the detection threshold of the ELISA assay at both time points (data not shown). CCL2 was secreted at higher levels than cytokines, although no differences were observed between infected and uninfected CTE or between productive vs. nonproductive CTE (Figure 4, **middle panel**). No differences were also observed for all these analytes in the different culture conditions after 12 days of virus exposure (data not shown).

Nonproductive CTE show increased CCL3 and CCL5 secretion in comparison to productive CTE

Next, we evaluated whether the differential permissiveness of CTE was associated with different levels of secreted CCR5 ligands (namely, CCL3, CCL4 and CCL5) into culture supernatants 3 and 12 days after virus inoculation or in donor-matched control tissues not exposed to HIV-1. In contrast to what observed for antiviral peptides and cytokines, nonproductive CTE were characterized by a higher release of CCL3 reaching statistical significance in uninfected conditions (103.7±13.9 vs. 175.5±32.7 pg/ml for productive and nonproductive CTE, respectively; $p<0.05$; Figure 4, **lower panel**). HIV-1 inoculation was associated with significantly higher levels of CCL5 in nonproductive vs. productive CTE (11,5±1.1 vs. 26.7±3.4 and 13.2±1.7 vs. 30.6±4.1 pg/ml for productive and nonproductive CTE infected in the absence or presence of 3TC, respectively, $p<0.05$; Figure 4, **lower panel**) 3 days after virus exposure. Moreover, we compared the levels of secreted CCL5 released from 4 non-productive tissues to those of the 8 productive ones after HIV-1 infection, all obtained from the donors in the secretory phase. On average, the levels of CCL5 secreted by non-productive tissue upon HIV-1 infection were twice as high than those of productive tissue although this difference did not reach statistical significance likely due to the small numbers of donors in these two groups (data not shown). These differences were not observed after 12 days of CTE cultures both in the presence or absence of HIV-1 (data not shown).

Only CTE from women in their secretory phase of the menstrual cycle became productively infected with R5 HIV-1

In order to better understand whether the productive or nonproductive outcomes of CTE infection correlated with a different tissue architecture, we performed a *post-hoc* histological analysis of the cervical tissues after surgery. In particular, the cervical tissues were fixed with 10% formaldehyde, stained with hematoxylin/eosin and were analyzed for the presence of koilocytosis (an epithelial cell transformation that may occur as a result of infection by the human papillomavirus), connective tissue sclerosis, glandular ectasia and metaplasia. These cervical pathological lesions were, however, similarly distributed between productive and nonproductive CTE, except for connective tissue sclerosis that was detected exclusively in 21% of nonproductive donors (Figure 5A).

Quite remarkably, the different outcomes of HIV-1 infection (i.e. productive vs. nonproductive infection in terms of $p24_{Gag}$ release in the culture supernatants) were strongly correlated with the different phases of the menstrual cycle of the donors at the time of surgical intervention. In fact, a *post-hoc* analysis revealed that all productive CTE were established from women in their secretory phase at the time of surgery (Figure 5B). Conversely, as shown in Figure 5B and 5C, nonproductive CTE were derived from women either in their proliferative (36%) or secretory (28%) phases of the menstrual cycle or histologically characterized by an atrophic endometrium (36%). Nonproductive CTE, however, showed low, but detectable levels of HIV-1 DNA accumulation above the 3TC controls (1.31±0.54, 0.64±0.01, 0.39±0.01 $\times 10^5$ copies of HIV-1_{gag} DNA for proliferative or secretory phases or endometrial atrophy, respectively; Figure 5D). No differences were observed in the age of donors from whom productive or nonproductive CTE were

established, except for those with an atrophic endometrium who were significantly older than the others, as detailed in Table 1.

Discussion

In order to infect its target cells in the course of male-to-female HIV-1 transmission, the virus must cross the mucosal barrier, consisting of an epithelial layer covered by mucus [8]. The exact mechanisms of this process and the nature of HIV-1 cell targets, especially the involvement of macrophages resident in the submucosa [7], remain largely speculative. Moreover, all these barrier elements are strongly influenced by the menstrual cycle that is under the control of sexual hormones [12–14]. Accordingly, susceptibility of women to viral infections depends on their hormonal status as experimentally demonstrated by SIV infection of NHP [17,18]. Of note, a significant increase of the risk of HIV-1 acquisition associated with a higher likelihood of female-to-male transmission has been reported in women taking or women using hormonal contraceptives systemically [19]. In order to decipher the mechanisms of HIV-1 transmission leading to the infection of target cells in the female lower genital tract, it is crucial to investigate these phenomena in an adequate laboratory-controlled system *ex vivo*, such as the R5 HIV-1 infection of CTE used in the present study. In fact, the cyto-architecture and at least some of the tissue cell functions are maintained in CTE for several days in culture; furthermore, CTE are permissive for productive R5, although not X4, HIV-1 infection without requiring exogenous stimulation by mitogens or cytokines [21,22].

In the present study, we have investigated the susceptibility to productive R5 HIV-1 infection of CTE established *ex vivo* from 22 HIV-1 seronegative women undergoing hysterectomy for the presence of non-malignant tumors. Here, we focused exclusively the exposure of CTE to R5, and not to X4 HIV-1, since *in vivo* R5 HIV-1 dominates the earliest stages of HIV infection following sexual transmission. Furthermore, *ex vivo* R5 HIV-1 but not X4 HIV-1 productively infects CTE, as we have reported earlier [22]. In the present study, the primary criterion for defining a productive infection was the release of $p24_{Gag}$ above the baseline release in donor-matched cultures inoculated with HIV-1 in the presence of the RT inhibitor 3TC. HIV-1 exposed cultures in the presence of 3TC indeed gradually released $p24_{Gag}$ in the supernatant that we considered as HIV-1 background. According to this criterion, 8 out of 22 CTE (36%) showed clear-cut evidence of virus replication, whereas in the remainder cultures the levels of total $p24_{Gag}$ released in the presence or absence of 3TC were superimposable. Thus, the outcome of HIV-1 inoculation of CTE fell into two clear-cut categories, i.e “productive” and “nonproductive” infections.

Both $CD4^+$ T cells and resident macrophages supported HIV-1 replication although macrophages (only a minority of which co-expressed CD4 and CCR5) to a lesser extent than T lymphocytes. At present, however, we cannot exclude the possibility that these resident macrophages merely took up virions earlier produced by $CD4^+$ T lymphocytes, rather than being productively infected. The different outcomes of infection (i.e. productive vs. nonproductive) did not correlate with the number of HIV-1 target cells; in fact, the relative numbers of CCR5-expressing $CD4^+$ T cells and macrophages were comparable in both productive and nonproductive CTE. Furthermore, the functional distributions of $CD4^+$ T

cells were similar in productive and nonproductive CTE, with $CD4^+$ T cells displaying an "effector memory" phenotype representing the dominant subset. In general, our findings are in agreement with the report of Shen and colleagues who isolated DC, $CD4^+$ T cells and macrophages from ectocervical tissue, infected them *in vitro* with an R5 HIV-1 strain and found that all these cells were productively infected although with different kinetics [27].

Concerning resident macrophages, while the percentage of those expressing CD206 (mannose receptor) was comparable in both productive and nonproductive CTE, there was a significantly higher percentage of cells expressing CD209 (DC-SIGN) on their surface in productive CTE than in nonproductive ones. In this regard, DC-SIGN has been described as an HIV-1 "capture" receptor capable of promoting infection of $CD4^+$ T cells in *trans* [28,29] as well as of influencing *cis* infection of DC via triggering of a signal transduction pathway involving Raf-1 [30]. Although the role of DC-SIGN in the biology of DC has been thoroughly investigated, the potential consequence of its expression by resident macrophages in the context of sexual HIV-1 transmission is much less understood. The observation of a more frequent expression of DC-SIGN by resident cervical macrophages of those CTE showing a productive profile upon infection suggests its potential contribution to HIV propagation in the tissue. In this regard, it is of interest that the potential efficacy of a candidate microbicide targeting DC-SIGN and preventing HIV-1 infection in cellular and human cervical explant models has been recently reported [31].

Searching for biological correlates of productive and nonproductive CTE infections, however, we did not observe significant differences in terms of secreted levels of either innate anti-viral peptides (β-defensins, SLPI), pro-inflammatory (TNF-α, IFN-γ, CCL2**)** or anti-inflammatory (IL-4, IL-10) cytokines. In contrast, some CCR5-binding chemokines were secreted at higher levels by nonproductive vs. productive CTE. In the case of CCL3, this difference was statistically significant in uninfected tissues, whereas for CCL5 the difference between productive and nonproductive CTE became evident after infection. Although this observation is only correlative, in consideration of the well-established role of CCR5-binding chemokines in inhibiting HIV-1 infection (in all the main routes of transmission, sexual, mother-to-child, blood-related [32]), we speculate that the higher levels of CCL3 and CCL5 released by nonproductive CTE might have contributed to prevent or curtail R5 HIV-1 infection in these tissues, including nonproductive CTE established from women in their secretory phase (in the case of CCL5), at least during the initial days following HIV-1 exposure, since no differences were observed after 12 days of culture. This interpretation fits with the observation of significantly higher levels of CCL5 in the genital secretion of female commercial sex workers (CSW) naturally resistant to HIV-1 infection compared to those of uninfected CSW [33]; however, no information on whether the levels of the chemokine varied according to the menstrual cycle was reported in this study [33]. A broader upregulation of chemokine release *in vitro* by either productive infection or by exposure to HIV-1 proteins (including Tat, Nef and $gp120_{Env}$) has been previously described in cultures of either activated PBMC, monocyte-derived macrophages or glial cell lines [34–37]. Thus, our findings sustain the potential relevance of strategies aimed at blocking R5 HIV-1 access to the CCR5 co-receptor in order to hamper early local replication in the female genital tract, an event also influenced by hormonally mediated modulation of the

CCL5/CCR5 axis [38]. In this regard, higher levels of CCR5 expression by T cells in the uterine cervix and blood obtained from post-menopausal women have been recently reported [39].

A *post-hoc* analysis of the *ex vivo* outcomes of HIV-1 exposure of CTE (productive vs. nonproductive) did not reveal a correlation with histological alterations of the mucosa and submucosa. In particular, we evaluated the tissues immediately after surgery for the presence of koilocytosis, connective tissue sclerosis, glandular ectasia and metaplasia. No differential distribution of these cervical lesions were observed with the exception of connective tissue sclerosis, a condition associated with an atrophic endometrium, typical of a menopausal state, that was observed exclusively in 21% of nonproductive CTE. In contrast, a strong correlation was observed between productive HIV-1 infection of CTE and the menstrual cycle phase of the donor at the time of surgery. Remarkably, all CTE that were productively infected were obtained from women in their secretory phase (dominated by progesterone), whereas non-productive tissues were obtained from women in different phases of their menstrual cycle, i.e., secretory (28%) or proliferative (36%) phases (the latter dominated by estrogens) or characterized by an atrophic endometrium (36%), a condition associated with the virtual absence of sexual hormones as typically observed in menopause. The strong association between the secretory phase of the menstrual cycle and the productive infection outcome of CTE was supported by the observation of higher levels of HIV-1 DNA accumulation in productive vs. nonproductive CTE.

The menstrual cycle and its related sexual hormones are known to be important factors in sexual HIV-1 transmission as highlighted by studies of SIV transmission in Rhesus macaques that are frequently treated with progesterone derivatives (such as Depoprovera) in order to increase the efficiency of intra-vaginal infection [40]. Although these studies indicate that progesterone did not influence the number of SIV target cells in the NHP female genital tract [41], the hormonal treatment induced an increased tightness of the vaginal epithelium [12], disruption of the integrity of epithelial tight junctions [14], and decreased the production of antimicrobial molecules, including SLPI [42] and β-defensins [43,44]. Concerning HIV-1 infection, Heffron and colleagues reported that the use of injectable formulation of hormonal contraceptives based on progestinic hormones in African women increased both their frequency of HIV-1 acquisition and the likelihood of transmitting the infection to their male partners [19]. In this regard, our results of *ex vivo* infection of CTE are in full agreement with both the epidemiological and the clinical experimental evidence that the secretory phase of the menstrual cycle (controlled by progesterone) is associated with the release of $p24_{Gag}$, likely reflecting virion production, and, consequently, with a higher likelihood of viral transmission by sexual intercourse.

In summary, our results demonstrate an association between the capacity of the cervical tissue to support productive HIV-1 infection, at least *ex vivo*, and the menstrual cycle phase of the donor, in particular with the progesterone-dependent secretory phase. Therefore, the menstrual cycle of women and/or their assumption of hormonal-based contraceptives should be taken into consideration when new preventative strategies against HIV transmission are developed and tested for their efficacy. Our study also emphasizes that CTE can represent an adequate experimental system to study the mechanisms underlying the different

susceptibility to HIV-1 infection and capacity to support its replication in different stages of the menstrual cycle. Finally, CTE could be adopted as a low-cost platform for testing novel antivirals and microbicides before moving towards more expensive NHP models of HIV-1 transmission and clinical trials in women.

Methods

Clinical diagnosis, histological analysis and determination of the menstrual cycle of the donors

Cervical tissues were obtained from 22 HIV-negative women (52.5±8.9 years-old, range: 40–76) undergoing hysterectomy for non-malignant indications (i.e., multiple leiomyomas, adnexal cysts or abnormal bleeding) in the Department of Obstetrics and Gynecology of the San Raffaele Scientific Institute, Milano, Italy. The histological analysis confirmed that all women were affected by multiple leiomyomas of the uterus. In addition, cervical tissues were evaluated for the presence of koilocytosis, connective tissue sclerosis, glandular ectasia and metaplasia. According to surgical protocols, women were asked to stop the assumption of any pharmacological agent and, in particular, of oral contraceptives at least one month before surgery. A short-term broad-spectrum antibiotic prophylaxis was administrated the day of the procedure.

The uterine/menstrual cycle stage of the donors was determined on the basis of medical records and upon endometrial dating (i.e. morphological evaluation of endometrium sections stained with haematoxylin/eosin**)**. This analysis indicated that, randomly, hysterectomy was performed in women either in their secretory (n=12) or proliferative (n=5) phases of the menstrual cycle; in addition, 5 cervical tissues were obtained from women characterized by an atrophic endometrium (Table 1).

Cervical tissue explant (CTE) culture and *ex-vivo* HIV-1 infection

The cervix was separated from the uterus within 60 min from the surgical removal and placed into cold RPMI-1640 (Lonza, Basel, Switzerland). Briefly, the mucosal epithelium and the underlying stroma of both ecto- and endo-cervix were separated from the muscular tissue and dissected into approximately 2-mm^3 blocks.

Fresh CTE from 22 donors were placed into culture and infected with CCR5-dependent (R5) HIV-1_{BaL}, according to a published protocol [22,45]; in addition, some tissue blocks from the same clinical samples were processed for phenotyping of T cells and resident macrophages. Sixteen tissue blocks per condition for each donor were incubated with 500 μl of undiluted R5 HIV-1_{BaL}, washed and placed on the top of collagen sponges at the liquid-air interface.

Viral stocks were prepared using the supernatants of peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA) [46] and IL-2 (400 U/ml; R&D Systems Minneapolis, MN) and infected with HIV-1_{BaL} collected at the peak of virus replication, typically occurring between the first and second week of culture, as determined by liquid phase reverse transcriptase (RT) activity [25]. The infectious titer of the viral stock (6.8×10^7 IU/ml) was determined on PHA-stimulated PBMC by applying the Reed and Muench formula [46].

CTE supernatant was replaced with fresh complete medium (RPMI, 15% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% MEM-non essential amino acids, 1% MEM-sodium pyruvate, 1% fungizone, 0,1% 1x gentamicin) every 3 days for up to 12 days of culture, after which time point the CTE no longer maintains their morphology and viability, as reported [22,47].

De novo productive infection of CTE was defined as the difference in $p24_{Gag}$ release into the culture medium in the absence and in the presence of the RT inhibitor lamivudine (3TC; 5 μM), as reported [22].

Real-time PCR for HIV-1 DNA_{gag} quantification

DNA was extracted from tissue blocks after 12 days of incubation with HIV-1 using a Nucleospin tissue kit from MN (Duren, Germany) following the provided protocol. Real-time-PCR was performed to quantify complete viral DNA transcripts using the following primer set that recognize the HIV-1_{gag} gene [48]: forward primer, 5′-ACATCAAGCAGCCATGCAAAT-3′; reverse primer, 5′-ATCTGGCCTGGTGCAATAGG-3′; and probe, 5′-(FAM) CATCAATGAG GAAGCTGCAGGAATGGGATAGA (TAMRA)-3′. The amount of cellular DNA was assessed by use of Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* as a reference gene using the following primes and probe: forward primer, 5′-ACCACAGTCCATGCATCACT-3′; reverse primer, 5′GGCCATCACGCCACAG ITT-3′; and probe, 5′-(FAM) CCCAGAAGACTGTG GATGGCCCC (TAMRA)-3′[49]. Samples were run on a Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA) using a Taqman® universal master mix (Life Technology).

Immunofluorescent cytometric bead assay for HIV-1 $p24_{Gag}$ quantification

Productive HIV-1 infection was assessed by measuring HIV-1 antigen $p24_{Gag}$ concentration in tissue culture medium using an immunofluorescent cytometric bead assay with minor modifications from a previously described protocol [50]. Briefly, carboxyl polystyrene particles (Spherotech, Lake Forest, IL) were coupled with $p24_{Gag}$ capture mAb (Immuno Diagnostics, MA) covalently using sulfo-NHS and EDC (Thermo scientific, Rockford, IL) according to the Luminex standard protocol. Coupled microspheres (4×10^3) were consequently mixed with 50 μl of standard (Perkin Elmer p24-alliance standard, Waltham, MA) or lysed culture medium and incubated overnight at 4°C. After washing (PBS plus 0.02% Tween 20, 0.1% BSA), an anti-$p24_{Gag}$-PE-conjugated detection monoclonal antibody (mAb) was added (Beckman Coulter, Brea, CA) for 2h at room temperature. Samples were washed and run on a LSR-Fortessa flow cytometer (BD Biosciences, San Jose, CA) using the DIVA 6.1.2 software; the events were analyzed with the FlowJo version 8.8.7 (Tree Star, Ashland, OR) and with the Prism 5.0 a program using a 5-parameter fitting algorithm.

Immunophenotyping of HIV-1 target cells present in CTE

Both T cells and macrophages were isolated after tissue digestion and staining by mAb directed against cell surface markers followed by cytofluorometric analysis. In particular, fresh CTE were digested with Collagenase IV (5 mg/mL; Invitrogen, Carlsbad, CA) in RPMI 5% FBS for 90 min. In order to characterize tissue T lymphocytes, single-cell

suspensions were washed in staining buffer (PBS plus 2% FBS) and stained with anti- CD3 (PE-labeled), anti-CD4 (APC), anti-CD8 (Pacific Blue), anti-CCR7 (PE-Cy7), anti-CD45RA (PE-TexasRed) and/or anti-CCR5 (PE-Cy7) mAbs. In order to identify macrophages, cell suspensions obtained by tissue disruption were washed in DMEM, 10% FBS, 5% human serum (HS) and then stained with FITC-labeled anti-CD3, anti-CD56, anti-CD19, anti-CD20 mAb in a single "dump" channel and with anti-CD14 (APC-Cy7), anti-CD11b (Pacific Blue), anti-CD4 (APC) and anti-CCR5 (PE-Cy7) mAbs. Macrophages were identified as lineage negative ($CD3^{neg}$, $CD19^{neg}$, $CD20^{neg}$, $CD56^{neg}$), $CD14^{+}$ $CD11b^{+}$ cells. In some experiments, macrophages were also stained with anti-CD206 (PE) and anti-CD209 (PerCP-Cy5.5). All the mAb were purchased from BD Biosciences except anti-CD8 (Pacific Blue) mAb that was purchased from Life Technology (Grand Island, NY, SA).

Cell suspensions obtained by tissue digestion of 22 CTE inoculated with HIV-1 were also collected and stained 6 days after infection with the anti-HIV-1 $p24_{Gag}$ KC57 mAb conjugated with FITC (Beckman Coulter, Fullerton, CA). The events were acquired by an LSR-Fortessa flow cytometer (BD Biosciences) equipped with 355, 407, 488, 532, and 638 nm laser lines using the DIVA™ 6.1.2 software and were then analyzed by FlowJo™ version 8.8.7. Dead cells were identified and excluded from the analysis using the LIVE/DEAD fixable Blue Dead Cell Stain™ kit (Invitrogen).

Multiplex chemokine and cytokine analysis

Supernatants from CTE were collected 3 and 12 days after infection in the presence or absence of 3TC and analyzed with a Fluorokine MAP Multiplex Cytokine Panel (R&D Systems). Samples were run on Bioplex 200 machine (Bio-Rad Laboratories, Hercules, CA) and analyzed by the Bioplex Manager 6 program. The CTE supernatants were tested for the presence of CCL2 (Monocyte Chemotactic Protein-1), CCL3, CCL4 (Macrophage Inflammatory Protein-1α and β, respectively), CCL5 (Regulated upon Activation Normal T cell Expressed and Secreted), IL-4, IL-10, TNF-α and IFN-γ.

β-defensin 1 and 2 and SLPI ELISA

CTE supernatants were analyzed for their β-defensin 1 and 2 content using an ELISA Development Kit (PeproTech, London, UK) according to the manufacturer's instructions. Briefly, plates were coated with anti-β-defensin 1 and 2 capture Ab. After washing the samples and the standards were added to the plate and the signal was detected with a detection Ab and Avidin-HRP conjugate. Plates were analyzed using an ELISA microplate reader Bio-Rad 680 (Bio-Rad Laboratories**)**.

CTE supernatants were also analyzed for SLPI content using the Quantikine immunoassay Kit (R&D Systems) according to the manufacturer's instructions. Briefly, supernatants were diluted 1:400 in diluent buffer. Plates were analyzed using an ELISA micro plate reader Bio-Rad 680 (Bio-Rad Laboratories**)**.

Statistical analysis

Each data point related to a single CTE represents the pooled value of 16 tissue blocks cultured under the same conditions. The statistical significance of differences between

results from different experimental groups was evaluated with the paired or unpaired Student's t-test where appropriate. The pooled data are presented as means ± SEM. All of the hypothesis tests were two-tailed, and a p value of ≤0.05 defined statistical significance.

Ethical declaration

This study was conducted in observation with the Declaration of Helsinki and after signing an informed consent form before the initiation of the study.

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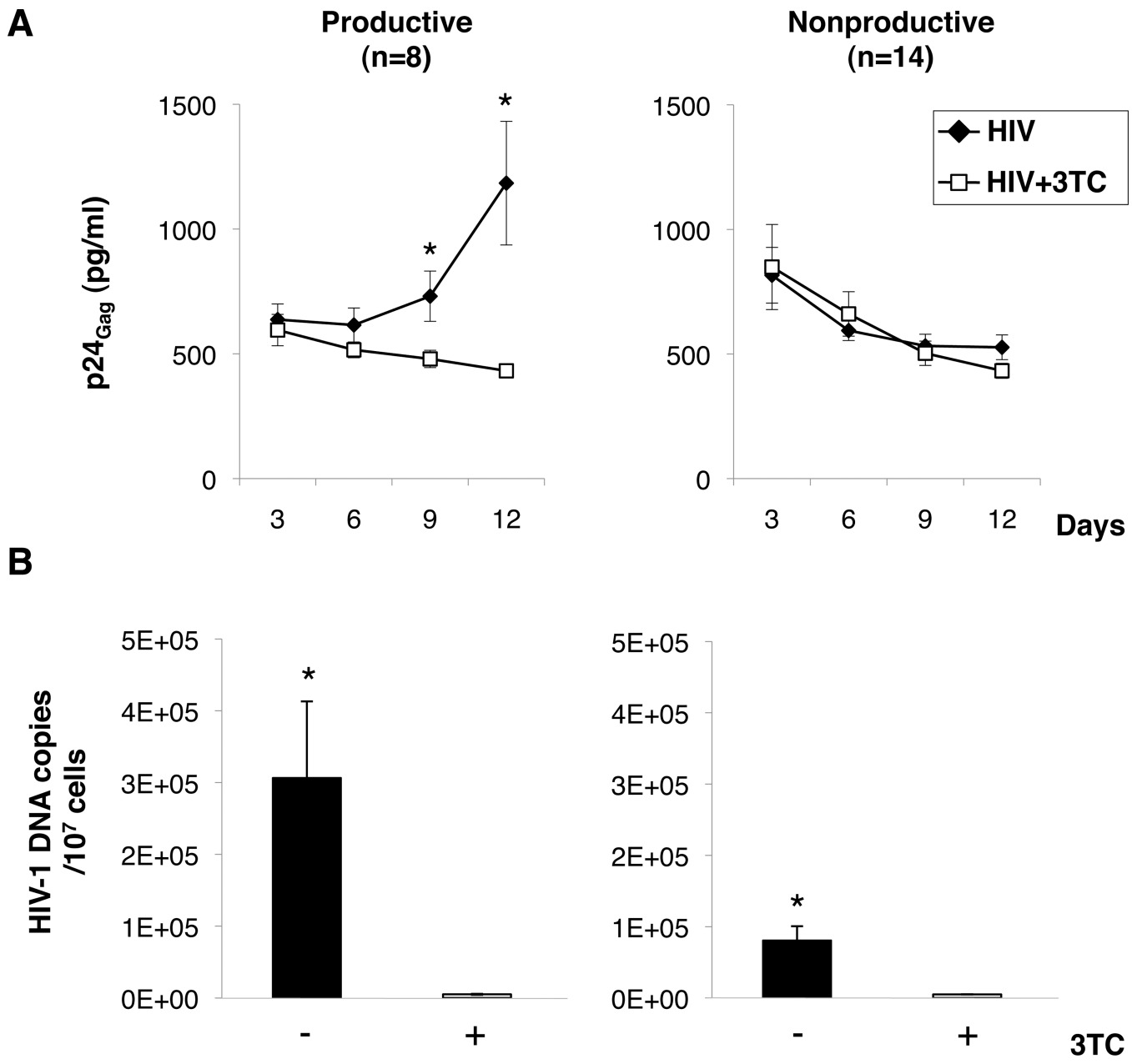

Figure 1. Productive and nonproductive R5 HIV-1 infection of CTE established from seronegative women

Donor-matched blocks of human cervical tissue were inoculated *ex vivo* with R5 HIV-1_{BaL} in the presence or absence of 3TC (5 μM). **A**. Kinetics of $p24_{Gag}$ release in culture supernatants of productive and nonproductive CTE, respectively (means ± SEM of 8 productive and 14 nonproductive tissues; *p≤0.05, Student's T test for 3TC-untreated vs. treated CTE). **B**. HIV-1_{gag}DNA levels determined in productive and nonproductive CTE 12 days post-infection in the presence or absence of 3TC (means ± SEM of 8 productive and 14 nonproductive CTE; *p≤0.05, Student's T test between productive and nonproductive CTE inoculated with HIV-1 in the absence of 3TC).

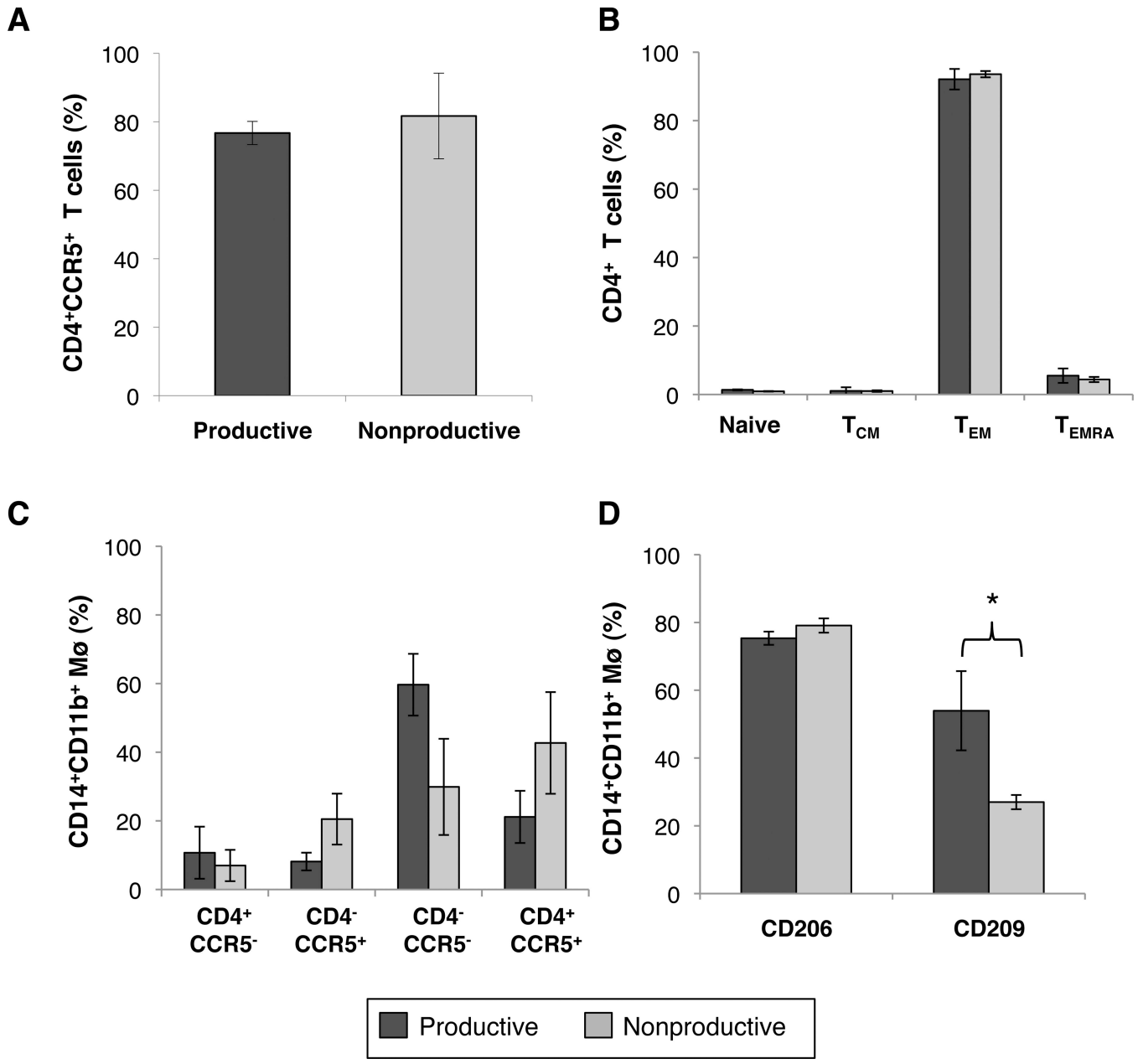

Figure 2. Phenotypic characterization of CD4$^+$ T cells and macrophages isolated from productive and nonproductive CTE. A

Most CD3$^+$CD4$^+$ T cells present in CTE expressed CCR5 irrespective of the productive or nonproductive outcomes of tissue infection (means ± SEM, n=7). **B**. CD3$^+$CD4$^+$ T cells were evaluated for the expression of CCR7 and CD45RA in order to define functional subsets. Most CD3$^+$CD4$^+$ T cells present in CTE showed an effector memory phenotype that were similarly represented in productive and nonproductive CTE. Furthermore, no differences were observed in the fraction of naive (CCR7$^+$CD45RA$^+$), central memory (CCR7$^+$CD45RA^{neg}), effector memory (CCR7^{neg}CD45RA^{neg}) or terminal effector memory (CCR7^{neg}CD45RA$^+$) CD3$^+$CD4$^+$ T cells between productive and nonproductive CTEs (means ± SEM, n=6). **C**. Only a fraction of resident macrophages, identified as

$Lin^{neg}CD14^{+}CD11b^{+}$, co-expressed CD4 and CCR5 irrespective of the infection outcomes of the CTE (means ± SEM, n=8). Also the remainder subsets as defined by either the expression or lack of expression of CD4 and/or of CCR5 showed a similar distribution in productive and nonproductive CTE. **D**. Resident macrophages were also positive for CD206 (means ± SEM, n=4) irrespective of the productive or nonproductive outcomes of tissue infection. Conversely, productive CTE were characterized by a higher fraction of $CD209^{+}$ macrophages compared to nonproductive CTE (means ± SEM, n=9, $p \leq 0.05$, Student's T test.).

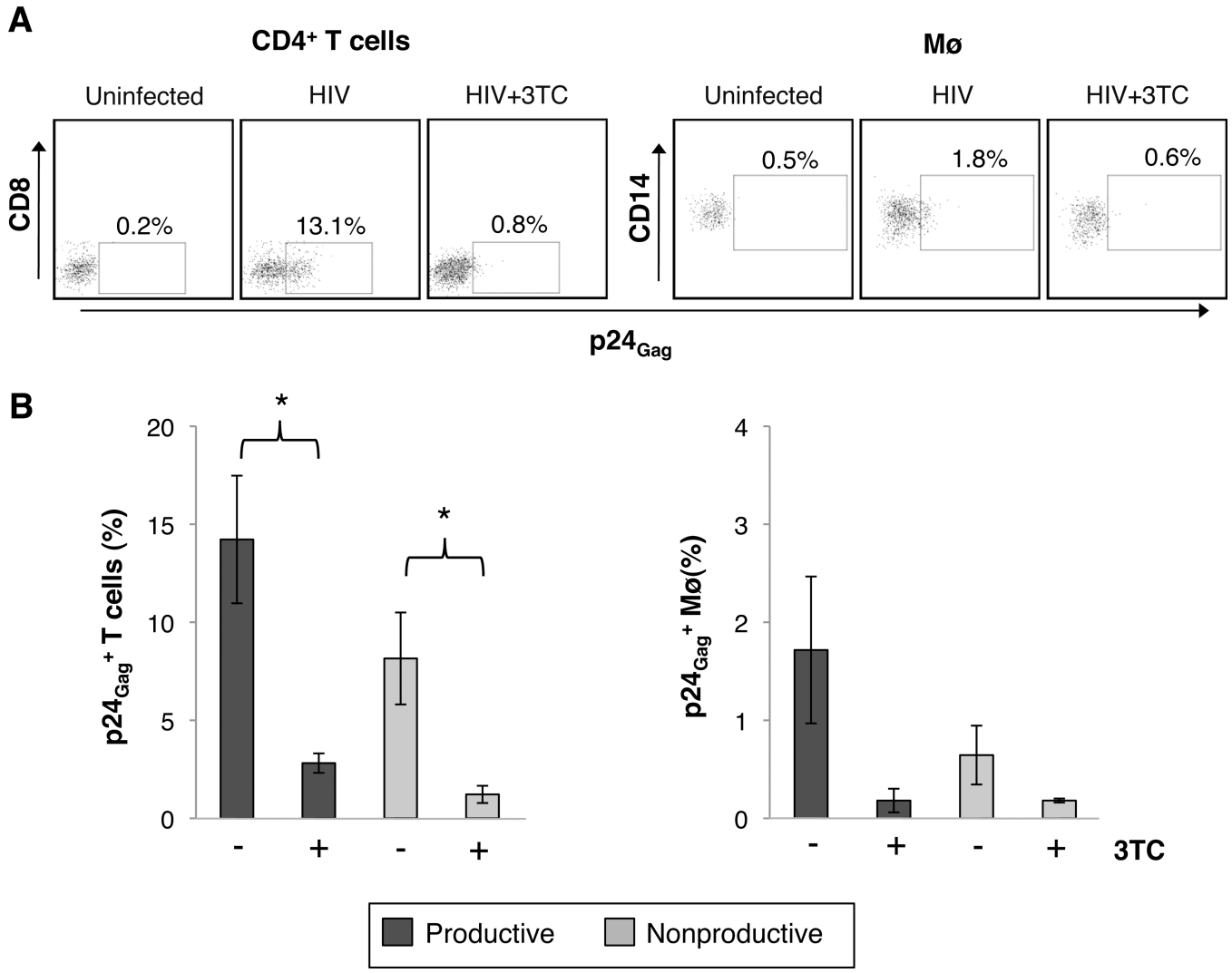

Figure 3. Both CTE-associated T cells and macrophages express HIV-1 $p24_{Gag}$

HIV-1 $p24_{Gag}^{+}$ $CD3^{+}CD8^{neg}$ T cells and $Lin^{neg}CD14^{+}CD11b^{+}$ macrophages were detected in donor-matched CTE infected with R5 HIV-1_{BaL} in the presence or absence of 3TC (5 μM) 6 days post-infection. **A**. Bivariate density plots of a single experiment with cells isolated from the CTE of 1 donor representative of 8 with a productive infection outcome. **B**. T cells and macrophages isolated from both productive and nonproductive CTE were positive for HIV-1 $p24_{Gag}$ expression at day 6 post-infection; presented are the mean ± SEM data from tissues of 8 productive and 14 nonproductive CTE. *p≤0.05, Student's T test.

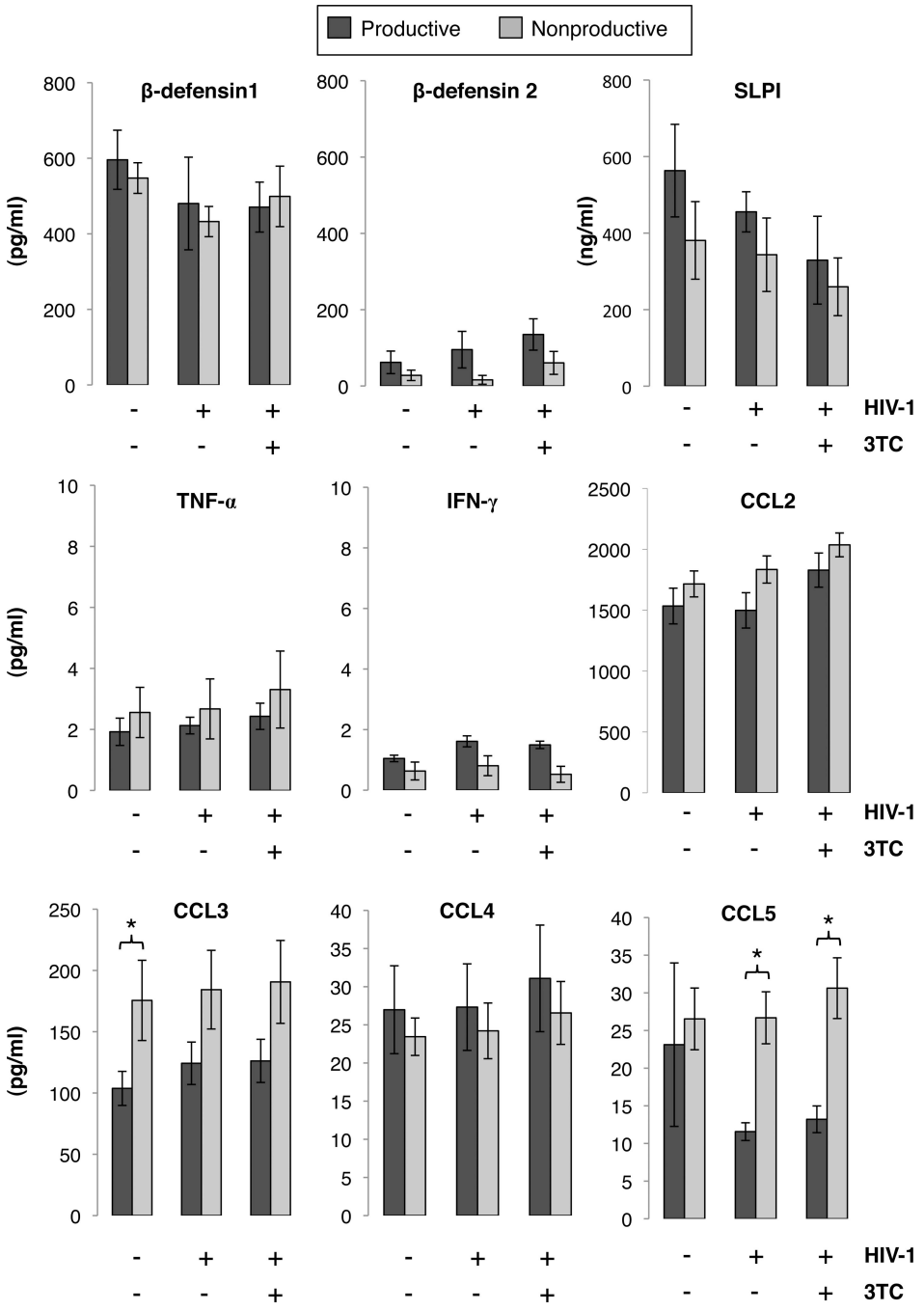

Figure 4. Selective upregulation of CCL3 and CCL5 release in the supernatant of nonproductive vs. productive CTE

CTE were exposed to R5 HIV-1_{BaL} in the presence or absence of 3TC (5 μM) and then cultured for 3 days; supernatants were collected and analyzed for the presence of the indicated analytes. The release of anti-viral peptides (β-defensins and SLPI; **upper panel**), pro-inflammatory cytokines (TNF-α, IFN-γ) or of CCL2 (**middle panel**) was not different in culture supernatants of productive and nonproductive CTE. Supernatants were also analyzed for the presence of CCR5 ligands (CCL3, CCL4, CCL5; **lower panel**). CCL3 production

was significantly higher in uninfected CTE showing a nonproductive profile upon HIV-1 exposure, whereas CCL5 was significantly more released by HIV-1 exposed nonproductive vs. productive CTE both in the presence or absence of 3TC. Presented are means ± SEM from 8 productive and 14 nonproductive CTE. *$p \leq 0.05$, Student's T test.

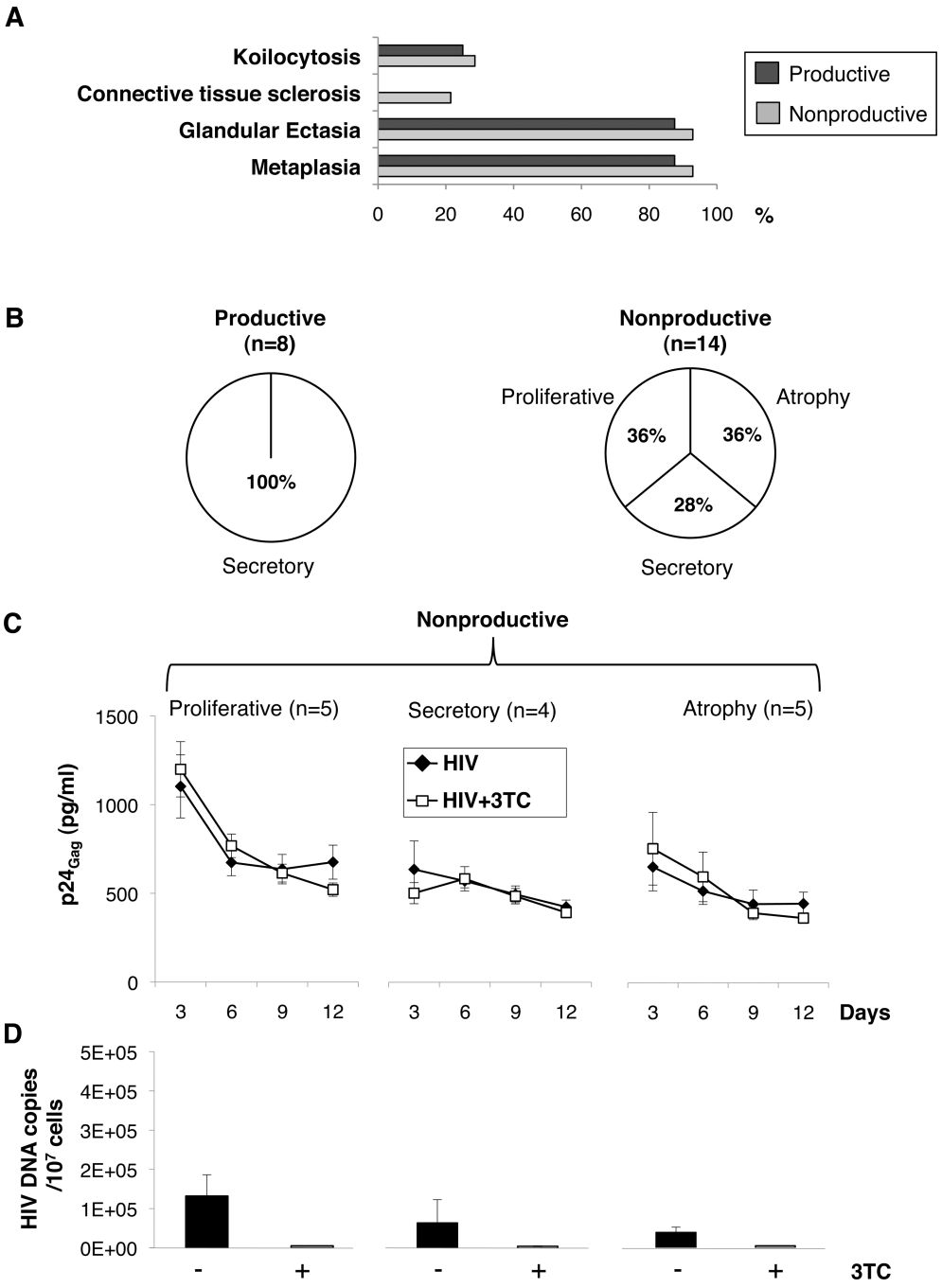

Figure 5. Association between the outcome of R5 HIV-1 exposure of CTE and the donor's menstrual cycle phase or the presence of endometrial atrophy at the time of hysterectomy. A Histological analysis of freshly isolated cervical tissue was performed in order to analyze the presence of lesions, which included koilocytosis, connective tissue sclerosis, glandular ectasia and metaplasia, associated with leiomyomas. The percentages of affected donors were similar, irrespective of the productive or nonproductive outcomes of CTE infection except for connective tissue sclerosis that was present in 21% of nonproductive CTE. **B**. Only tissues from women in their secretory phase were productively infected by R5

HIV-1_{BaL}, as shown in Figure 1A. **C**. Undistinguishable $p24_{Gag}$ release between untreated and 3TC-treated CTE by nonproductive CTE. CTE from donors in proliferative phase showed a higher initial level of $p24_{Gag}$ than those from donors in secretory phase or with an atrophic endometrium, likely as a reflection of the different mucus composition. **D**. HIV-1 DNA accumulation occurred in nonproductive CTE; no significant differences were noted in nonproductive CTE in terms of HIV-1 DNA levels according to the menstrual cycle phase or the presence of endometrial atrophy.

Table 1

Endometrial histology and HIV-1 infection outcomes

		Endometrial histology		
HIV infection*	**Number of donors**	**Proliferative phase**	**Secretory phase**	**Atrophic endometrium**
Productive	8	0	8	0
Age (range)	48±2 (41–54)	n.a.°	48±2 (41–54)	n.a.°
Nonproductive	14	5	4	5
Age (range)	55±3 (40–76)	50±2 (40–56)	50±6 (47–51)	65±9 (55–76)

* The HIV-1 infection outcome was based on the net release of $p24_{Gag}$ in culture supernatants over 3TC-treated CTE, as shown in Figure 1A.

°n.a.: not applicable.