BIOLOGY OF REPRODUCTION (2015) **92**(3):68, 1–10
Published online before print 14 January 2015.
DOI 10.1095/biolreprod.114.122887

Progesterone-Based Intrauterine Device Use Is Associated with a Thinner Apical Layer of the Human Ectocervical Epithelium and a Lower ZO-1 mRNA Expression[1]

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ABSTRACT

Currently, whether hormonal contraceptives affect male to female human immunodeficiency virus (HIV) transmission is being debated. In this study, we investigated whether the use of progesterone-based intrauterine devices (pIUDs) is associated with a thinning effect on the ectocervical squamous epithelium, down-regulation of epithelial junction proteins, and/or alteration of HIV target cell distribution in the human ectocervix. Ectocervical tissue biopsies from healthy premenopausal volunteers using pIUDs were collected and compared to biopsies obtained from two control groups, namely women using combined oral contraceptives (COCs) or who do not use hormonal contraceptives. In situ staining and image analysis were used to measure epithelial thickness and the presence of HIV receptors in tissue biopsies. Messenger RNA levels of epithelial junction markers were measured by quantitative PCR. The epithelial thickness displayed by women in the pIUD group was similar to those in the COC group, but significantly thinner as compared to women in the no hormonal contraceptive group. The thinner epithelial layer of the pIUD group was specific to the apical layer of the ectocervix. Furthermore, the pIUD group expressed significantly lower levels of the tight junction marker ZO-1 within the epithelium as compared to the COC group. Similar expression levels of HIV receptors and coreceptors CD4, CCR5, DC-SIGN, and Langerin were observed in the three study groups. Thus, women using pIUD displayed a thinner apical layer of the ectocervical epithelium and reduced ZO-1 expression as compared to control groups. These data suggest that pIUD use may weaken the ectocervical epithelial barrier against invading pathogens, including HIV.

epithelial junction proteins, female reproductive tract, HIV receptors, hormonal contraception, in situ staining

INTRODUCTION

Hormonal contraceptive usage is common in both developed and developing countries. Approximately 14% of women of reproductive age around the world who are married or in a union use intrauterine devices (IUDs), while 9% use oral contraception, and 4% use injectable contraception [1]. It has been suggested that hormonal contraceptives may affect human immunodeficiency virus (HIV) susceptibility within the vaginal vault by modulating the genital immune system, regulating genital epithelial cells, inducing cervical ectopy, and affecting the local microflora [2–6]. Data regarding vaginal epithelial thickness differences between hormonal contraceptive users and nonusers are however indecisive, and the clinical relevance of some of these limited changes is unclear [7–12]. Thus, the global impact of the biological effects of hormonal contraceptives needs to be explored in detail to understand how they may affect susceptibility to HIV acquisition and transmission. In nonhuman primate (NHP) studies, progesterone-based hormonal contraceptives were clearly shown to enhance Simian immunodeficiency virus (SIV) vaginal transmission whereas human HIV epidemiological observation studies have proved inconclusive [13–22]. For instance, World Health Organization guidelines conclude that the most current clinical data neither establish a clear causal association with progesterone-based injectables and HIV acquisition, nor definitively rule out the possibility of an effect [23].

Progesterone-based intrauterine device (pIUD) use suppresses the endometrial glands accompanied by stromal decidualization [24–26]. The main mechanism of action seems to be prevention of cervical mucus sperm penetration and inhibition of sperm transport. Combined oral contraception (COC) consists of an estrogen (estradiol) and a progesterone (progestin) component, and works by inhibiting follicular development and preventing ovulation as a primary effect. Thus, hormonal contraceptives contain different mixtures of sex hormones and therefore induce various molecular changes in the female genital tract (FGT) mucosa that in turn may affect susceptibility to genital infections.

The multistratified epithelium of the ectocervix and vaginal mucosa are structurally similar and line the vaginal vault, thereby resulting in direct exposure to seminal fluid and possible pathogens during sexual intercourse. The stratum corneum is the apical layer of the epithelium and covers the viable epithelial layers, the stratum malpighii [27]. The thickness and barrier function of the epithelium is important for inhibiting pathogens from entering the host via the submucosa. Epithelial junction proteins may play a pivotal role in protection against viral ingress as they interact with

[1]Supported by the Swedish Society for Medical Research (A.T.), the Swedish Research Council (K.B.), SIDA/SAREC (K.B.), National Institute of Allergy and Infection Diseases grant R33 AI076968 (T.J.H.), and National Institutes of Health grant P01AI082971 (T.J.H.).
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Received: 2 July 2014.
First decision: 3 August 2014.
Accepted: 6 January 2015.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

similar proteins on adjacent cells to form a robust barrier against pathogenic invasion. E-cadherin is the core membrane protein of the adherens junction, which is localized immediately below the tight junction [28]. Adherens junction is important for the initiation and stabilization of cell-cell adhesion, regulation of the actin cytoskeleton, intracellular signaling, and transcriptional regulation [29]. Tight junctions prevent the mixing of membrane proteins between the apical and basolateral membranes (fence function) and control the paracellular passage of ions and solutes between cells (gate function) [29]. Claudins form the protein strands of the tight junction and, together with occludins, regulate the paracellular permeability barrier between cells. The tight junction protein ZO-1 forms a link between the adherens (E-cadherin) and tight junctions [29]. Down-regulation of epithelial junction proteins has been associated with a number of severe clinical disorders, including disseminated herpes simplex virus type-1 (HSV-1) infection [30], hence, further suggesting that epithelial junction proteins may play a role in HIV transmission.

Immune cells, including HIV target cells, are also present in the multistratified epithelium of the ectocervix and may be differentially distributed under the influence of exogenous sex hormones [3, 9, 31–33]. Thus, superficially located T cells and Langerhans cells expressing CD4, CCR5, and Langerin can potentially bind HIV particles in the presence of small breaches or in ectocervical and vaginal tissue affected by inflammation [34]. A relocation of these target cells under the influence of hormonal regulation may thus play a role for HIV susceptibility. Here we investigated whether hormonal contraception use produces a thinning effect on the epithelium, down-regulates epithelial junction proteins, or alters HIV target cell distribution in the ectocervix by assessing human ectocervical tissue samples ex vivo.

MATERIALS AND METHODS

Study Populations and Sample Collection

Healthy premenopausal volunteers were recruited based on long-term hormonal contraceptive use: pIUD based on levonorgestrel (n = 24), COC (n = 23), and no hormonal contraceptive (noHC) (n = 15). Volunteers were examined by a gynecologist at the Women's Clinic at Karolinska University Hospital, Stockholm, Sweden. Cervical swabs and urine samples were obtained to test for *Chlamydia trachomatis* and *Neisseria gonorrhea*. Human papilloma virus (HPV) was diagnosed with Pap smears and the Linear Array HPV genotyping test. Blood was drawn for Herpes simplex virus type 2 (HSV-2) serological testing. All the tests were performed by the accredited microbiological laboratory service at the Karolinska University Hospital. Women using systemic immunosuppressive therapy or who had a history of cervical pathology were excluded. Written informed consent was obtained from all the patients, and ethical approval was obtained from the Regional Ethical Review Board of Stockholm.

Two ectocervical tissue biopsies were collected from each woman. The biopsies (3 mm^2) were collected from the superior portion of the ectocervix with Schubert biopsy forceps (B. Braun Aesculap AG, Tuttlingen, Germany). One biopsy was placed in RNAlater solution (QIAGEN Inc., Valencia, CA), and the other was snap frozen in liquid nitrogen and cryopreserved at −80°C until use.

Quantitative Measurement of the Epithelial Thickness

Eight micrometer thick sections of the cryopreserved cervical biopsies were mounted on SuperFrost glass slides (Histolab Products AB, Gothenburg, Sweden), fixed in 2% formaldehyde, and stained with hematoxylin. The tissue sections were scanned into digital images using a Hamamatsu Slide Scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan). The NDP viewer software was used to assess epithelial thickness in individual cervical samples. The height of the epithelium was measured by drawing lines from the basal membrane (perpendicular) to the last cell layer of the ectocervical epithelium. The height measurements were done in intervals of approximately 100 μm along the entire length of the epithelium (Fig. 1A).

Quantitative Measurement of the Epithelial Thickness of Individual Cell Layers of the Ectocervix

Eight micrometer sections were mounted on SuperFrost glass slides (Histolab Products AB), fixed in methanol/acetone, and blocked with normal donkey serum prior to staining. For the identification of adherens junction proteins, anti-E-cadherin (1:200 dilution of HECD1 hybridoma, a gift from the laboratory of Dr. Kathy Green at Northwestern University, Chicago, IL) was utilized along with secondary antibody, Rhodamine Red X (1:500 dilution, 715-295-150; Jackson ImmunoResearch, West Grove, PA). Lastly, Hoechst 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA) was used for staining of DNA. After staining, mounting medium (DakoCytomation, Stockholm, Sweden) and coverslips were applied to sections and sealed with clear nail polish until imaged. Antibody specificity was determined by incubating in the presence of an immunoglobulin G (IgG) isotype control antibody (1:200 dilution, MCA929A647; AbD Serotec, Raleigh, NC).

Images were obtained by deconvolution microscopy on a DeltaVision RT system and collected on a digital camera (CoolSNAP HQ; Photometrics, Tucson, AZ) using a 40× objective. To assess epithelial thickness in individual cervical samples, seven to ten 3×5 panel images of each donor were analyzed (Fig. 2A). By utilizing an antibody specific for E-cadherin, the stratum corneum equivalent of each sample was distinguished from the stratum malpighii of the stratified squamous epithelium, thereby allowing for easy measurement of the squamous epithelial thickness of each individual layer. Next, automated three-dimensional Z-stack analysis using IDL was performed that utilized algorithms written to quantify distance measurements and the total thickness of the cervical epithelium, including the thickness of the stratum malpighii and stratum corneum. By allowing the user to manually draw lines along the edges of the different layers, algorithms were used to calculate the epithelial thickness along every individual point of one line to another and vice versa (Fig. 2A). Measurement values were recorded in individual text files and then transferred to Prism 6.0 for data and statistical analysis.

Detection and Quantification of mRNA

Quantification of mRNA expression was performed as previously described [35]. The ABI PRISM 7700 sequence detection system and FAM dye-labeled TaqMan minor groove binder probes and primers (Applied Biosystems, Foster City, CA) were used to detect, amplify, and quantify the following targets: Ubiquitin C (UBC), E-cadherin, ZO-1, claudin-1, and occludin. Samples were run in duplicate and Ct values for each target gene were normalized to UBC. Samples with undetectable target cDNA were assigned a reference value greater than Ct = 40. Fold change of the target genes was calculated using the 2^{-dCT} equation.

Detection of HIV Receptors by In Situ Staining

Immunohistochemistry was performed on sections of the cryopreserved cervical biopsies. Tissue sections were fixed in 2% formaldehyde, and the peroxidase-labeled streptavidin-biotin amplification method was used as previously described [31, 36]. Monoclonal mouse IgG antibodies detecting human CD4 (1:10 dilution, clone SK3; BD Biosciences, San Jose, CA), CCR5 (1:100 dilution, clone MC-5; kindly provided by Professor M. Mack from the University of Regensburg, Germany), and DC-SIGN (1:750 dilution, clone 120507; R&D Systems, Minneapolis, MN). A polyclonal goat antibody against Langerin (1:250 dilution, AF2088; R&D Systems) was also used. Secondary biotinylated polyclonal rabbit anti-mouse IgG or rabbit anti-goat antibody (1:500 dilution, E0143 and E0466, respectively; Dako Sweden AB, Stockholm, Sweden) was then added. Thereafter, the peroxidase-based Vectastain kit (Vectastain Elite Standard; Vector Laboratories, Burlingame, CA) was used, and staining reactions were developed with diaminobenzidine tetrahydrochloride (Vector Laboratories). The sections were counterstained with hematoxylin. Negative control staining consisted of incubation with isotype control antibodies (1:200 dilution, clone X40; BD Biosciences and 1:500 dilution, SC-2028; Santa Cruz Biotechnology, Inc., Dallas, TX).

Quantitative Analysis of In Situ-Stained HIV Receptors

The stained tissue sections were scanned into digital images using a Hamamatsu Slide Scanner (Hamamatsu Photonics K.K.). The computerized image analysis software Panoramic Viewer with the image analysis module DensitoQuant (3DHistotech Ltd., Budapest, Hungary) was used to analyze the stained tissue sections. For each individual, a median of five (range = 3–5 fields) 40× magnification fields of the cervical epithelium were analyzed. Each field represents an average tissue area of 1.2×10^5 μm^2 (range = $0.5–1.9 \times 10^5$ μm^2) of epithelium. Thus, a total area of 5.6×10^5 μm^2 (range = $2.8–7.5 \times 10^5$

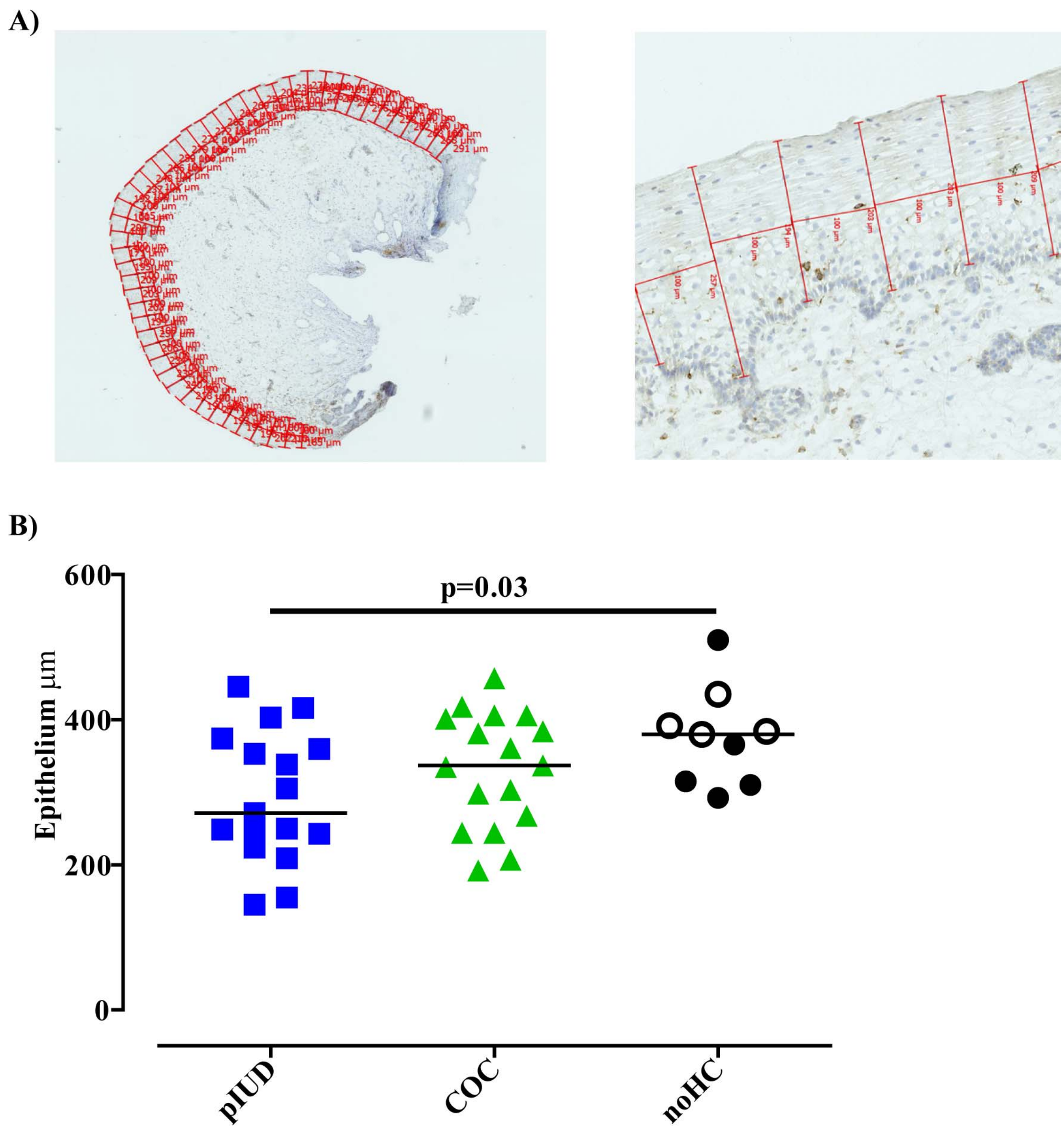

FIG. 1. Users of pIUDs display a thinner ectocervical epithelium. **A**) Bright field images of ectocervical tissue sections showing how the height of the epithelium was measured by drawing lines from the basal membrane to the top/last cell layer of the epithelium. The height measurements were taken in intervals of approximately 100 µm along the entire length of the epithelium. The image on the left was collected with a 2.5× objective while the image on the right was captured with a 20× objective. **B**) Graphs showing the distribution and median of epithelial thickness (µm) in the three study groups. For the noHC group, empty symbols represent women in the early stage of the menstrual cycle and filled symbols represent women in the late stage of the menstrual cycle. A nonparametric, two-tailed Mann-Whitney *U*-test was used to compare pIUD versus COC and pIUD versus noHC; $P < 0.05$ was considered statistically significant.

μm^2) of epithelium tissue was analyzed. The frequency of positively stained cells ($CD4^+$, $CCR5^+$, DC-SIGN$^+$, and Langerin$^+$ cells) is expressed as a percentage of stained area relative to the total epithelial tissue area. All the imaging analysis was performed blind by an investigator.

Statistical Analysis

All the analyses were performed using GraphPad Prism 5.0 or 6.0 (GraphPad Software Inc., La Jolla, CA). Nonparametric comparisons were performed between the pIUD group versus the COC or noHC group (e.g., the statistical method of choice was for comparison of two groups: pIUD vs. COC and pIUD vs. noHC). Furthermore, all the immunological parameters were also compared between women who were uninfected or infected with HPV. Statistical significance was calculated using the Mann-Whitney *U*-test for comparisons of continuous variables. The Fisher's exact test was applied to examine categorical variables between the study groups. All the tests were two-sided, and a $P < 0.05$ was considered statistically significant.

RESULTS

Description of the Study Cohort

Healthy premenopausal volunteers were recruited based on their regular and long-standing use of either pIUDs (n = 24) or COCs (n = 23). Women with a regular menstrual cycle who did not use any hormonal contraceptives (noHC, n = 15) were also included. The latter group represented women in different stages of their menstrual cycle. Because sex hormones can influence the genital epithelium, results from this study group are presented for the early- and late-phases of the menstrual cycle (n = 5 and n = 10, respectively) as a rough estimate of the monthly variations. Clinical and laboratory characteristics comparing the pIUD group to the COC group and the pIUD

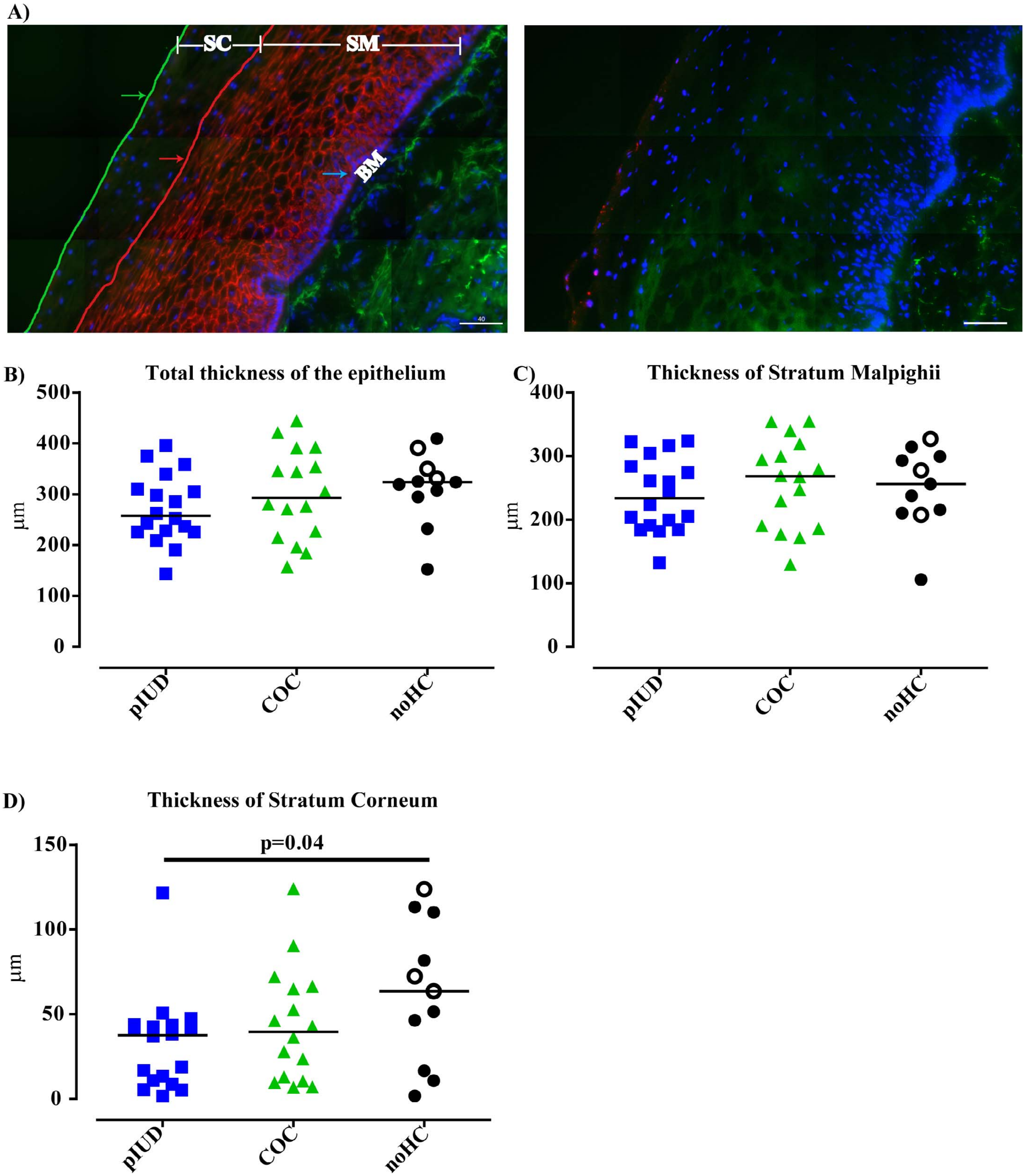

FIG. 2. The apical layer of the ectocervical epithelium is thinner in pIUD users. **A**) Immunofluorescence images of ectocervical tissue sections stained for the adhesion junction protein E-cadherin (red) (left image) or stained with IgG isotype control (right image) was used to demonstrate the localization of the different layers of ectocervical epithelium. 4′,6-Diamidino-2-phenylindole (blue) was used as a counterstain for visualization of cell nuclei. The total height of the ectocervical epithelium was measured from the basal membrane (BM; indicated by the blue arrow) to the last cell layer of the ectocervical epithelium (indicated by the green arrow). The height of the stratum malpighii (SM) was measured from the basal membrane to the end of the E-cadherin-expressing layer (indicated by the red arrow). The stratum corneum (SC) was measured from the E-cadherin-expressing layer to the last cell layer of the ectocervical epithelium. The images were collected with a 40× objective. Bar = 40 μm. The graphs show the distribution and median of the total epithelial thickness (**B**), the thickness of the stratum malpighii (**C**), and the thickness of the stratum corneum (**D**) in the three study groups. For the noHC group, empty symbols represent women in the early stage of the menstrual cycle and filled symbols represent women in the late stage of the menstrual cycle. A nonparametric, two-tailed Mann-Whitney *U*-test was used to compare pIUD versus COC and pIUD versus noHC; $P < 0.05$ was considered statistically significant.

TABLE 1. Clinical characteristics of study subjects at date of biopsy.

Characteristic	pIUD (n = 24)[a]		COC (n = 23)[b]		noHC (n = 15)		*P* value[c]
	Median or number (range or %)		Median or number (range or %)		Median or number (range or %)		
Age (yr)	27	(18–41)	26	(20–43)	35	(23–49)	NS
Steady partner[d]	17	71%	14	61%	13	87%	NS
Sexual partners last year	1	(1–11)	2	(0–12)	1	(1–2)	*[g]
Bacterial vaginosis	1	4%	1	4%	0	0%	NS[f] NA[g]
Yeast	5	21%	0	0%	0	0%	NA
C. trachomati[e]	0	0%	0	0%	0	0%	NA
N. gonorrhea	0	4%	0	0%	0	0%	NA
HPV	10	42%	12	52%	4	27%	NS
HSV-2 seropositive	3	13%	3	13%	3	15%	NS

[a] Mirena: n = 17; Levonova: n = 5; not known: n = 2; the dose of progesterone (levonorgestrel) is 0.02 mg/24 h.
[b] Yasmin: n = 8; Neovletta: n = 5; Cilest: n = 2; Trinovum: n = 2; Prionelle: n = 1; Yasminelle: n = 1; not known: n = 4. The COCs include etinylestradiol (0.02–0.035 mg) combined with either 3 mg drospirenon (Yasmin, Yasminelle), 0.15 mg levonorgestrel (Neovletta, Prionelle), 0.25 mg norgestimat (Cilest), or 0.5 mg noretisteron (Trinovum).
[c] Statistical analyses were performed by comparing the [f]pIUD versus COC group and the [g]pIUD versus noHC group. NS, not significant; NA, not applicable; *$P < 0.05$, Mann-Whitney *U*-test.
[d] Monogamous relationship status.
[e] One patient treated for *Chlamydia* infection in the pIUD group during the past 3 mo.

group to the noHC group are presented in Table 1. The groups were comparable with regard to age, monogamous relationship status, presence of bacterial vaginosis (BV), yeast, *C. trachomatis*, *N. gonorrhea*, HPV, and HSV-2 seropositivity ($P > 0.05$). However, women in the pIUD group had significantly more sexual partners over the last year than those in the noHC group ($P = 0.02$).

The Stratum Corneum Equivalent of the Ectocervical Epithelium Is Slightly Thinner in pIUD Users

Some human studies have illustrated little to no variability in vaginal epithelial thickness with exogenous or endogenous progesterone exposure [3, 8, 10, 11, 32]. In these studies, researchers focused on counting individual cell layers to determine epithelial thickness without taking into account the variability in the distance from the basal layer to the lumen and the sinuous patterning of the epithelial rete ridges that extend into the dermal papillae. To address this issue, we first measured the total epithelial thickness from the basal membrane to the luminal border with a mean number of 37 ± 12 single measurements per biopsy. Only samples with an intact epithelium of a defined length were included in the assessment (Fig. 1A). No statistical differences were seen between the pIUD (n = 17, median = 272 μm, range = 145–446 μm) and COC (n = 17, median = 337 μm, range = 192–457 μm) study groups (Fig. 1B). However, when comparing the pIUD group (n = 17) against the noHC group (n = 9, median = 380 μm, range = 293–510 μm), the pIUD group exhibited significantly thinner epithelium ($P = 0.03$).

Next, to further characterize the thickness of the layers, the epithelium was stained for an adherens junction protein, E-cadherin (Fig. 2A). The distribution of these cellular junctions allowed for the nonviable stratum corneum to be distinguished from the stratum malpighii. By using this technique, a pattern similar to that described above was observed with the total thickness being lowest in the pIUD group, followed by the COC group, and the noHC group (pIUD: n = 18, median = 257 μm, range = 143–396 μm; COC: n = 16, median = 292 μm, range = 157–444 μm; noHC: n = 11, median = 323 μm, range = 152–409 μm) (Fig. 2B). However, the differences between the groups were not statistically significant ($P > 0.05$). Also, a high quality of the tissue was required for a valid assessment to ensure that only intact epithelium was analyzed. Thus, different sections from each tissue sample were evaluated with the two independent methods.

The stratum malpighii (the E-cadherin-positive stratum layer) was also evaluated and found to be comparable between the groups (pIUD: median = 234 μm, range = 132–324 μm; COC: median = 268 μm, range = 129–355 μm; noHC: median = 257 μm, range = 106–327 μm) (Fig. 2C). However, after measuring the thickness of only the stratum corneum (the apical layer), thus bypassing any possible measurement skewing from the randomly sinuous-shaped basal rete ridges, the pIUD group displayed a significantly thinner stratum corneum (median = 38 μm, range = 2–122 μm) as compared to the noHC group (median = 64 μm, range = 2–124 μm; $P = 0.04$). However, no difference in thickness of the stratum corneum was seen when comparing the pIUD group to the COC group (median = 39 μm, range = 7–124 μm; $P > 0.05$) (Fig. 2D). The possible effect of HPV infection on the total epithelial thickness was assessed for each study group, but no statistical significant differences were observed (data not shown). The women scoring positive for HSV-2, yeast, or BV were not among the outliers in Figures 1 and 2 (data not shown). However, one exception was a woman in the COC group who was both HSV-2- and BV-positive and displayed the thickest stratum corneum in the study.

Lower Expression of ZO-1 in pIUD Users

The mRNA level of several epithelial junction markers, namely E-cadherin, ZO-1, claudin-1, and occludin, was measured by quantitative RT-PCR (Fig. 3). Among these four markers, the level of ZO-1 mRNA was significantly lower when comparing the pIUD users (n = 23; relative quantification (RQ): median = 0.040, range = 0.000001–0.162) to COC users (n = 23, RQ: median = 0.080, range = 0.20–0.162) ($P = 0.009$), and trended lower as compared to the noHC users (n = 12; RQ: median = 0.09, range = 0.010–0.127) ($P = 0.054$). Two women, both in the pIUD group, had undetectable ZO-1 mRNA and were plotted as outliers in Figure 3B. One of these outliers was HPV-positive (genotypes 44 and 55), but additional analyses revealed no difference in ZO-1 mRNA expression between women who were uninfected or infected with HPV (data not shown). Furthermore, no specific patterns were detected in women diagnosed with HSV-2, yeast, or BV infection (data not shown). The other three epithelial markers

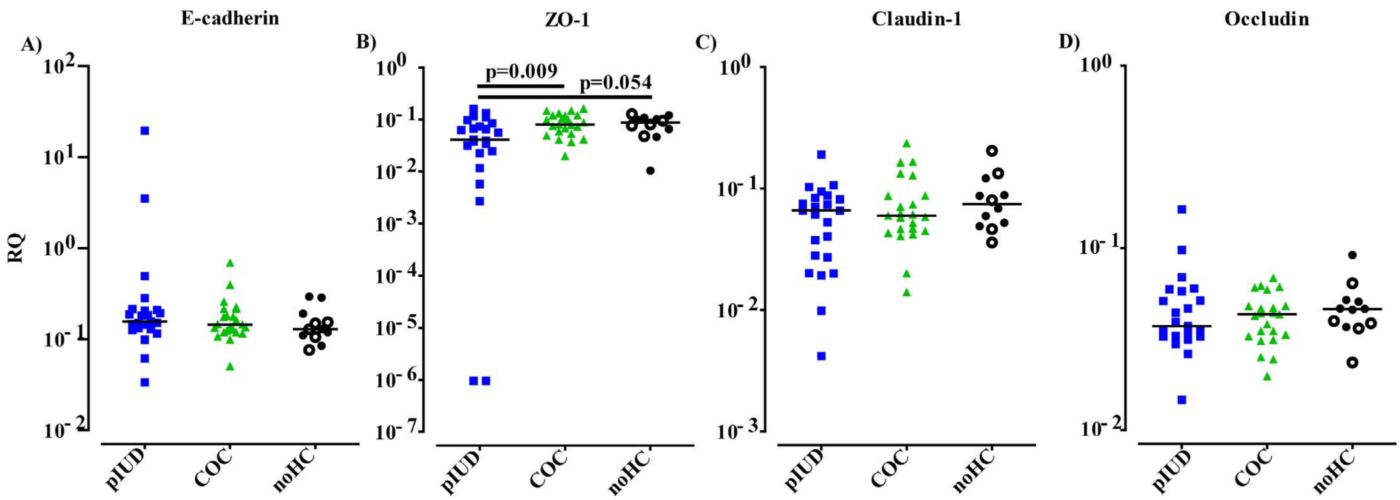

FIG. 3. Use of pIUDs lowers the level of ZO-1 mRNA. Quantitative RT-PCR was performed to determine the distribution and median of relative quantification (RQ) of the mRNA expression levels of the epithelial junction markers E-cadherin (**A**), ZO-1 (**B**), claudin-1 (**C**), and occludin (**D**). The cycle threshold (Ct) values for the selected markers were normalized to UBC by using the 2^{-dCT} method. The two outliers exhibited undetectable ZO-1 expression level and were assigned a reference value above Ct 40. For the noHC group, empty symbols represent women in the early stage of the menstrual cycle and filled symbols represent women in the late stage of the menstrual cycle. A nonparametric, two-tailed Mann-Whitney *U*-test was used to compare pIUD versus COC and pIUD versus noHC; $P < 0.05$ was considered statistically significant.

were expressed similarly across the study groups (Fig. 3, A, C, and D) and infection with HPV, HSV-2, yeast, or BV had no effect (data not shown).

HIV Receptor Expression Does Not Differ Between the Groups

Epithelium thickness and epithelial junction protein expression are not the only determinants for the ability of HIV to penetrate the squamous epithelium to reach the submucosa for further dissemination. HIV target cell density and HIV receptors within the epithelium also influence virus uptake. To assess this, HIV receptors and the coreceptors CD4, CCR5, DC-SIGN, and Langerin were stained by immunohistochemistry. Our data demonstrate that $CD4^+$, $CCR5^+$, and $Langerin^+$ cells were present in all the individual tissue samples representing the three study groups (pIUD: n = 16; COC: n = 16; noHC: n = 9), while a few sporadic DC-$SIGN^+$ cells were detected in the basal membrane of a few samples from the pIUD and COC groups, but not in the noHC group (Fig. 4). Next, the frequencies of these cells were assessed by computerized image analysis. Similar expression of $CD4^+$ cells (pIUD: median = 1.2%, range = 0.2%–7.0%; COC: median = 1.1%, range = 0.5%–8.2%; noHC: median = 3.3%, range = 0.4%–5.7%), $CCR5^+$ cells (pIUD: median = 0.8%, range = 0.2%–2.4%; COC: median = 1.4%, range = 0.2%–2.6%; noHC: median = 1.0%, range = 0.4%–1.5%), DC-$SIGN^+$ cells (pIUD: median = 0.0%, range = 0.0%–0.2%; COC: median = 0.0%, range = 0:0%–0.1%; noHC: median = 0.0%, range = 0.0%–0.0%), and $Langerin^+$ cells (pIUD: median = 1.1%, range = 0.3%–2.3%; COC: median = 0.8%, range = 0.3%–2.3%; noHC: median = 1.1%, range = 0.6%–2.2% of stained area relative to the total epithelial tissue area) were observed (Fig. 5).

DISCUSSION

Most studies have focused on the vaginal epithelium with regard to the influence of hormonal contraceptives on sexual HIV transmission. Here, we extended this knowledge to the ectocervical region, which is similarly exposed to HIV infection [34, 37, 38]. We thus observed that the epithelial thickness of women using pIUDs was comparable to COC users, but significantly lower than noHC women. To better understand the functional relevance of our findings, we characterized the thickness of individual layers of the epithelium. The thinner epithelial layer of the pIUD group could therefore be specified to the apical layer of ectocervix (stratum corneum), whereas the combined basal, spinosum, and granulosum layers (stratum malpighii) were comparable between the study groups. In terms of functional relevance, a thin apical layer could theoretically result in critical differences in HIV susceptibility as viral particles and other molecules can penetrate the outermost layer from the luminal side by passive diffusion to depths up to 50 μm and reach HIV target cells, which are located in the epithelium [37, 39]. Furthermore, because potential intraepithelial target cells are primarily located within the viable stratum malpighii, these data suggest an easier route for infectious virus to penetrate the squamous epithelium and encounter intraepithelial target immune cells in pIUD users [37].

In NHP models, the vaginal epithelial thickness went from greater than 25 cell layers to less than 10 cell layers after progesterone treatment, which translated into an almost eightfold increase in experimental SIV transmission in progesterone-treated animals [18]. In another study of NHP using progesterone levels comparable with those in humans being injected with progesterone-based hormonal contraceptives (i.e., depot medroxyprogesterone acetate), significant thinning of the vaginal epithelium was also observed; however, the thinning was reversed after cessation of the treatment [40]. Conversely, estrogen treatment enhanced vaginal keratinization and thickness, and this correlated with protection against SIV mucosal challenges [41, 42]. However, results from NHP studies are in contrast to analogous research conducted on human samples. Previous studies assessing the human vaginal epithelium have shown that both short-term and long-term oral contraceptive use resulted in changes of only two to three cell layers [3, 7, 8, 10, 11]. Such variations or lack thereof have also been noted throughout the course of a normal menstrual cycle [3, 43]. The progesterone dose, which varies between different hormonal contraceptives, may affect the FGT in

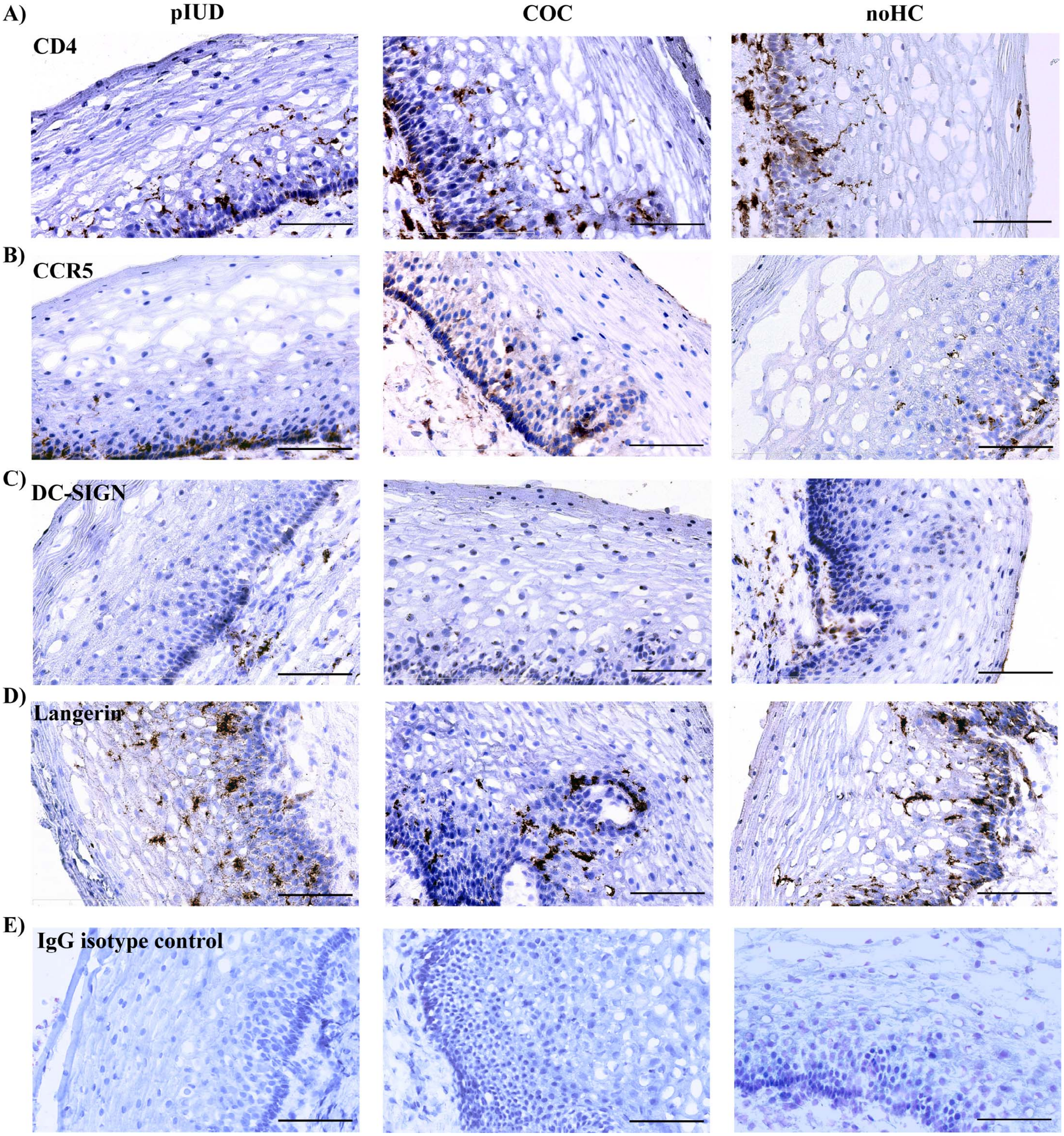

FIG. 4. Similar expression of HIV receptor in the ectocervical epithelium in the three study groups. Bright field images of ectocervical tissue sections showing the in situ expression of HIV receptors. The tissue sections were stained with hematoxylin (blue) for visualization of cell nuclei and stained brown for CD4 (**A**), CCR5 (**B**), DC-SIGN (**C**), Langerin (**D**), and IgG isotype control (**E**). The images in the first, middle, and right columns represent the pIUD, COC, and noHC groups, respectively. The negative controls did not contain any positively stained cells. The images were collected with a 40× objective. Bar = 100 μm.

diverse ways. For example, long-acting injectable progesterone-based hormonal contraception contains about 150 mg depot medroxyprogesterone acetate per injection and serum concentrations generally plateau at about 1 ng/ml for 3 mo [44]. While the local genital progesterone concentration is not clearly defined, it causes the endometrium to become atrophic. The pIUDs have mainly local progesterone effects in the uterine cavity with an in vivo release rate of approximately 20 μg/24 h and declines to 10 μg/24 h after 5 yr of use. The corresponding serum concentration of levonorgestrel declines from about 200 pg/ml to approximately 130 pg/ml after 5 yr of use.

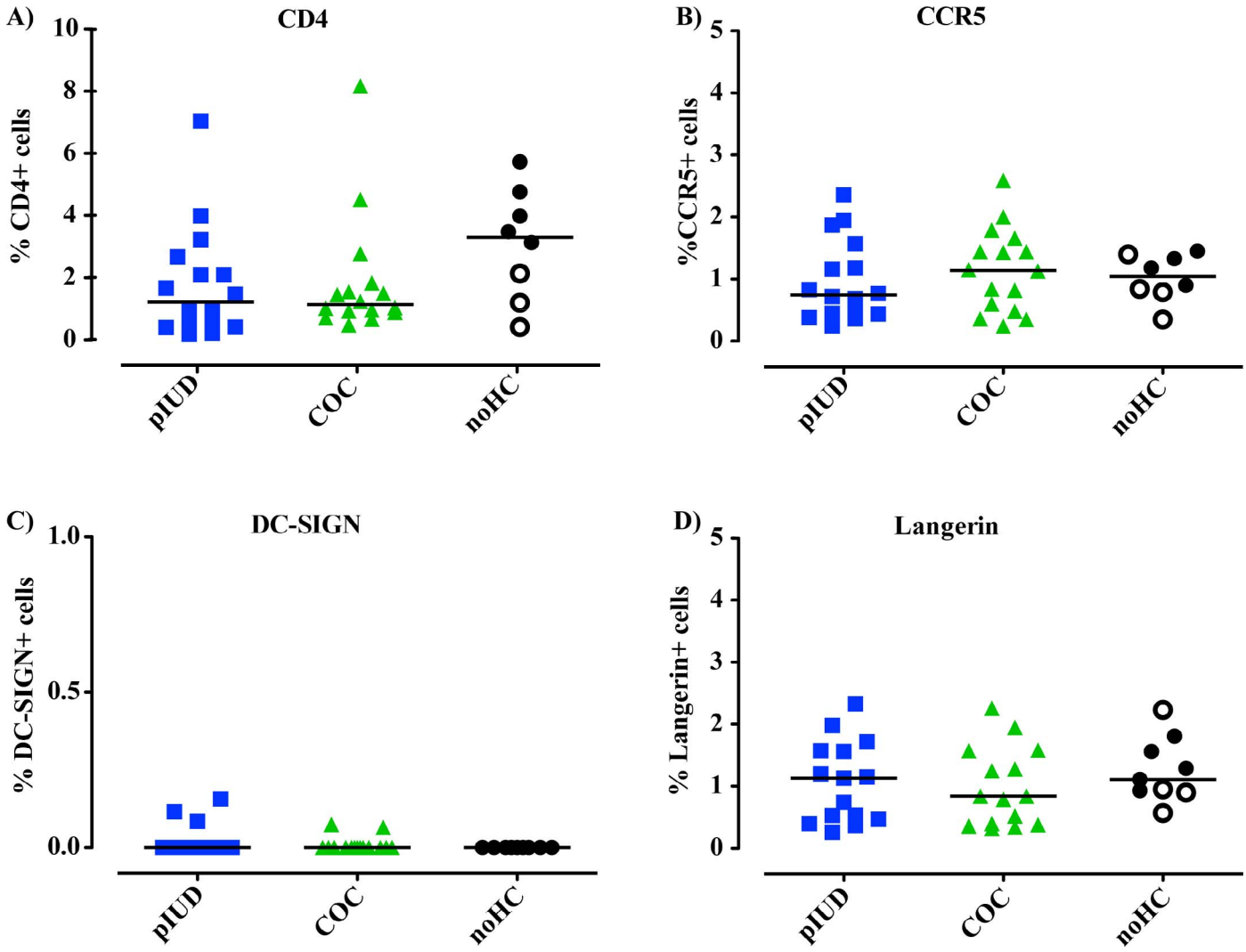

FIG. 5. Similar frequencies of HIV receptor expression in the ectocervical epithelium in the three study groups. The graphs show the distribution and median of the percentage of $CD4^+$ (**A**), $CCR5^+$ (**B**), DC-$SIGN^+$ (**C**), and $Langerin^+$ (**D**) cells relative to the total epithelial tissue analyzed. For the noHC group, empty symbols represent women in the early stage of the menstrual cycle and filled symbols represent women in the late stage of the menstrual cycle. Note that one data point is missing for the frequency of $CD4^+$ and $CCR5^+$ cells in the noHC group due to technical difficulties. A nonparametric, two-tailed Mann-Whitney *U*-test was used to analyze statistical significance between the study groups; $P < 0.05$ was considered statistically significant.

Deeper into the epithelium, the virus can be effectively hindered by epithelial junction proteins that form a tight and adherent barrier against invading pathogens. Here we chose to study four major epithelial junction markers (E-cadherin, ZO-1, claudin-1, and occludin) and found that ZO-1 mRNA was significantly lower in pIUD users than in women using COCs. Although down-regulation of a single protein may be compensated by higher expression of other proteins, this relative ZO-1 deficiency may affect the resistance of the mucosal barrier against HIV penetration because an isolated defect in only one of the epithelial junction proteins can still cause severe disease [30]. Some pathogens may also directly affect the integrity of the epithelial junctions and exposure to the HIV envelope glycoprotein gp120, resulting in disruption of several tight junction proteins, including occludin and claudin-1 in columnar epithelial cell cultures [45]. Unfortunately, it was not possible to quantify ZO-1 at the protein level due to lack of material. However, the correlation between mRNA and protein expression of epithelial junction markers has previously been shown to correlate in another virus model of the human mucosa [46]. The hormonal influence on genital epithelial junction markers has not been well studied in humans and should thus be subject to further characterization both during the normal menstrual cycle and as a result of using hormonal contraceptives. Sex hormones can also change the vaginal microflora [2, 6], which in turn may affect the integrity of epithelial junction proteins.

Studies in NHP and human explant models indicate that sexual transmission of HIV might occur across the vaginal and cervical epithelial lining [34, 37, 38]. Dendritic cells, including Langerhans cells, residing in the epithelium can bind HIV. SIV transmission studies suggest that viral replication occurs predominantly in $CD4^+$ T cells present in the female genital mucosa [47]. Within the epithelium of the human ectocervix, the main dendritic cell subset has been defined as Langerhans cells, whereas myeloid dendritic cells are found to be present in both the epithelium and submucosa [31]. Because $CD4^+$ T cells and dendritic cells can bind HIV, we determined the expression and localization of their major HIV receptors and coreceptors, namely CD4, CCR5, DC-SIGN, and Langerin, throughout the epithelium, including cells distributed in close proximity to the luminal side. Similar frequencies of these

target cells, as well as comparable distribution patterns, were observed in the three study groups, suggesting that the specific types of hormonal contraceptives evaluated here did not have a major impact on these cell types. Previous studies evaluating the effect of other exogenous and endogenous sex hormones on these markers on genital epithelial cells have shown that expression of CCR5 and CD4 can vary with the menstrual cycle [48]. Moreover, CCR5 was up-regulated on T cells in the cervical epithelium of oral contraceptive users [49]. In contrast, other studies have shown that immune cell populations in vaginal and cervical tissues appeared stable throughout the menstrual cycle [3, 32, 50, 51]. While the distribution of HIV receptors was comparable, we hypothesize that the thinner epithelium and decreased expression of epithelial junction proteins observed in pIUD users could be potential markers of HIV susceptibility.

A caveat when performing studies evaluating the effect of different hormonal contraceptives on HIV susceptibility is the selection of control groups because it is complicated by two major facts: 1) women using all types of hormonal contraceptives lack a normal menstrual cycle because the exogenous sex hormones cause a dysregulation of the endogenous hormone balance and 2) women who do not use hormonal contraceptives will vary in all study parameters as a result of their menstrual cycle stage. Thus, two control groups were therefore included in our study to evaluate the effect of pIUD use, namely one using another type of hormonal contraceptive (i.e., COCs consisting of both estrogen and progesterone components), and one representing women at different stages of the menstrual cycle. The results from the latter study group were divided into the early- and late-menstrual stages although an even more detailed characterization would have been preferable if the study group had been larger. A limitation of working with tissue biopsies obtained from the FGT is that they are rather small (3 mm^2), and unfortunately not all specimens were evaluated in all our analysis due to lack of material. In addition, one should keep in mind that the parameters we investigated here (epithelial thickness, expression of epithelial junction markers, and expression of HIV receptors) might not be evenly distributed throughout the ectocervical epithelium. Furthermore, genital infections may affect the FGT mucosal lining and the cells located within the mucosa and may thus be considered as confounders. Our investigation of women diagnosed with HPV, HSV-2, yeast, or BV infection did not reveal any significant trends in the immunological parameters studied. However, a few women were positive for these conditions, but the study was not designed to address these issues.

Within this study, we explored the influence of pIUD use on ectocervical epithelial thickness and barrier function as well as the distribution of potential HIV target cells. These parameters could all have the capacity to affect mucosal susceptibility to viral infections. Although these ex vivo observations cannot be directly translated to the natural setting, our results indicate that pIUD use may weaken the ectocervical epithelial barrier. Future studies using larger study cohorts as well as experimental studies assessing cervical explant models to evaluate the effect of exogenous sex hormones are warranted.

ACKNOWLEDGMENT

The authors would like to thank the study participants and staff at the Women's Clinic at Karolinska University Hospital, Stockholm, Sweden. We also acknowledge Dr. Tove Kaldensjö for clinical assistance and Mr. Axel Frisell for technical assistance.

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