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*Immunopathology and Infectious Diseases*

# Abundant Expression of HIV Target Cells and C-Type Lectin Receptors in the Foreskin Tissue of Young Kenyan Men

Taha Hirbod,* Robert C. Bailey,† Kawango Agot,‡ Stephen Moses,§ Jeckoniah Ndinya-Achola,¶ Ruth Murugu,‡ Jan Andersson,‖ Jakob Nilsson,* and Kristina Broliden*

*From the Department of Medicine, Center for Molecular Medicine,* Infectious Disease Unit, Solna, Karolinska Institutet, Stockholm, Sweden; the Division of Epidemiology,† School of Public Health, University of Illinois at Chicago, Chicago, Illinois; the Impact Research and Development Organization,‡ Kisumu, Kenya; the Departments of Medical Microbiology, Community Health Sciences, and Medicine,§ University of Manitoba, Winnipeg, Canada; the Department of Medical Microbiology,¶ University of Nairobi, Nairobi, Kenya; and the Department of Medicine,‖ Huddinge, Karolinska Institutet, Stockholm, Sweden*

**A biological explanation for the reduction in HIV-1 (HIV) acquisition after male circumcision may be that removal of the foreskin reduces the number of target cells for HIV. The expression of potential HIV target cells and C-type lectin receptors in foreskin tissue of men at risk of HIV infection were thus analyzed. Thirty-three foreskin tissue samples, stratified by Herpes simplex virus type 2 status, were obtained from a randomized, controlled trial conducted in Kenya. The samples were analyzed by confocal *in situ* imaging microscopy and mRNA quantification by quantitative RT-qPCR. The presence and location of T cells ($CD3^+CD4^+$), Langerhans cells ($CD1a^+$Langerin/$CD207^+$), macrophages ($CD68^+$ or $CD14^+$), and submucosal dendritic cells ($CD123^+$BDCA-$2^+$ or $CD11c^+$DC-$SIGN^+$) were defined. C-type lectin receptor expressing cells were detected in both the epithelium and submucosa, and distinct lymphoid aggregates densely populated with $CD3^+CD4^+$ T cells were identified in the submucosa. Although the presence of lymphoid aggregates and mRNA expression of selected markers varied between study subjects, Herpes simplex virus type 2 serostatus was not the major determinant for the detected differences. The detection of abundant and superficially present potential HIV target cells and submucosal lymphoid aggregates in foreskin mucosa from a highly relevant HIV risk group demonstrate a possible anatomical explanation that may contribute to the protective effect of male circumcision on HIV transmission.** *(Am J Pathol 2010, 176:2798–2805; DOI: 10.2353/ajpath.2010.090926)*

Randomized, controlled trials in Africa have shown that male circumcision reduces HIV-1 (HIV) acquisition in men by approximately 60%.[1–3] Circumcision is now recommended as a component of HIV prevention strategies, particularly in countries with endemic, generalized HIV epidemics. One of the biological explanations for the reduction in HIV acquisition could be that removal of the foreskin reduces the number of target cells for HIV.

In uncircumcised men, the foreskin is retracted over the shaft during intercourse, exposing the inner mucosa to genital secretions of the partner. Genital secretions contain HIV particles, and higher viral loads of HIV in blood and semen correlate with increased risk of transmission to the sexual partner.[4] After intercourse, the subpreputial penile moistness in uncircumcised men may also increase the risk of HIV acquisition,[5] whereas the relative dryness in the absence of this pocket-formed area in circumcised men is a less optimal environment for HIV viability. Furthermore, the foreskin tissue is vulnerable to trauma during intercourse, providing a portal for virus entry. Inflammatory conditions of the foreskin, including sexually transmitted diseases (STDs), may also act as cofactors for HIV transmission[3] (reviewed in Gray et al[6]). Even though the unkeratinized urethral meatus may still be vulnerable to HIV after circumcision, this surface area is much smaller relative to the foreskin.

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Address reprint requests to Taha Hirbod, Ph.D., Center for Molecular Medicine, Infectious Disease Unit, Department of Medicine, Solna, Karolinska Institutet, 171 76 Stockholm, Sweden. E-mail: taha.hirbod@ki.se.

Many subpopulations of mononuclear phagocytes have been defined in skin and mucosal tissue based on surface markers.[7] Among these, dendritic cells (DCs) and C-type lectin receptor (CLR) expressing $CD68^+$ macrophages are likely initial target cells for HIV in the genital tract mucosa, in addition to $CD4^+$ T lymphocytes.[8–11] Foreskin mucosa has indeed a high density of such cells under the lightly keratinized surface.[12] Langerhans cells (LCs) are characterized based on the expression of Langerin/CD207 (Langerin), an endocytic CLR that localizes to and forms Birbeck granules. These cells also express CD1a, a HLA-class I-like molecule that presents glycolipids.[13] In the stratified squamous epithelium of skin and genital mucosa, the LCs overlie interstitial or submucosal DCs. So-called myeloid or conventional DCs in many tissues are often identified on the basis of high expression of HLA-DR antigen-presenting molecules and the CD11c integrin. Dermal or submucosal DCs also express pattern recognition receptors such as DC-SIGN and mannose receptor (MR). HIV has been found to bind to the CLRs Langerin, DC-SIGN, and MR, in addition to the classical HIV receptors CD4 and CCR5, which can be expressed by the same cells.

Previous studies have defined target cell populations in foreskin tissue of relevance for HIV transmission.[12,14–19] To improve the biological understanding of the decreased HIV acquisition rates after male circumcision, we have here extended previous studies with a more detailed phenotypic characterization of both potential HIV target cells as well as CLRs in foreskin tissue. We selected a highly relevant study group by obtaining cryopreserved tissue samples representing young men of high risk for HIV infection participating in the Kenyan circumcision clinical trial.[2] Presence of STDs was carefully controlled for because the distribution and expression of phenotypic markers of mucosal cell populations is highly affected by inflammatory activity in the tissue. This also allowed a comparison between immune markers in asymptomatic Herpes simplex virus type 2 (HSV-2) seropositive versus seronegative men.

## *Materials and Methods*

### *Study Population and Sample Collection*

Foreskin tissues were obtained from a randomized, controlled trial conducted in Kisumu, Kenya, through the Universities of Nairobi, Illinois, and Manitoba Collaborative Research Project. Men aged 18 to 24 years were recruited into an unblinded trial with two arms: the circumcision arm and the delayed circumcision (control) arm to examine whether male circumcision reduces HIV incidence.[2] For the present study, samples were randomly selected on the basis of a negative STD history during a 3-month period before circumcision. The STD screening included a history of discharge and previously diagnosed sexually transmitted infections, a clinical examination 2 weeks before and at the time of circumcision, as well as laboratory analysis of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Haemophilus ducreyi* (chancroid), and *Treponema pallidum* (syphilis).[2] A total of 33 specimens were selected, stratified by HSV-2 serological status. Approximately equal numbers of specimens were randomly selected from each stratum.

Informed consent was obtained from all study subjects, and ethical approval was obtained from the ethical review boards at the University of Nairobi, University of Illinois, University of Manitoba, and the Karolinska Institutet, respectively.

### In Situ *Detection of Cellular Markers and CLRs by Immunostaining and Confocal Microscopy*

The foreskin tissues were processed immediately in a laboratory at the study clinic by making two horizontal cuts 4 mm apart approximately 1.0 cm from distal end of intact foreskin oriented with the frenulum up center. Tissue material ($4 \times 5$ mm) was embedded in optimal cutting temperature medium and stored at −75°C. Cryopreserved biopsies were sectioned to 15 $\mu$m and fixed in 2% formaldehyde. Selected anti-human monoclonal antibodies were used to detect the immune markers of interest: mouse anti-human Human Leukocyte Antigen DR (HLA-DR; clone: L243), CD14 (clone: M5E2), CD11c (clone: B-ly6), CD3 (clones: SK7 and HIT3a), CD4 (clone: SK3), CD123 (clones: 9F5 and 7G3), CD144 (clone: 55-7H1; Becton Dickinson, Franklin Lakes, NJ), Podoplanin/gp36 (clone: 18H5), MR/CD206 (clone: 15.2; Abcam, Cambridge, UK), CD68 (clone: EBM11; Dako, Stockholm, Sweden), DC-LAMP/CD208 (clone: 104.G4; Immunotech, Prague, Czech Republic), DC-SIGN/CD209 (clone: 120507), DLEC (BDCA-2 clone: AF1376; R&D Systems, Minneapolis, MN), CD1a (clone: NA1/34-HLK; AbD Serotec, Oxford, UK), rat anti-human CD4 (clone: YNB46.1.8; AbD Serotec), and goat anti-human Langerin/CD207 (clone: aa64-328; R&D Systems).

The staining reactions for CD144 and Podoplanin were developed red by using Vulcan Fast Red Chromogen (Biocare Medical, Concord, CA), and nuclear counterstaining was performed with hematoxylin. Digital images were transferred from a DMR-X microscope (Leica, Wetzlar, Germany) into a computerized image analysis system, Quantimet, Q 550 IW (Leica Imaging Systems, Cambridge, UK). For 1- and 2-color fluorescent staining, combinations of relevant antibodies were used followed by the appropriate Alexa Fluor–conjugated secondary antibody. Negative control staining consisted of irrelevant mouse, rat, or goat IgG (Dako). Whole section scans were performed, and fluorescently labeled cells were evaluated by using the Qwin 550 software and a filter-free spectral confocal microscope (Leica TCS SP2 AOBS).

$CD3^+$ cell counts were calculated for epithelial and submucosal compartments separately. Scores were assigned for the number of $CD3^+$ cells in approximately $5 \times 10^5$ $\mu m^2$ within 700 $\mu$m from the tissue surface: fewer than 50 cells = 1; 50 to 100 cells = 2; and more than 100 cells = 3. The size of the submucosal lymphoid aggregates containing $CD3^+$ cells were scored as follows: clusters containing fewer than 20 cells = 0, and at least one cluster per $5 \times 10^5$ $\mu m^2$ tissue containing more than

20 cells = 1. The specimens were evaluated by an experienced technician, blinded to HSV-2 and subject data.

### Detection and Quantification of mRNA

All 33 optimal cutting temperature embedded foreskin biopsies were thawed and disrupted in lysis buffer by using a mechanical rotor. RNA was extracted according to the manufacturer's protocol (RNeasy, QIAgen; Hilden, Germany) and converted in equal dilutions to cDNA in a single reverse transcriptase reaction using superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexanucleotide primers (Roche, Basel, Switzerland). DNA was stored at −20°C. Amplification of ubiquitin C (UBC), HLA-DR$\alpha$, CD3, CD4, Langerin, MR, DC-SIGN, CD68, CD14, CD1a, CD11c, CD123, BDCA-2, and DC-LAMP cDNA was performed by using the ABI PRISM 7700 sequence detection system and commercial FAM dye-labeled TaqMan MGB probes and primers (Applied Biosystems, Foster City, CA). UBC was chosen as endogenous control after a dilution series testing UBC, 18sRNA, and glyceraldehyde-3-phosphate dehydrogenase revealing UBC as the most constantly highly expressed reference gene in our samples. Each sample and control was run in triplicates. Relative quantity of target cDNA was computed by using the comparative threshold (Ct) method.[20] Ct values for target cDNA were normalized to UBC by using the normalized expression ratio $2^{-dCT}$, thus amounts are described as a relative quantity to UBC.

### Statistical Analysis

Amounts expressed mRNA did not follow a Gaussian distribution, thus intergroup variations of the markers of interest were analyzed by unpaired two-tailed Mann–Whitney test and nonparametric Spearman correlation tests. All analyses were performed with SPSS version 16.0 software (SPSS, Inc., Chicago, IL) and GraphPad Prism 4.00 (GraphPad Software, Inc., La Jolla, CA).

## Results

### Distribution of Blood and Lymphatic Vessels

Presence of vascular endothelial −cadherin (CD144) and Podoplanin was examined for detection of blood and lymphatic vasculature, respectively, in the human foreskin (Figure 1, A–D). Both markers were first investigated by using the Fast Red Chromogen (Biocare Medical) for immunohistochemical staining. However, the presence of dark melanocytes complicated the detection initially of positive staining in the epithelial layer; thus, fluorescently labeled markers were assessed thereafter. CD144$^+$ and Podoplanin$^+$ cells were consequently detected both in the epithelium and in the submucosa (Figure 1).

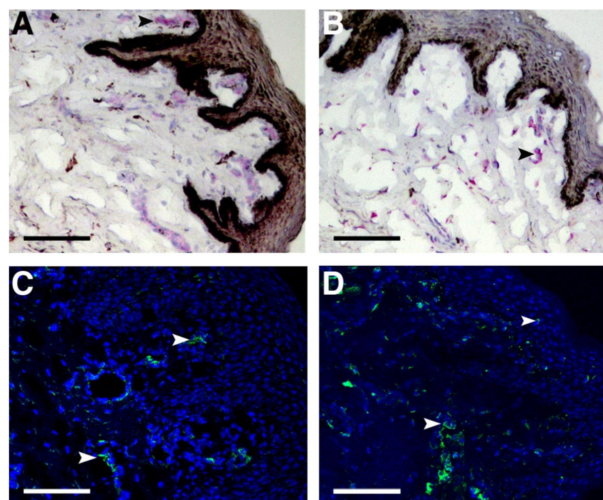


**Figure 1. A:** CD144 and (**B**) Podoplanin were solely visible in the submucosa when using the Fast Red Chromogen (Biocare Medical) due to the presence of dark melanocytes in the epithelium. However, when fluorescently labeled markers were used, both (**C**) CD144 and (**D**) Podoplanin were detected also in the epithelium (minimum distance to tissue surface [MDTS]: 111 $\mu$m and 55 $\mu$m, respectively). **Black** and **white arrowheads** indicate positive staining. Scale bars = 75 $\mu$m. Results are from one of at least four tissue samples yielding similar results.

### Distribution of Langerhans Cells, Macrophages, and Subepithelial Dendritic Cells

LCs, defined here by the detection of CD1a and/or Langerin, were present in both the epithelial and submucosal compartments of the foreskin tissue (0.015 cells/100 $\mu m^2$ tissue, 60% and 40% detected in the epithelial and submucosal compartment, respectively; Figure 2, A–P). CD1a was also expressed on CD4$^+$ cells (19% and 7% of total CD4$^+$ cells in the epithelial and submucosal compartment, respectively) and co-expression of CD11c and CD1a was found (3% and 76% of total CD1a$^+$ cells in epithelium and in submucosa, respectively). LCs expressing DC-LAMP were solely present in the submucosal compartment (Figure 2). Although present within the submucosa, the CD1a$^+$Langerin$^+$ LCs were strictly diverse from other cellular markers (CD68, CD14, and CD123) and CLRs (DC-SIGN and MR) commonly expressed in the submucosa (data not shown).

Macrophages, defined here as CD68 and/or CD14 positive cells, were exclusively present in the submucosa of the human foreskin (Figure 2). CD68$^+$ cells were detected more frequently as compared with CD14$^+$ cells (0.06 vs. 0.004 cells/100 $\mu m^2$ tissue), and double positive cells were rare (0.002 cells/100 $\mu m^2$ tissue). A disparity was detected between the two macrophage markers because only CD68$^+$ cells expressed MR (>80% of cells) or DC-SIGN (approximately 15% cells; Figure 2). Neither CD14$^+$ nor CD68$^+$ cells expressed DC-LAMP or Langerin (data not shown).

Submucosal myeloid cells, here defined as CD11c positive cells, co-expressed a number of the investigated cellular markers (Figure 2). Approximately 25% of the submucosal CD11c$^+$ cells expressed CD4 and CD11c$^+$CD1a$^+$ cells, presumably LCs, were detected in the same range (29% of submucosal CD11c$^+$ cells expressed CD1a).

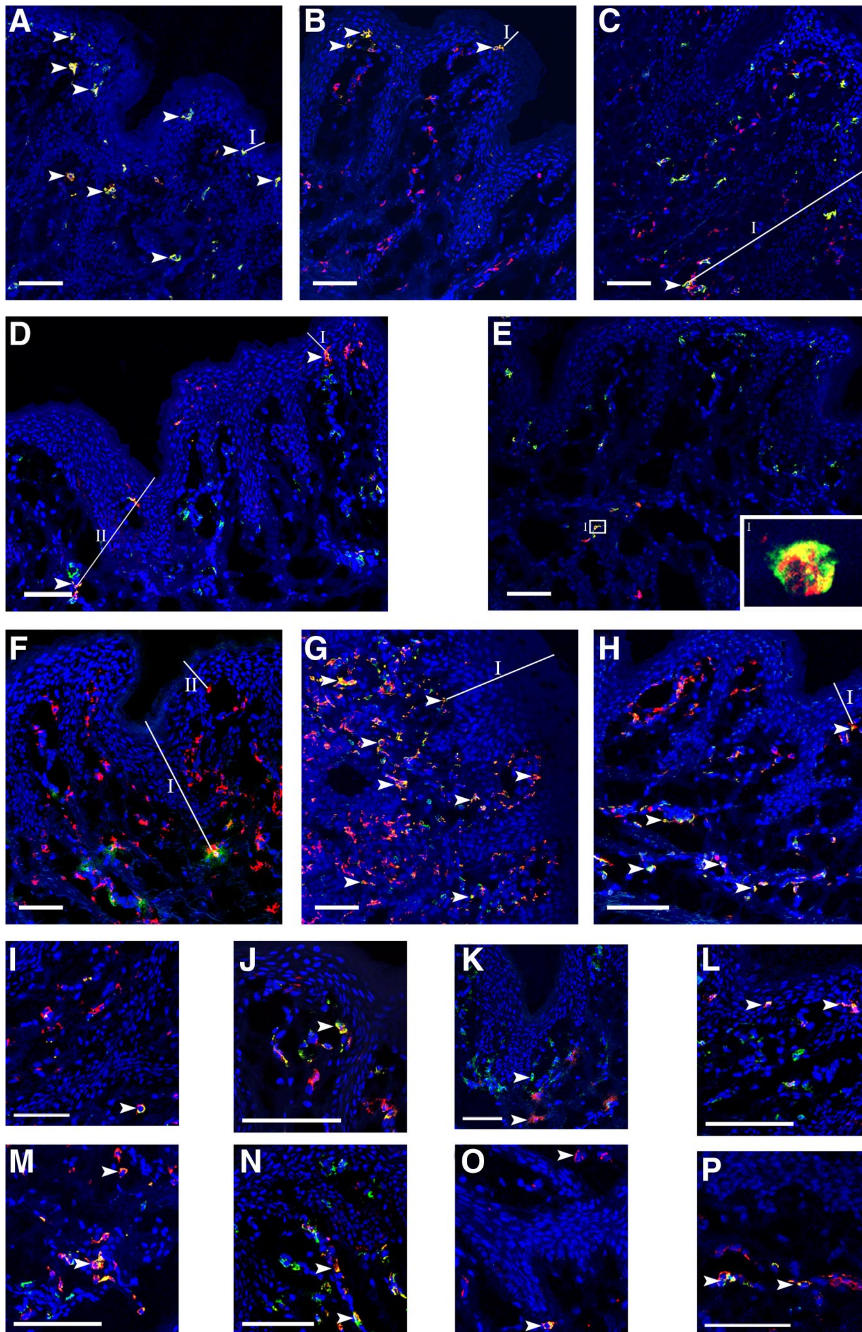


**Figure 2.** **A:** LCs were defined here as CD1a$^+$ (green) and/or Langerin$^+$ (red), and these markers co-localized on the majority of cells (yellow; I: MDTS: 24 $\mu$m). **B:** CD1a$^+$ (green) LCs within the epithelium frequently expressed CD4 (red; yellow cells: double positive; I: MDTS: 37 $\mu$m). **C:** CD1a$^+$ CD4$^+$ (yellow) LCs were also present deep within the submucosa (I: MDTS: 359 $\mu$m). **D:** CD1a$^+$ (red) LCs expressed CD11c (green), and double positive (yellow) cells were found both within the epithelium (I: MDTS: 45 $\mu$m) and the submucosa (II: MDTS: 226 $\mu$m). **E:** CD1a$^+$ (green) LCs expressing DC-LAMP (red) were solely present in the submucosal compartment (I: magnification of double positive cell). **F:** Macrophages were defined here as CD68$^+$ (red) and/or CD14$^+$ (green) cells. Double positive cells (yellow) were rare and located further from the tissue surface as compared with single positive CD68 cells (MDTS I: 248 $\mu$m and II: 63 $\mu$m). CD68$^+$ (green) cells expressed (**G**) MR (red; I: MDTS: 198 $\mu$m), and (**H**) CD68$^+$DC-SIGN (yellow) cells were also present, although less frequent (I: MDTS: 54 $\mu$m). CD4 (green) was present on submucosal CD11c$^+$ (red) cells (**I**). CD11c$^+$CD68$^+$ (yellow) cells were frequent in the submucosa (**J**), whereas CD11c$^+$ (green) cells did not express CD14 (red; **K**). CD11c$^+$ (green) cells co-localized (yellow) with the CLRs (red): (**L**) Langerin, (**M**) MR, and (**N**) DC-SIGN. **O:** The pDCs specific marker BDCA-2 was rarely present in the foreskin tissue, when present BDCA-2 always co-localized with CD123$^+$ (red) cells. The pDCs markers used here did not co-express any of the other investigated markers with one exception; (**P**) CD123$^+$CD4$^+$ (yellow) cells were detected in the submucosal compartment. **White arrowheads** indicate positive staining. Scale bars = 75 $\mu$m. Results are from one of at least four tissue samples yielding similar results.

CD11c$^+$CD68$^+$ cells, presumably myeloid dendritic cells or macrophages, were detected in high numbers (65% of submucosal CD11c$^+$ cells), whereas CD14 was not detected on CD11c$^+$ cells. The submucosal CD11c$^+$ cells also expressed all investigated CLRs (Figure 2).

Plasmacytoid dendritic cells (pDCs) were here defined as CD123 and BDCA-2$^+$ positive cells (Figure 2). The pDCs did not express CD11c, CD68, CD14, or any investigated CLRs (data not shown). CD4 was, however, occasionally expressed on pDCs (approximately 13% of CD123$^+$ cells).

### *Distribution of T Cells and Presence of Lymphoid Aggregates*

All foreskin samples examined contained lymphoid, CD3$^+$CD4$^+$, and CD3$^+$CD4$^-$ cells scattered throughout the epithelium and submucosa (Figure 3, A–D). The density of these cells in both the foreskin submucosa and epithelium was quantified. All individuals had fewer than 50 CD3$^+$ cells in the epithelial compartment, whereas 13 of the 33 individuals had more than 100 CD3$^+$ cells in the submucosal compartment (analysis performed in 5 $\times$ 10$^5$

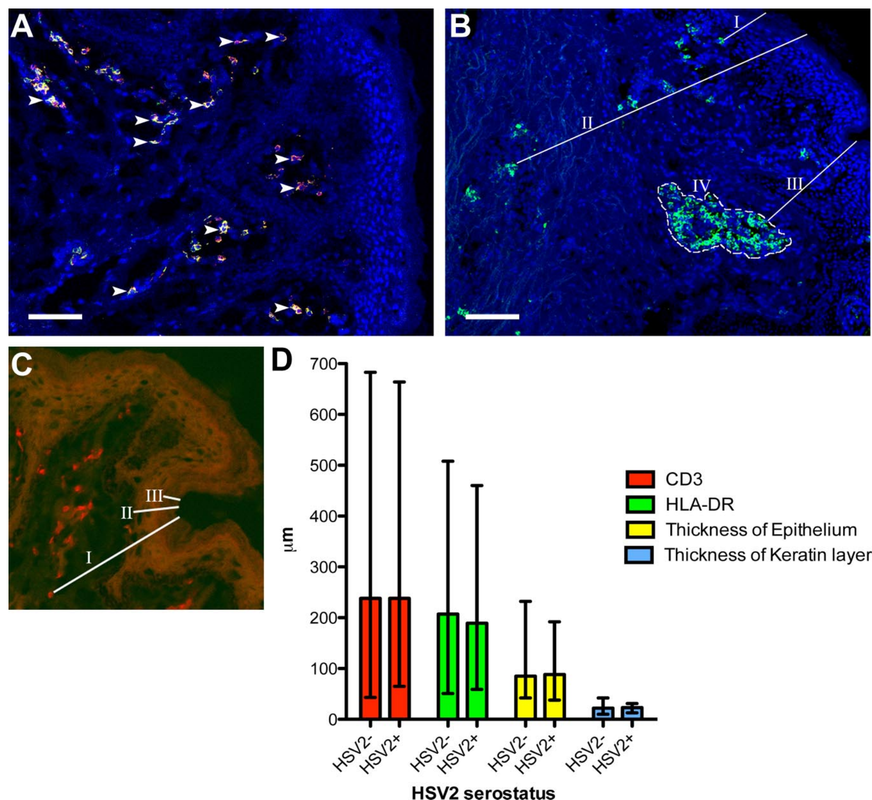


**Figure 3. A:** $CD4^+$ (green) and $CD3^+$ (red) frequently co-localized (yellow) within the submucosa of the human foreskin (0.02 cells/100 $\mu m^2$ tissue). **B:** The $CD4^+$ cells were observed both within the epithelium (I: MDTS: 92 $\mu$m) and deep in the submucosa (II: MDTS: 609 $\mu$m). A vast number of $CD4^+$ cells were also detected within lymphoid aggregates, which were present below the basal membrane (III: MDTS: 231 $\mu$m; IV: area 10,587 $\mu m^2$). **White arrowheads** indicate positive staining. Scale bars = 75 $\mu$m. Results are from one of at least four tissue samples yielding similar results. **C:** Measurements of (I) distance of cell body to tissue surface; (II) epithelial thickness; and (III) thickness of keratin layer were performed on five randomly selected HSV-2 seronegative (HSV-$2^-$) individuals and five randomly selected HSV-2 seropositive (HSV-$2^+$) individuals. All measurements were performed in approximately $5 \times 10^5$ $\mu m^2$ tissue within 700 $\mu$m from the tissue surface. Results are presented in **D**. No differences were detected between HSV-$2^-$ and HSV-$2^+$ individuals regarding distance of $CD3^+$ (red bars) or HLA-$DR^+$ (green bars) cells to tissue surface; median (range) in micrometers were as follows: 238 (43 to 683) vs. 238 (65 to 664) and 207 (51 to 508) vs. 189 (59 to 460), respectively. Neither were differences in epithelial thickness (yellow bars) or thickness of keratin layer (blue bars) detected; median (range) in micrometers were as follows: 85 (42 to 232) vs. 88 (38 to 192) and 22 (10 to 42) vs. 23 (13 to 31), respectively. Bars show the medians and error bars the range.

$\mu m^2$ tissue within 700 $\mu$m from the tissue surface). Although large individual variations were noted, there was no difference between HSV-2 seronegative versus seropositive men in neither the epithelial nor the submucosal compartments (data not shown).

In addition to the presence of scattered $CD3^+$ cells, $CD3^+$ cells were also detected in formations of lymphoid aggregates in the foreskin submucosa just beneath the basal membrane. These were also characterized by staining for antigen-presenting cells (HLA-DR) and a subgroup of HIV target cells (CD4; Figure 3). The distribution and size of the lymphoid aggregates were analyzed to allow for a comparison between the HSV-2 negative and seropositive individuals. Seven of 17 HSV-2 negative and 8 of 16 HSV-2 positive samples had at least one aggregate (cluster) containing more than 20 cells in the submucosal compartment (analysis performed in $5 \times 10^5$ $\mu m^2$ tissue within 700 $\mu$m from the tissue surface). Thus, no significant difference was detected.

No differences were detected in randomly selected HSV-2 seronegative ($n$ = 5) versus seropositive ($n$ = 5) men when measuring the distance of $CD3^+$ or HLA-$DR^+$ cells to tissue surface. Neither did we detect any differences in epithelial thickness or thickness of the keratin layer when comparing these men (Figure 3).

### *HSV-2 Seropositivity Can Influence the Expression of Immune Markers in the Foreskin*

HSV-2 seropositivity can either indicate a latent HSV-2 infection or active viral shedding. Even in the absence of visible lesions, the mucosal tissue can show histological signs of inflammation that may affect HIV susceptibility. We therefore attempted to quantify immune markers in the foreskin tissue of asymptomatic HSV-2 seropositive ($n$ = 16) versus seronegative ($n$ = 17) men. Exact quantifications of immunohistochemical staining with a large number of fluorescently labeled markers in a high number of individuals may be difficult. Thus, quantification of mRNA expression of the markers calculated in relative quantity to the reference gene UBC was considered to be a good complement. Expression of HLA-DR mRNA was measured to assess the presence of antigen presenting cells in addition to measurement of the cellular phenotypic markers CD3, CD4, CD68, CD11c, CD1a, CD123, the CLRs Langerin, MR, DC-SIGN, BDCA-2, and the maturation marker DC-LAMP. HSV-2 seropositive individuals had significantly higher levels of MR ($P$ = 0.046), CD14 ($P$ = 0.046), and CD123 ($P$ = 0.0002) in their foreskin, whereas the other markers did not differ significantly between the two groups (Figure 4).

## *Discussion*

Here we present a detailed characterization of the distribution of HIV target cells and expression of CLRs in the foreskin tissue of a highly relevant study group of young Kenyan men at high risk of HIV exposure. Previous reports have provided information on a limited set of phenotypic markers in foreskin tissue.[12,14–16,18,19,21] The present characterization revealed that intraepithelial LCs

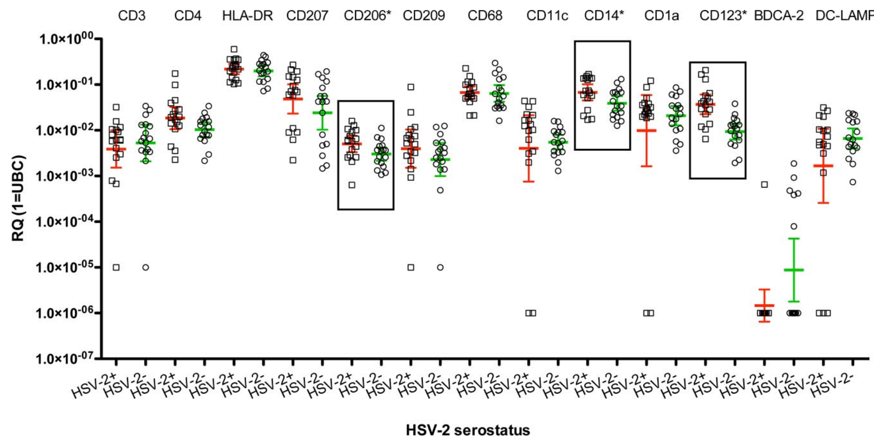


**Figure 4.** Expression of CD3, CD4, HLA-DR, Langerin/CD207, MR/CD206, DC-SIGN/CD209, CD68, CD11c, CD1a, CD123, BDCA-2, and DC-LAMP mRNA was measured by quantitative PCR. Lines and bars depict geometric mean with 95% confidence interval (**asterisk**) and **boxes** indicate statistical significant differences in expression between HSV-2 seropositive individuals (HSV-$2^+$) and HSV-2 seronegative individuals (HSV-$2^-$; statistical test: Mann-Whitney). HSV-$2^+$ individuals had significantly higher levels of MR/CD206 ($P$ = 0.046), CD14 ($P$ = 0.046), and CD123 ($P$ = 0.0002) in their foreskin.

($CD1a^+Langerin^+CD4^+$) were localized as close as 24 $\mu$m to the outer surface of the foreskin mucosa; close enough for their dendrites to theoretically reach out and sample HIV particles from genital secretions of a sexual partner. Furthermore, a number of activated LCs ($CD1a^+Langerin^+DC\text{-}LAMP^+$) was seen in the submucosa. Some of these intraepithelial and submucosal $CD1a^+$ LCs also expressed CD11c. Additional HIV target cells include subpopulations of the $CD11c^+$ mononuclear phagocyte system, and no markers clearly distinguish dendritic cells from macrophages, often defined as CD14 and CD68 positive.[22] Nevertheless, abundant submucosal $CD68^+$ cells were found, whereas $CD14^+$ cells were rare. The $CD68^+$ cells frequently expressed MR and occasionally DC-SIGN, thus indicating their susceptibility to HIV attachment. Submucosal myeloid cells, defined here as $CD11c^+$, expressed CD4 and all investigated CLRs. Given that one of these CLRs was the specific DC marker DC-SIGN, these $CD11c^+DC\text{-}SIGN^+$ cells are here defined as submucosal myeloid DCs. pDCs ($CD123^+BDCA^-2^+CD11c^-$) were not found in the foreskin epithelium, but a few single cells were found in the submucosa. CD123 is not specific for pDCs, and the expression of this marker in the absence of BDCA-2 may represent other cell types including endothelial cells.

Abundant numbers of $CD4^+$ cells (presumably including T cells, macrophages, and dendritic cells) were predominantly found in the submucosal compartment underlying the epithelium of the foreskin. These potential HIV target cells could thus function as early sites of viral replication before further dissemination, resembling the initial massive simian immunodeficiency virus replication occurring in a few genital $CD3^+CD4^+$ T cells before spreading to the lymphoid compartment in experimental monkey models.[23]

An abundant and superficial distribution of the CLRs was found with both intraepithelial expression of Langerin and submucosal expression of DC-SIGN and MR close to the epithelial basal membrane. HIV binding to CLRs can result in both enhancement and limitation of viral transmission.[8,11,24–27] The receptors can also be differentially expressed depending on cellular maturation and mobilization within the tissue. The density of the CLRs has to our knowledge only been described in the genital tract of women[28] and only one of these CLRs, DC-SIGN, in foreskin tissue.[17] With regard to the spread of incoming HIV particles, the foreskin tissues were also found to be vascularized with superficial blood and lymph vessels in close proximity to the HIV target cells. Breaches in the epithelial layer through trauma or inflammation, dendrites reaching out to the foreskin surface,[15,16] and passive diffusion of HIV particles through intact epithelium[29] may all contribute to foreskin susceptibility to HIV infection.

The degree of keratinization and distribution of CD1a, CD4, and CD68 expressing cells in inner versus outer foreskin mucosal surfaces have shown contradictory results in different models.[12,14–16,18,29] The discrepancies are likely dependent on age, STD history, tissue sampling techniques (including variable distances of the measurements with regard to the sulcus border), type of *ex vivo* explant models, fixation techniques, or use of cadaver tissue. In the present study, the foreskin tissue samples were collected at the time of circumcision and were immediately frozen, thus preserving the cellular structures in an optimal manner. Furthermore, the foreskin tissue samples were collected from sexually active young men from an HIV endemic geographic area, thus representing a highly relevant group of individuals at high risk of acquiring HIV infection. We have also presented the exact location of the tissue section, which is likely to influence any possible differences between the degree of keratinization and cellular distribution when comparing inner versus outer foreskin susceptibility to HIV infection. A decrease in the number of HIV target cells and an increase in keratinization would suggest better resistance against HIV penetration. However, no anatomical differences between the inner and outer foreskin mucosa with regard to $CD4^+$ or CLR expression, epithelial thickness, or thickness of keratin layer could be noted in our material collected 1 cm from the distal end of the intact foreskin regardless of HSV-2 serostatus.

About 40% of our study population, independent of HSV-2 serostatus, had abundant presence of scattered $CD3^+$ cells in the submucosal compartment. This indication of submucosal inflammation corresponds to the findings of Johnson et al[19] who studied foreskin inflammation in an Ugandan population. Also, Zhu et al[30] observed histological inflammatory markers, in this case at sites of

HSV-2 reactivation months after healing genital skin lesions. Interestingly, in our material we also observed distinct lymphoid aggregates just beneath the basal membrane, which were densely populated with $CD3^{+}CD4^{+}$ T cells. To our knowledge, this is the first time that such distinct densely populated lymphoid aggregates have been seen in human male genital tissue, and due to the superficial location and abundance their presence may be highly relevant for HIV susceptibility. Similar structures have been reported in the female genital tract of monkeys and HSV-2 immunized mice.[31,32] Also, in the human female genital tract, small focal accumulations of lymphocytes without evidence of organized lymphoid nodules have been described.[33] However, the observed aggregates did not appear to have been transiently induced by any known active genital infections because only men who lacked STDs (other than being HSV-2 seropositive) were eligible for tissue sampling, and the aggregates were present in 45% of study participants, irrespective of HSV-2 serostatus. It is important to point out that the intrasubject variations regarding size of aggregate may indeed be attributable to undiagnosed subclinical genital infections, including human papilloma virus (factors that were not included in the STD screening of this high risk study population). The lymphoid aggregates may be directly accessed after ulceration or trauma and serve as local sites for rapid expansion of immune effector cells, as potential HIV target cells, and may also provide a target for HIV containing migrating LCs or other dendritic cells.

In a previous study on foreskin tissue from our cohort, men with or without a history of active STDs were compared demonstrating a higher number of $CD1a^{+}$ and $CD68^{+}$ cells (but the same number of $CD4^{+}$ cells) in men with previous STDs. In the present study, the influence of HSV-2 seropositivity on expression of several cellular markers and CLRs was quantified by mRNA analysis. This analysis was a relevant complement to our immunohistochemical studies on the mucosal distribution of the corresponding structures. We found that MR and CD14 expression was significantly higher in the HSV-2 seropositive group. However, our immunohistochemistry findings depict that MR was not expressed on $CD14^{+}$ cells but rather on $CD68^{+}$ cells. Because the expression of CD68 did not vary between the two study groups, our finding may merely indicate that more MR is expressed per cell in the HSV-2 seropositive individuals and that the up-regulation of MR and CD14 mRNA expression are unrelated. In fact, a similar observation was made by our group when studying the expression of CLRs in the ectocervix of women of risk of HIV infection.[28] Given that the statistical values for these differences were borderline ($P = 0.046$), we believe more and larger studies are needed to confirm this. We also found that up-regulated CD123 mRNA expression was not accompanied by up-regulated BDCA-2. Because CD123 may be expressed on other cellular phenotypes apart from pDCs, such as endothelial cells, our finding illustrates the importance of additional markers, such as BDCA-2, when investigating the presence of pDCs. Overall, although HSV-2 seropositivity in men without reactivated clinical lesions for at least 3 months was not associated with a general up-regulation of inflammatory markers, the detected up-regulation of these selected markers indicates that some long-term persistence causing alterations may exist.[30]

The endorsement of male circumcision as a potential HIV preventions strategy is partially dependent on an understanding of the biological mechanisms involved in HIV transmission through mucosal surfaces. In addition, to rationally design a microbicide or mucosal vaccine, it is important to define the key cellular phenotypes to target to prevent transmission. The present findings of abundant and superficial HIV target cells and lymphoid aggregates provide possible anatomical explanations for the protective effect against HIV infection of male circumcision.

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