Foreskin T-cell subsets differ substantially from blood with respect to HIV co-receptor expression, inflammatory profile, and memory status

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The foreskin is the main site of heterosexual human immunodeficiency virus (HIV) acquisition in uncircumcised men, but functional data regarding T-cell subsets present at this site are lacking. Foreskin tissue and blood were obtained from Ugandan men undergoing elective adult circumcision. Tissue was treated by mechanical and enzymatic digestion followed by T-cell subset identification and assessment of cytokine production using flow cytometry. Foreskin CD4^{+} T cells were predominantly an effector memory phenotype, and compared with blood they displayed a higher frequency of CCR5 expression (42.0% vs. 9.9%) and interleukin-17 production. There was no difference in T-regulatory cell frequency, but interferon-γ and tumor necrosis factor-α production were increased in foreskin CD8^{+} T cells. These novel techniques demonstrate that the foreskin represents a proinflammatory milieu that is enriched for HIV-susceptible T-cell subsets. Further characterization of foreskin T-cell subsets may help to define the correlates of HIV susceptibility in the foreskin.

INTRODUCTION

As of 2009 there were 33.3 million people infected with human immunodeficiency virus (HIV)-1, and only a third of those requiring treatment were receiving it.[1] In addition, there were an estimated 2.6 million new infections in that year, the majority transmitted through heterosexual sex, emphasizing the urgent need for better HIV prevention strategies. Clinical trials have demonstrated that circumcision reduces HIV acquisition by 50–60% in heterosexual men, proving that the foreskin is the site of most acquisition in uncircumcised men exposed to HIV during insertive vaginal sex.[2–4] Although other penile sites such as the urethra may also have a role,[5] the central role of the foreskin in HIV acquisition was further supported by the observation that an increased foreskin surface area correlated with increased risk of HIV acquisition.[6] However, the immune events that surround acquisition and establishment of productive infection in the foreskin are poorly defined.[7] Understanding the immunopathogenesis of HIV acquisition in the foreskin remains an important priority for the development of new prevention modalities, despite

the efficacy of male circumcision, as evidenced by the fact that only a third of eligible men opted to avail themselves of free male circumcision during a recent HIV vaccine trial in South Africa.[8]

In the cervix, HIV and SIV infection is initiated by a small founder population of infected CD4^{+} T cells that expands through the local production of chemoattractant cytokines, followed by subsequent recruitment of activated memory CD4^{+} T cells.[9,10] It is likely that the efficiency with which this founder virus population expands depends on the immune milieu in the genital mucosa at the time of exposure to HIV.[11] While resting CD4^{+} T cells can be infected, viral replication within such cells is less efficient, and HIV propagation and dissemination from the site of initial infection are driven by the rapid recruitment of activated CD4^{+} T cells, in which the virus can more readily replicate.[9,12] Recruitment of these activated CD4^{+} T cells to the initial site of exposure may be assisted by HIV-induced changes in the local immune milieu, including the expression of chemokines such as MIP-3α and MIP-1β by epithelial and plasmacytoid dendritic cells.[9,13]

The presence or absence of certain T-cell subsets at the mucosal site of HIV exposure may be an important determinant of HIV susceptibility. Genital herpes is associated with an increase in activated CD4^{+} T cells within the foreskin and female genital tract,[14–16] perhaps contributing to the three-fold increase in HIV susceptibility associated with this infection.[17] The proinflammatory Th17 cells that normally protect skin and mucosal sites against bacterial and fungal infection are present at high frequency in the female genital mucosa and display enhanced HIV susceptibility.[18,19] Interleukin (IL)-22 is an important effector molecule of Th17 cells, having a role in epithelial integrity and repair. IL-22 is also produced by proinflammatory Th22 cells, which may be preferentially infected by HIV.[20] Conversely, CD25^{+}/FoxP3^{+} (Forkhead box P3) T-regulatory cells (Tregs) have an important role in controlling inflammation, and higher Treg frequencies in the blood have been linked to reduced HIV susceptibility.[21] Furthermore, individuals who are HIV exposed but seronegative show a quiescent immune phenotype with reduced basal T-cell cytokine production and lower proportions of activated T cells.[21–24]

Although immunohistochemistry is able to demonstrate the tissue position of specific cells in three dimensions, the ability of this technique to define cellular immune function is very limited. Therefore, we have developed techniques to isolate a single-cell suspension from fresh foreskin tissues, and to characterize the frequency and function of foreskin T-cell subsets using multiparameter flow cytometry. Our results indicate that the foreskin constitutes a proinflammatory immune environment that is enriched for HIV-susceptible T-cell subsets.

RESULTS
Study population
Participants were recruited from a longstanding community cohort in Rakai, Uganda.[25] Foreskin and whole blood were collected from 46 men between the ages 15 and 49 years, who had requested elective circumcision at the Rakai Health Sciences Program clinic in Kalisizo, Uganda, and who had provided written informed consent. All men were free of symptomatic sexually transmitted diseases at the time of surgery.

T-cell proportions in the blood and foreskin
Foreskin T cells were identified based on the expression of CD3, and comprised between 0.1 and 0.6% of total recorded events from digested, filtered foreskin tissue (**Supplementary Material** online). Upon permeabilization, many contaminating events (non-CD3^{+}) were removed from the cell solution, so that CD3^{+} events constituted 10–15% of total recorded events, allowing for easier identification of lymphocytes based on forward and side scatter alone. Owing to differences in the size of foreskin samples and to variation in tissue physical properties leading to differential cell loss during the digestion procedure, the absolute number of CD3 cells per foreskin was not defined. Rather, we report proportions of cells, standardized to CD3^{+}, CD3/4^{+}, or CD3/8^{+}. The majority of foreskin CD3^{+} cells were found to express either CD4 (mean, 51.4% of CD3^{+} cells) or CD8 (mean, 35.1%). Peripheral blood cells isolated from the same participants in parallel contained a higher proportion of CD4^{+} cells (63.4%, $P = 0.0001$) and slightly lower proportion of CD8^{+} cells (31.8%, $P = 0.005$; **Figure 1a**), resulting in a substantially reduced CD4/CD8 ratio in the foreskin compared with blood (1.53 vs. 2.27; $P < 0.0001$). A small proportion of CD3^{+} cells in both the foreskin and peripheral blood were found to express both CD8 and CD4 (0.41% and 0.84%, respectively, not significantly different). Of note, the foreskin contained more than twice as many CD4^{−}/CD8^{−} (double negative) CD3^{+} cells as the blood (12.4% vs. 5.1%, $P < 0.0001$; **Figure 1b**).

CCR5 expression and CD4^{+} Th17 and T-regulatory subsets in the foreskin
The great majority of sexually transmitted viruses use CCR5 as a co-receptor.[26] Therefore, we examined the expression of CCR5 on CD3/CD4^{+} T cells isolated from the foreskin and the blood of study participants (**Figure 2a**). The proportion of foreskin CD4^{+} T cells expressing CCR5 was over four-fold higher than that in blood (41.7% in the foreskin vs. 9.9% in peripheral blood mononuclear cells (PBMCs), $P < 0.0001$; **Figure 2b**).

Th17 cells may be preferentially infected by HIV[18] and the ratio of mucosal Th17/Treg cells is important in HIV immunopathogenesis.[27] Tregs were defined as CD3^{+}/CD4^{+} cells that
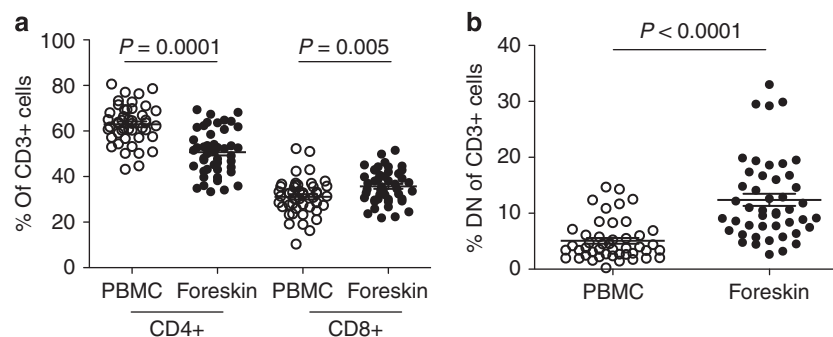

Figure 1 CD4^{+} and CD8^{+} T-cell subsets within the foreskin and peripheral blood. Peripheral blood mononuclear cells (PBMCs) and foreskin cells from 46 men were stained with CD3-FITC, CD4-PE, and CD8-PerCP. Graphs show percentages of CD3^{+} cells within PBMCs or foreskin cells that co-express (**a**) either CD4 or CD8, or (**b**) expressed neither CD4 nor CD8 (double-negative, DN, T cells).
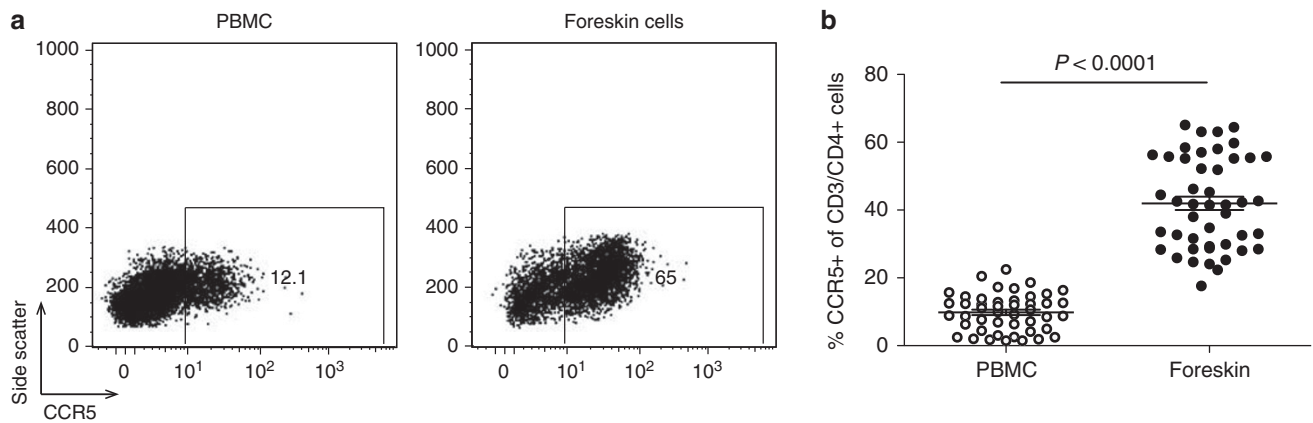
Figure 2 CCR5 expression on CD4+ T cells from the foreskin and peripheral blood. Peripheral blood mononuclear cells (PBMCs) and foreskin cells from 46 men were stained with CD3-APC, CD4-PE, and CCR5-FITC. Plots in panel **a** were created by gating on CD3+/CD4+ events. The gate defining CCR5+ events was created based on PBMC staining for this marker and applied to foreskin plots. (**b**) Proportions of CD3+/CD4+ cells in PBMCs and foreskin cells co-expressing CCR5.
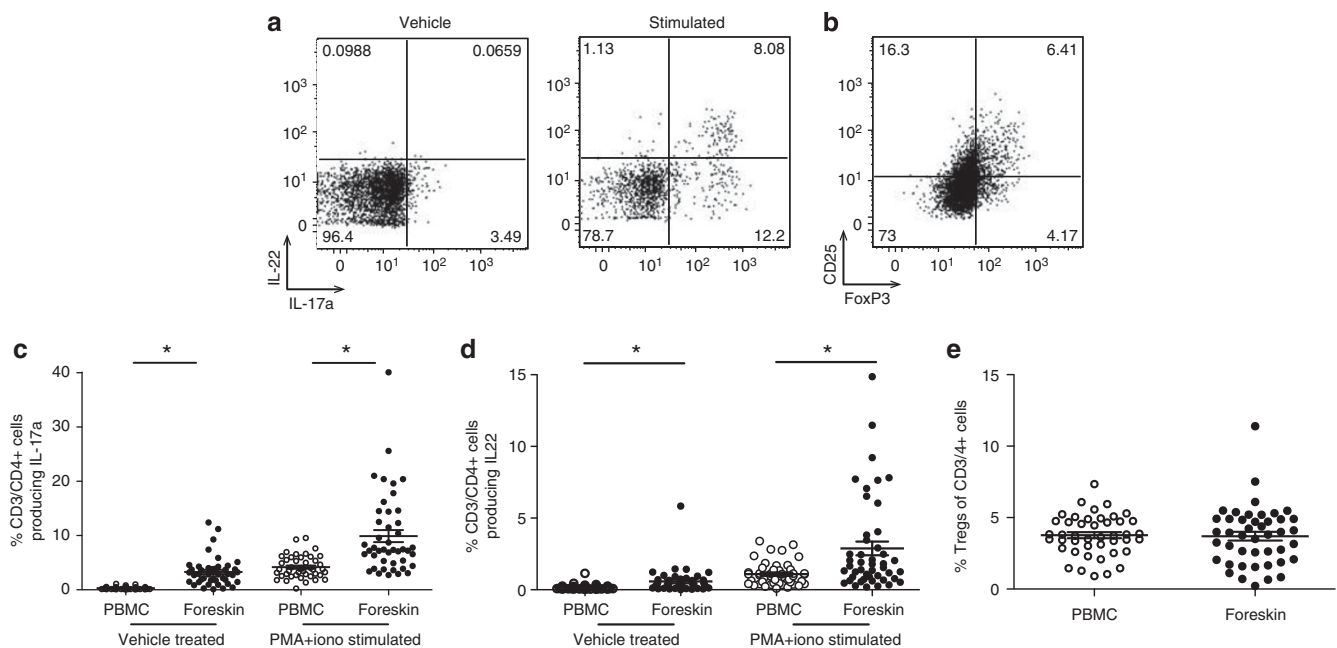

Figure 3 Increased production of interleukin (IL)-17a and IL-22 by foreskin CD4+ T cells, with no increase in T-regulatory (Treg) frequency. Peripheral blood mononuclear cells (PBMCs) and foreskin cells from 46 men were either left unstimulated (vehicle/Treg) or treated with phorbol-12-myristate-13-acetate (PMA)–ionomycin (stimulated). Representative plots of foreskin cells are shown (**a, b**). The gates in panel **a** defining IL-17a+ and IL-22+ events were created based on unstimulated PBMC staining for each patient, and then applied to stimulated PBMC and foreskin plots. The gate defining CD25+ events in panel **b** was created based on CD25-FMOs (fluorescence minus one = CD3, CD4, and FoxP3). (**c**) Proportions of Th17 cells in PBMC and foreskin samples (CD3+/CD4+/IL17a+). (**d**) IL-22 production in stimulated PBMC and foreskin CD4 T cells. (**e**) Proportions of PBMC and foreskin CD3+/CD4+ cells that are Tregs. *P < 0.0001.

co-expressed CD25 and the transcription factor FoxP3, and Th17 cells were defined as CD4+ T cells producing IL-17a, either at rest or upon stimulation with phorbol-12-myristate-13-acetate–ionomycin. Substantial differences in Th17 subsets were seen between foreskin and blood. Specifically, a much higher proportion of isolated foreskin CD4+ T cells produced IL-17a, both unstimulated (3.3% of CD3/4+ cells vs. 0.30%, P < 0.0001) and after stimulation (7.4% vs. 3.8%, P < 0.0001; **Figure 3a** and **c**). In addition, a higher proportion

of CD4+ T cells from the foreskin produced the Th17-associated cytokine IL-22 than in the peripheral blood, both at rest (0.166% vs. 0.579%; P < 0.0001) and after stimulation (1.09% vs. 2.88%, P < 0.0001; **Figure 3a** and **d**). However, no difference was observed in the frequency of Tregs between the foreskin and blood (3.9% of foreskin CD3/4+ cells vs. 3.7% in blood; **Figure 3b** and **e**). As a consequence, the Th17/Treg ratio was considerably higher in the foreskin that in the blood (4.1% vs. 1.3%, respectively; P < 0.0001).
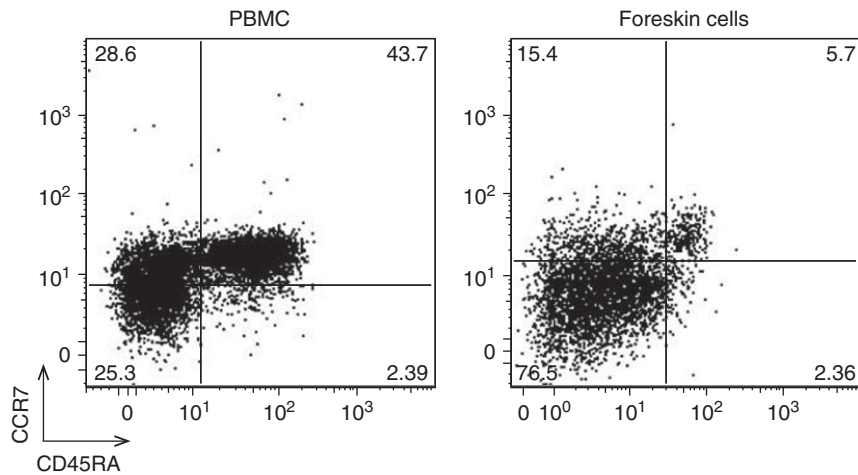
Figure 4 The foreskin contains primarily effector memory CD4 T cells (T_{EM}). Memory phenotype of peripheral blood mononuclear cells (PBMC) and foreskin mononuclear cells was assessed on a subgroup of three men by staining with CD3-FITC, CD4-PerCP, CCR7-PE, and CD45RA-APC. Representative plots were created by gating on CD3$^+$/CD4$^+$ events. The gates defining CD45RA$^+$ and CCR7$^+$ events were created based on FMO (fluorescence minus one) staining for each cell type.

Foreskin CD4$^+$ T cells display a predominantly effector memory phenotype

The memory phenotype of foreskin and blood CD4$^+$ T cells was assessed in a subset of three individuals (representative plots; **Figure 4**) by staining with CD45RA to distinguish naive (CD45RA$^+$) from memory T cells (CD45RA$^-$), and CCR7 to further delineate central (T_{CM}; CD45RA$^-$/CCR7$^+$) and effector (T_{EM}; CD45RA$^-$/CCR7$^-$) memory cells.[28] While blood contained approximately equal proportions of naive and memory T cells, the foreskin contained few naive T cells (ranging from 1.2 to 5.8%). Of the memory CD4$^+$ T cells in the foreskin, the majority were of the T_{EM} phenotype (72.6–89.5%).

Capacity of foreskin T cells to produce proinflammatory cytokines

As an inflammatory mucosal immune milieu may enhance HIV acquisition,[11] we next assessed the production of the cytokines interferon (IFN)γ and TNFα by CD8$^+$ and CD8$^-$ T-cell subsets, both at rest and after stimulation (**Figure 5a**). A relatively low frequency of foreskin T cells produced proinflammatory cytokines prior to stimulation, but this frequency was higher than blood T cells for both TNFα (2.8% vs. 0.47%, $P < 0.0001$; **Figure 5b**) and IFNγ (0.33% vs. 0.19%, $P = 0.048$; **Figure 5c**). Similarly, a higher frequency of foreskin CD8$^+$ T cells produced proinflammatory cytokines after mitogen stimulation: this was the case for both TNFα (45.3% vs. 39.4%, $P = 0.0029$; **Figure 5b**), IFNγ (48.2% vs. 41.3%, $P = 0.025$; **Figure 5c**), and for bifunctional cells coproducing both cytokines (35.2% vs. 28.6%, $P = 0.035$; **Figure 5d**).

While both peripheral blood and foreskin contained a small proportion of "double-negative" (CD4$^-$/CD8$^-$) T cells, the great majority of CD3$^+$/CD8$^-$ cells were CD4$^+$ T cells (**Figure 1**). Therefore, we also quantified TNFα and IFNγ production in these CD8$^-$ T cells as a proxy for CD4$^+$ T cells. A greater frequency of foreskin CD8$^-$ T cells than blood produced proinflammatory cytokines prior to stimulation (0.98% of foreskin cells produced TNFα vs. 0.29% of those from blood, $P < 0.0001$; 0.48% of foreskin cells produced IFNγ vs. 0.18% of blood, $P < 0.01$). After mitogen stimulation, the foreskin contained more CD8$^-$ T cells producing IFNγ (45.1% of foreskin T cells vs. 40.9% of blood T cells, $P = 0.0006$) and more bifunctional cells (20.6% vs. 15.9%, $P < 0.0027$), although no difference in the frequency of cells producing TNFα was apparent between compartments.

DISCUSSION

While circumcision reduces the incidence of HIV by up to 60% in heterosexual African men,[2–4] providing strong evidence that the foreskin is the main site of male HIV acquisition during vaginal sex,[29] the immunobiology of HIV acquisition in the foreskin is poorly understood. Previous studies of genital immunology, as it relates to the sexual acquisition of HIV, have focused on the female genital tract and gut, as samples are more easily obtained from these sites.[30,31] While results of the recent circumcision trials have focused interest on the foreskin, immunology studies have often used cadaveric or fixed/cryopreserved tissues, precluding functional immune studies.[14,32–37] In collaboration with a clinical site providing safe and free male circumcision as an HIV prevention tool,[4] we have developed field techniques utilizing expedited tissue processing and use of collagenase I for tissue digestion to isolate viable T cells from foreskin tissue with retention of the expression of T-cell markers and the functional ability to produce multiple cytokines. This has allowed for the characterization of functional foreskin T-cell subsets.

Several clear differences were evident between foreskin and blood T-cell subsets, both in terms of proportions, expression of HIV co-receptor CCR5, memory phenotypes, and the production of proinflammatory cytokines. There was a relative
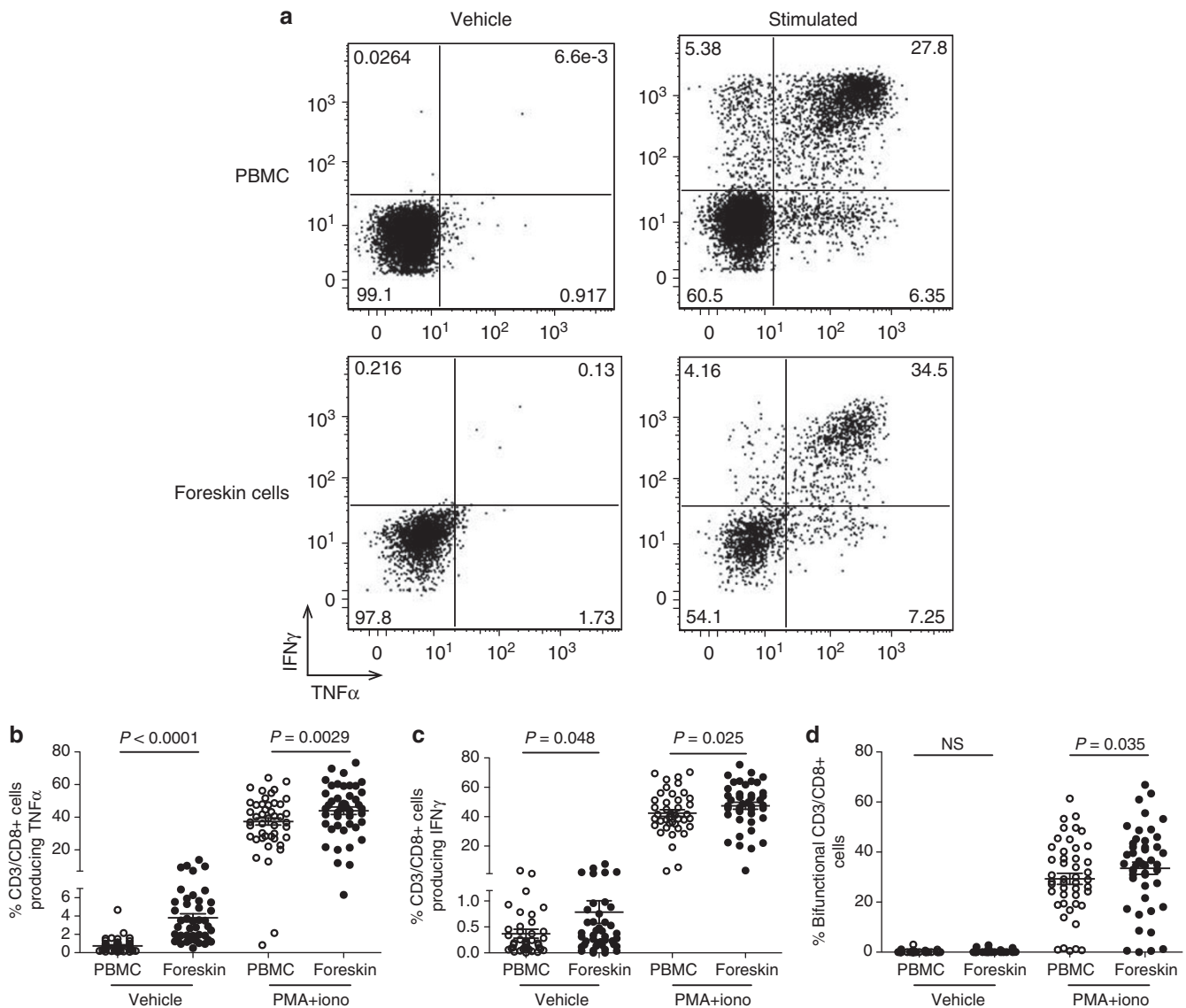
Figure 5 Enhanced production of proinflammatory cytokines by foreskin CD8$^+$ T cells. Peripheral blood mononuclear cells (PBMCs) and foreskin cells from 46 men were either left unstimulated (vehicle) or treated with phorbol-12-myristate-13-acetate (PMA)–ionomycin. Cells were then stained with CD3-FITC, CD8-PerCP, TNFα-PE, and IFNγ-APC. Plots in panel **a** were created by gating on CD3$^+$/CD8$^+$ events. The gates defining TNFα$^+$ and IFNγ$^+$ events were created based on unstimulated PBMC staining for each patient, and then applied to stimulated PBMC and foreskin plots. (**b**) Proportions of CD8 T cells producing TNFα, (**c**) IFNγ, and (**d**) of bifunctional CD8 T cells (producing both TNFα and IFNγ). IFN, interferon; NS, nonsignificant; TNF, tumor necrosis factor.

enrichment of CD8$^+$ T cells in the foreskin compared with the blood, contributing to a significantly reduced CD4/CD8 ratio in the foreskin. However, although the proportion of CD4$^+$ T cells was reduced, the proportion of CD4 T cells in the foreskin that co-expressed CCR5 was over four times higher than in blood, potentially enhancing susceptibility to HIV infection. HIV strains that use CCR5 as an entry co-receptor (R5 strains) are almost always responsible for sexual HIV transmission *in vivo*,[26] and an *ex vivo* model has demonstrated that the foreskin is susceptible to infection with R5-tropic but not X4-tropic viruses.[38,39] These results indicate that the substantial enhancement of CCR5 expression on foreskin-derived T cells may have direct implications for HIV acquisition.

Interestingly, the proportion of double-negative T cells (i.e., CD3$^+$ but CD4$^-$/CD8$^-$) was twice as high in the foreskin than in blood. Various CD3$^+$ T-cell populations may be contained within this subset, including natural killer T cells[40] and T cells bearing the variant T-cell receptors γδ[41] or the regulatory TCR αβ$^+$.[42] Double-negative CD3$^+$ T cells have been associated with protection against SIV immunopathogenesis in some primate species.[43] Elucidating the identity of these double-negative cells using multiparameter flow cytometry and investigating their possible relevance for HIV transmission will be important areas for future study.

While phenotypic characterization of foreskin T cells has been possible using fixed or cryopreserved tissues, we were

particularly interested to define their function directly *ex vivo*. Two CD4$^+$ T-cell subsets that may be particularly relevant to HIV acquisition and pathogenesis are Th17 and Tregs.[27] Th17 cells are CD4$^+$ T cells producing the cytokine IL-17a, and have a prominent proinflammatory role in mucosal immune defense against invading bacterial and fungal pathogens through the IL-17–mediated recruitment of neutrophils, induction of antimicrobial peptides, and maintenance of epithelial integrity.[44] Th17 cells display enhanced susceptibility to HIV *in vitro*[18,45] and are preferentially depleted from the blood and particularly the mucosa of HIV-infected individuals,[18,30] suggesting their enrichment at mucosal surfaces might enhance HIV acquisition. Tregs have immunomodulatory effects that are thought to have an important role in counterbalancing Th17-induced inflammation, despite sharing a common precursor, chemokine receptors, and mucosal homing properties with Th17 cells.[27] We found that Th17 proportions were substantially increased in the foreskin compared with blood in the absence of any corresponding enrichment in Treg cells. This increased Th17/Treg ratio in the foreskin suggests that this tissue is biased toward a predominantly proinflammatory immune environment, which could enhance HIV acquisition.[11]

Our data showing enhanced production of the cytokines TNFα and IFNγ by foreskin T cells, both at rest and after non-specific stimulation, support the concept of the foreskin as a proinflammatory tissue. This enhanced cytokine production is likely to be related to the high proportion of effector memory T cells (T$_{EM}$) found in the foreskin tissues, as this cell subset is primed to migrate to tissue sites and to carry out immediate effector functions.[28]

It is likely that both the function and proportions of T-cell subsets in the foreskin would be impacted by common bacterial and viral genital coinfections,[14,15] and any such differences might well have implications for HIV susceptibility. While men with symptomatic genital infections were excluded from male circumcision due to the potential increased risk of post-surgical infection and other complications, asymptomatic genital infections such as HSV-2 and HPV are common in these men.[46,47] The purpose of our initial analysis was to compare T-cell subsets in the foreskin and blood, but recruitment of a larger participant sample size is ongoing with the goal of characterizing the immune impact of these infections.

While our study examined pooled T lymphocytes derived from both the inner and outer foreskin, there is *in vitro* evidence to suggest that HIV acquisition may be more efficient across the inner surface of the foreskin,[48] defined as the portion of the foreskin that sits against the glans on the non-erect penis, but is exposed on the erect penis during intercourse. It was initially assumed that this increased susceptibility was due to a thinner keratin layer on the inner foreskin,[36,48] but studies using freshly processed foreskin samples have shown no difference in this layer between the inner and outer foreskin.[34,37] Although reports of differences in the density of HIV target cells between these two sites have been contradictory,[35,36,38,39,49] it does seem that cells of the inner foreskin may be functionally different to those of the outer foreskin, both in their responsiveness to

cytokines such as TNFα and MIP1α[49] and in their production of chemokines after HIV exposure.[48–50] Better elucidation of the functional differences between T cells derived from the inner and outer foreskin will constitute an important area for future research.

In summary, we have developed novel techniques to purify a single-cell suspension from fresh foreskin tissues, and to characterize the functional characteristics of foreskin T-cell populations. Compared with blood, the foreskin manifested a proinflammatory immune environment that was enriched for highly HIV-susceptible CD4$^+$ T-cell subsets, such as Th17 cells and those expressing the HIV co-receptor CCR5. These observations have important implications for HIV susceptibility in the foreskin, and will inform larger immuno-epidemiology field studies aiming to define the immune correlates of HIV susceptibility in the foreskin.

METHODS

Participants. Participants were men recruited from an established community cohort in Rakai, Uganda,[25] who had elected to undergo adult circumcision at the Rakai Health Sciences Program in Kalisizo, Uganda. Foreskins and whole blood samples were obtained from 46 HIV-negative men between the ages of 15 and 49 years. All participants provided written informed consent, and ethical approval was obtained through the research ethics boards of collaborating institutions (the University of Toronto, Uganda Virus Research Institute, and Western IRB). Surgery was deferred in the context of urethral discharge or clinically apparent genital ulceration. Participants were confirmed to be antibody-negative for HIV-1 and HIV-2 using two enzyme-linked immunosorbent assays (Murex HIV-1.2.O, Abbott, Abbott Park, IL; and Vironistika HIV Uni-Form II plus O Mircoelisa System, bioMerieux; Marcy l'Etoile, France). Discordant results were confirmed by western blot (GS HIV-1 Western Blot, BioRad, Hercules, CA). All participants were also screened for acute HIV infection by real-time polymerase chain reaction. RNA was extracted from plasma samples using the Sample Preparation System (Abbott), and amplification was performed using the Real Time HIV-1 Amplification Reagent Kit (Abbott) run on the M2000rt (Abbott).

T-cell isolation from the foreskin and blood. Foreskins were collected into RPMI 1640 media supplemented with: 10% heat-inactivated fetal bovine serum, $10 \, U \, ml^{-1}$ penicillin, $10 \, \mu g \, ml^{-1}$ streptomycin, $250 \, ng \, ml^{-1}$ amphotericin B, and 2 mM L-glutamine (all from Gibco, Invitrogen, Carlsbad, CA; henceforth referred to as R10 medium). Foreskin samples were always processed within 15 min of surgery, as additional time caused the dermal morphology to change substantially, with gross macroscopic tissue edema (data not shown). Tissue was first sectioned into longitudinal strips including both inner and outer foreskin and containing both epidermal and dermal tissue. These strips were then further sectioned to create pieces of approximately 0.25 cm^2. Each piece was placed in a 1.5-ml conical tube containing 1.0 ml of $500 \, U \, ml^{-1}$ Collagenase Type I (Gibco) and $42.5 \, U \, ml^{-1}$ of DNAse (Invitrogen) in RPMI 1640 media supplemented with $10 \, U \, ml^{-1}$ penicillin, $10 \, \mu g \, ml^{-1}$ streptomycin, $250 \, ng \, ml^{-1}$ amphotericin B, and 2 mM L-glutamine (henceforth referred to as RPMI, all from Gibco). Initial immune studies have used dispase for foreskin tissue digestion, but we found that treatment with as little as $1.0 \, U \, ml^{-1}$ of dispase (Gibco) for 30 min at 37°C led to the loss of CD4 expression and decreased CD8 expression in both peripheral blood and foreskin-derived T cells (**Supplementary Figure S1** online). Scissors were used to mechanically disrupt each piece of tissue, and tubes were then placed on a shaker (Eppendorf Thermomixer; Hamburg, Germany) for 30 min of enzymatic digestion at 37°C with shaking at 900 r.p.m. The cellular suspension obtained from each tube was pooled and collagenase activity

was inhibited by the addition of fetal bovine serum to a final concentration of 10%. This cell suspension was then filtered through a 100-µm cell strainer (BD Biosciences, Franklin Lakes, NJ,) to remove any remaining undigested tissue. Filtered cells were washed once, resuspended in R10, and allowed to rest under normal growth conditions (37°C 5% CO_2, humidified atmosphere) for 3–7 h. This combination of collagenase 1 and gentle mechanical digestion allowed for the retention of CD4 expression and gave a single-cell suspension containing $CD3^+$ T cells that showed a similar CD4 and CD8 expression profile to PBMCs from the same individual (**Supplementary Figure S2** online).

PBMCs were isolated by density gradient centrifugation (Ficoll-Paque Plus; Amersham Biosciences, Uppsala, Sweden).

Characterization of $CD4^+$ T-cell subsets. Both PBMC and foreskin cell numbers were determined by trypan blue exclusion. PBMCs (1×10^6) and foreskin (10 to 20×10^6) cells (depending on yield) were plated in 500 µl R10 and stimulated with either 1 ng ml^{-1} phorbol-12-myristate-13-acetate and 1 µg ml^{-1} ionomycin (both from Sigma, St Lousi, MO) or vehicle (0.1% dimethylsulfoxide) with 5 µg ml^{-1} Brefeldin A (GolgiPlug, BD Biosciences) for 9 h at 37°C. Samples were then washed with cold 2% fetal bovine serum in phosphate-buffered saline and stained with fluorochrome-labeled monoclonal antibodies specific for CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), CCR5 (2D7/CCR5), and CD25 (M-A251; all BD Biosciences). Excess surface antibody was removed by washing with 2% fetal bovine serum in phosphate-buffered saline. Samples for intracellular staining were permeabilized using either the eBioscience fixation/permeabilization solution for Treg identification (eBiosciences, San Diego, CA) or the BD Cytofix/Cytoperm solution (BD Biosciences) for all others. Cells were washed in permeabilization wash buffer and stained with fluorochrome-labeled monoclonal antibodies specific for combinations of the following intracellular cytokines/transcription factors: TNFα (MAb11; BD Biosciences), IFNγ (B27; BD Biosciences), IL-17a (eBio64DEC17; eBioscience), IL-22 (22URTI; eBioscience), and FoxP3 (PCH101; eBioscience). Samples were acquired using a FACSCalibur flow cytometer (BD Systems) and data analysis performed using FlowJo analytical software version v.9.3 (Treestar, Ashland, OR).

Unpermeabilized foreskin cells were gated based on forward and sideward scatter (**Supplementary Figure S2** online). This gate was created based on the location of $CD3^+$ T cells in the PBMC sample from the same patient. Back gating was used to confirm that this gate corresponded with the location of $CD3^+$ cells in the foreskin sample ($CD3^+$ cells representing approximately 0.1–0.6% of total events in the foreskin sample). A clearly visible population of $CD3^+$ cells in the foreskin samples could then be identified. For unpermeabilized foreskin cells, 10^6 events were recorded, while only 10^5 events were recorded for PBMC and permeabilized foreskin cells, due to the large amount of other cell types present in unpermeabilized foreskin samples. After permeabilization the T-cell population was enriched (3–5% of total events) and could be directly identified on the forward by side scatter plot (**Supplementary Figure S2** online).

Statistical analysis. T-cell populations were compared between blood and foreskin by paired Wilcoxon rank-sum test. Statistical tests were run on SPSS v.17.0 for Mac (IBM; New York, NY). Flow cytometry data were analyzed in FlowJo v.9.3 and Excel (Microsoft; Redmond, WA) prior to statistical testing.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

DISCLOSURE
The authors declared no conflict of interest.

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