

ABSTRACT

Objectives:

To study the distribution of HIV-1 receptors and degree of keratinisation in the human penis.

Design:

Formalin-fixed penises were obtained from 9 uncircumcised cadavers.

Foreskins were obtained from 21 healthy adult men undergoing elective circumcision for social reasons. Uncircumcised penises were obtained within 24 hours of death from 8 men. All tissues were stained for keratin and HIV-1 receptors.

Methods:

Penises from 9 formalin fixed cadavers aged 64-80 were obtained from the Dept. of Anatomy, University of Melbourne. Foreskins were obtained from 21 men aged 18-64 following circumcision performed at either the Freemason's or Mercy Private Hospitals, Melbourne, Australia. Fresh penile necropsy specimens from 8 uncircumcised men aged 23-63 were obtained from the Victorian Institute of Forensic Medicine, Melbourne. The degree of keratinisation was scored, and the distribution of HIV-1 susceptible cells was mapped in the glans penis, penile urethra, urethral meatus, frenulum and foreskin.

Results:

Cells with HIV-1 receptors were present in all penile epithelia, but Langerhans cells were most superficial in the inner foreskin and frenulum. The inner foreskin had a significantly thinner keratin layer (1.8 ± 0.1 units), than the outer foreskin (3.3 ± 0.1), or glans penis (3.3 ± 0.2), $p < 0.05$.

Conclusions:

Superficial Langerhans cells on the inner aspect of the foreskin and frenulum are poorly protected by keratin and thus could play an important role in primary male infection. These findings provide a possible anatomical explanation for the epidemiologically observed protective effect of male circumcision.

TITLE: Potential HIV-1 target cells in the human penis.

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KEYWORDS

Circumcision

HIV

Langerhans cells

Mucosa

Penis

INTRODUCTION

The most recent World Health Organisation estimates show that there are currently 18.7 million men infected with Human Immunodeficiency Virus Type 1 [1]. Approximately 80 to 90% of these men were infected following heterosexual intercourse [1, 2], yet very little is known about the precise routes of HIV-1 entry into the male reproductive tract. Previous studies [3-6] have observed HIV-1 susceptible cell populations in the foreskin of adult men, but to date there have been no studies of the other penile epithelia.

It is generally agreed that keratin provides an impermeable barrier to HIV-1 [7]. Two studies have shown that the glans penis is heavily keratinised in both circumcised and uncircumcised men [2, 8]. Therefore, it is unlikely to be involved in primary infection unless the keratin layer is compromised by lesions, inflammation or microtrauma. The inner aspect of the foreskin is poorly keratinised [4, 5], but to date there have been no studies on keratinisation of the urethral meatus, penile urethra or frenulum.

Recent epidemiological evidence collected from over 37 observational studies proposes that male circumcision reduces the relative risk of acquiring HIV-1 by 1.8 to 8.2 fold [9-12]. The protective effects of circumcision still remain even when potentially confounding social practices such as religion, number of sexual partners and condom usage are taken into consideration [13]. Perhaps the most compelling evidence for the protective benefit of male circumcision was obtained from a recently completed prospective randomised control trial in South Africa. Following over 3000 participants for 21 months, Auvert and colleagues observed a 65% protective benefit amongst the

men they circumcised compared to the uncircumcised control group [14]. Thus it seems likely that the foreskin plays a major role in HIV-1 transmission in uncircumcised men.

Potential HIV-1 target cells in the mucosa include Langerhans cells, subepithelial dendritic cells, macrophages and CD4 T cells [5, 15]. The role of Langerhans cells in the mucosa is to sample foreign antigens and migrate to regional lymph nodes where they present the processed antigens to naïve T cells [16, 17]. Langerhans cells are the most superficial of all HIV-1 susceptible cells in the absence of disease or trauma, and have also been shown to express the c-type lectin langerin, which may play a complimentary role in HIV-1 dissemination to regional lymph nodes [18]. It is probable that Langerhans cells whose dendritic processes are closest to the epithelial surface will be first to come in contact with HIV-1 in vaginal secretions from the man's infected partner. This primary infection is most likely to occur when there is little or no overlying protective layer of keratin.

Other cells with HIV-1 receptors including T cells, dendritic cells and macrophages are all present in the inner and outer foreskin [4, 5], but are commonly found deeper within the sub-mucosa. Therefore, HIV-1 is less likely to encounter these cell types in the healthy male genital mucosa. If however there is a loss of epithelial integrity by trauma during intercourse, ulcerative STI or inflammation then these cells are much more likely to encounter Infectious HIV-1 virions. It is well recognized that some sexually transmitted infections show an epidemiological synergy with HIV-1 and may result in a significant increase in susceptibility to HIV-1 infection [19]. This is especially true of infections that cause epithelial lesions such as syphilis, chancroid and genital

herpes[19, 20], which expose the underlying target cells deeper within the epidermis and dermis.

The purpose of this study was to locate HIV-1 susceptible cells in all the epithelia of the human penis and to quantitate the thickness of the overlying layer of keratin in order to evaluate potential sites of HIV-1 entry into the penis.

METHODS

Tissue:

The formalin fixed penises of 9 uncircumcised cadavers of mean age 77.4 years were obtained from the Department of Anatomy, The University of Melbourne. Fresh foreskins were obtained from 21 healthy, consenting men of mean age 28.9 years following elective male circumcisions performed at either the Freemasons Hospital or the Mercy Hospital in Melbourne, Australia. Penile necropsy specimens were obtained within 18 hours of death from 8 men of mean age 30.9 years from the Victorian Institute of Forensic Medicine with next-of-kin consent. All samples were obtained from HIV seronegative individuals. The study was approved by all relevant institutional ethics committees.

Keratin Staining:

1cm thick cross-sections were taken from the midpoint of each glans penis from the 9 cadavers, and embedded in paraffin prior to sectioning and staining. These were then sectioned at 8µm and stained for keratin with both the Hematoxylin–Eosin and the Ayoub-Shklar methods. Sections were examined under light microscopy at 200X to 400X magnification.

Immunocompetent Cell Staining:

Immediately following circumcision or autopsy, fresh tissue was immersed in sterile saline and transported on ice to the laboratory. Each foreskin was separated using blunt dissection into inner and outer aspects. The frenulum, urethra, urethral meatus and glans penis were dissected out from the autopsy specimens. All tissues were then snap frozen in Jung tissue freezing medium (Leica Microsystems, Wetzlar, Germany).

Frozen blocks were sectioned by cryostat and stained for immunocompetent cells using monoclonal antibodies targeting CD1a, CD4, HLA-DR, DC-SIGN, CXCR4 and CCR5. Sections were stained specifically for Langerhans cells using anti-CD1a hybridoma supernatant (OKT6, American Type Culture Collection, Manassas, VA, USA). Other potential HIV-1 target cells were specifically stained using anti-CD4 and anti HLA-DR hybridoma supernatants (American Type Culture Collection, Manassas, VA, USA), and anti-DC-SIGN, anti-CCR5 and anti-CXCR4 (BD Biosciences). Sheep anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (Silenus Labs Pty. Ltd., Boronia, Australia) was diluted 1:100 and used to label all positively stained cells. Nuclear counterstaining with 0.25µg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) enabled cell position within the epithelium to be examined. Digital images were obtained with a Zeiss Axioplan 2 confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA) attached to a µ-Radiance confocal scanning system (Bio-Rad Laboratories, Hercules, CA, USA). Images were collected at 100X to 200X magnification for density analysis and under oil immersion at 630X to 1000X for individual morphological analysis. Lasersharp 2000 software (Bio-Rad Laboratories, Hercules, CA, USA) was used for all measurements.

Statistical Analyses:

Post-mortem autolysis prior to perfusion fixation of the cadavers made it difficult to obtain objective quantitative measurements of keratin thickness. Therefore, three experienced microscopists subjectively estimated keratin thickness in randomised sections. Three microscopic fields of each glans penis, inner foreskin, and outer foreskin were randomly selected from each of the 9 penis samples. Keratin thickness was subjectively assessed on a scale of 0 to 5 arbitrary units, where 0 corresponded to

no keratin, and 5 to maximum keratinisation (keratin thickness $\geq 20\mu\text{m}$). Two-tailed, two-sample unequal variance t-tests were used to analyse the data.

Cellular densities were calculated for all samples by averaging the number of positively stained cells within three $500\mu\text{m}^2$ fields from the epithelial surface to the dermis. Values were converted into cells per square millimeter (cells/mm^2). Individual cellular analysis was performed on twenty randomly chosen cells from all fresh-frozen sections. A cell was included only if the nucleus was visible. Three-dimensional digital reconstructions of individual Langerhans cells showed the number of dendritic processes originating from each cell, and how close to the epithelial surface these processes extended. Two-tailed, paired t-tests were used to determine differences between Langerhans cell populations in the various penile epithelia tested.

RESULTS

Keratin Measurements:

In the cadaveric samples, the inner foreskin (mean thickness of 1.8 [SE 0.1] units) was significantly less keratinized than the outer foreskin (3.3 [SE 0.1]) or glans penis (3.3 [SE 0.2]), $p < 0.05$. There was no difference in keratinisation between the outer aspect of the foreskin and the glans penis. These results were confirmed by studying the distribution of keratin in all penile epithelia collected at autopsy. The frenulum and the urethral meatus were poorly keratinized, and there was no keratin observed in the penile urethra.

Immunocompetent cell localisation:

CD1a positive Langerhans cells were clearly visible in the outer and inner foreskin, the glans penis, urethral meatus and frenulum, although none were observed in the penile urethra (Figure 1). The majority were observed in the superficial layers of the epidermis. Most Langerhans cells had dendritic projections extending up between the keratinocytes towards the epithelial surface (Figure 2).

CD4 positive cells including T-cells, macrophages and dendritic cells were found in all epithelia but were generally located within the dermis. CCR5 and CXCR4 were expressed in a minority of superficial Langerhans cells but were found on a greater proportion of cells deeper in the dermis. DC-SIGN was expressed in low levels in dermal dendritic cells and was localised predominantly near the basal lamina.

Cell density and distribution:

The highest density of Langerhans cells was in the outer foreskin (85.5 [SE 4.1] cells/mm²), followed in descending order by the inner foreskin (61.3 [SE 5.0]), frenulum (56 [SE 8.3], glans penis (41 [SE 10.0]), and urethral meatus (14 [SE 6.4]). None were observed in the penile urethra. Densities in each epithelium for CD4, CXCR4, CCR5 and DC-SIGN can be seen in Table 1.

The depth of each HIV susceptible cell type from the epithelium was measured (Table 2). Measurements were taken from the cell surface or dendritic process nearest the epithelial surface. Langerhans cells (61µm) were significantly more superficial across all epithelia than CD4 (102µm), CCR5 (94µm), CXCR4 (92µm), DC-SIGN (77µm) or HLA-DR (128µm) expressing cell types $p < 0.0001$. Dendritic processes from Langerhans cells were particularly superficial on the inner aspect of the foreskin (23µm) and frenulum (34µm). In the inner foreskin, dendritic processes came within 4.8µm of the epithelial surface, whereas in the outer foreskin they rarely came within 20µm of the epithelial surface due to the thicker layer of keratin.

DISCUSSION

This study has shown that both the inner aspect of the foreskin and the frenulum are poorly keratinized, and are richly supplied with HIV-1 susceptible cells. Of the cell types tested, Langerhans cells are the most likely to be encountered as they are most superficial and have dendritic processes sampling a large epithelial surface area. Langerhans cells of the inner foreskin and frenulum are protected by a much thinner layer of keratin than those in the glans penis or outer foreskin. The urethral meatus and penile urethra, although poorly protected by keratin, contained very few Langerhans cells. The highest density of Langerhans cells was in the outer foreskin, but these were covered by a thicker protective layer of keratin. The dendritic processes of the Langerhans cells in the inner foreskin were significantly more superficial due to decreased epithelial keratinisation. The presence of c-type lectins in the male genital mucosa was also observed and may play an important role in the binding, internalisation and subsequent transport of HIV-1 to regional lymph nodes. DC-SIGN was confined to dendritic cells near the basal lamina. These findings provide a possible anatomical explanation for the protective effect of male circumcision against HIV-1 infection.

The position of T-cells, macrophages and dendritic cells other than Langerhans cells suggested that in healthy individuals they are less likely to be involved in HIV-1 sexual transmission as they are predominantly dermal. Many of these cells contain the specific receptors required for HIV-1 infection, and are likely to become involved in viral entry if the integrity of the overlying epithelium is disrupted by sexually transmitted infections or trauma. The presence of these cells in the dermis may therefore help to explain the increased susceptibility to HIV-1 infection in men with ulcerative STI's [19].

During penile erection, the turgid glans penis is well protected by its thick overlying layer of keratin. However, the delicate, vascular inner aspect of the foreskin is stretched halfway down the penile shaft (Figure 3), further attenuating its thin protective layer of keratin that is directly exposed to vaginal secretions. The highly keratinized outer foreskin is reflected down to the base of the penis and much of it may not even come in contact with the vaginal epithelium. In circumcised men where the foreskin has been removed and the frenulum has atrophied, the whole of the penile shaft is covered with a thickly keratinized epithelium. Following intercourse, the preputial cavity may also provide an environment conducive to increased viral survival and thus increase transmission in uncircumcised men. Male circumcision has been shown to confer protection against genital herpes, syphilis, candidiasis, gonorrhoea and genital ulcer disease [20, 21]. Male circumcision also provides significant protection to men, and their female partners against human papilloma virus infection, and the resultant penile and cervical carcinoma [22]. With the South African randomised control trial showing a 61% protective benefit of male circumcision against HIV infection [14], the procedure must now be seriously considered as an adjunct to existing prevention strategies. To be most beneficial, the procedure must be conducted in a safe and sterile manner and involve thorough education of the participants to minimise the effects of disinhibition.

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REFERENCES

1. UNAIDS, *2004 report on the global HIV/AIDS epidemic : 4th global report*. 2004.
2. Szabo, R. and R.V. Short, *How does male circumcision protect against HIV infection?* *Bmj*, 2000. **320**(7249): p. 1592-4.
3. Weiss, G.N., M. Sanders, and K.C. Westbrook, *The distribution and density of Langerhans cells in the human prepuce: site of a diminished immune response?* *Isr J Med Sci*, 1993. **29**(1): p. 42-3.
4. Hussain, L.A. and T. Lehner, *Comparative investigation of Langerhans' cells and potential receptors for HIV in oral, genitourinary and rectal epithelia*. *Immunology*, 1995. **85**(3): p. 475-84.
5. Patterson, B.K., et al., *Susceptibility to human immunodeficiency virus-1 infection of human foreskin and cervical tissue grown in explant culture*. *Am J Pathol*, 2002. **161**(3): p. 867-73.
6. Soilleux, E.J. and N. Coleman, *Expression of DC-SIGN in human foreskin may facilitate sexual transmission of HIV*. *J Clin Pathol*, 2004. **57**(1): p. 77-8.
7. de Vincenzi, I. and T. Mertens, *Male circumcision: a role in HIV prevention?* *Aids*, 1994. **8**(2): p. 153-60.
8. Cold, C.J. and J.R. Taylor, *The Prepuce*. *Br. J. Urol.* , 1999(83 (Supplement)): p. 1-12.
9. Bailey, R.C., F.A. Plummer, and S. Moses, *Male circumcision and HIV prevention: current knowledge and future research directions*. *Lancet Infect Dis*, 2001. **1**(4): p. 223-31.
10. Weiss, H.A., M.A. Quigley, and R.J. Hayes, *Male circumcision and risk of HIV infection in sub-Saharan Africa: a systematic review and meta-analysis*. *Aids*, 2000. **14**(15): p. 2361-70.
11. O'Farrell, N. and M. Egger, *Circumcision in men and the prevention of HIV infection: a 'meta-analysis' revisited*. *Int J STD AIDS*, 2000. **11**(3): p. 137-42.
12. Siegfried, N., et al., *HIV and male circumcision--a systematic review with assessment of the quality of studies*. *Lancet Infect Dis*, 2005. **5**(3): p. 165-73.
13. Reynolds, S.J., et al., *Male circumcision and risk of HIV-1 and other sexually transmitted infections in India*. *Lancet*, 2004. **363**(9414): p. 1039-40.

14. Auvert, B., et al., *Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial*. PLoS Med, 2005. **2**(11): p. e298.
15. Zaitseva, M., et al., *Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection*. Nat Med, 1997. **3**(12): p. 1369-75.
16. Soto-Ramirez, L.E., et al., *HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV*. Science, 1996. **271**(5253): p. 1291-3.
17. McLellan, A.D., et al., *Dermal dendritic cells associated with T lymphocytes in normal human skin display an activated phenotype*. J Invest Dermatol, 1998. **111**(5): p. 841-9.
18. Turville, S.G., et al., *Diversity of receptors binding HIV on dendritic cell subsets*. Nat Immunol, 2002. **3**(10): p. 975-83.
19. Fleming, D.T. and J.N. Wasserheit, *From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection*. Sex Transm Infect, 1999. **75**(1): p. 3-17.
20. Cook, L.S., L.A. Koutsky, and K.K. Holmes, *Circumcision and sexually transmitted diseases*. Am J Public Health, 1994. **84**(2): p. 197-201.
21. Lavreys, L., et al., *Effect of circumcision on incidence of human immunodeficiency virus type 1 and other sexually transmitted diseases: a prospective cohort study of trucking company employees in Kenya*. J Infect Dis, 1999. **180**(2): p. 330-6.
22. Castellsague, X., et al., *Male circumcision, penile human papillomavirus infection, and cervical cancer in female partners*. N Engl J Med, 2002. **346**(15): p. 1105-12.

Table 1: Mean Density of HIV-1 susceptible cell types in penile epithelia (Cells/mm²)

Tissue type	CD1a	CD4	CCR5	CXCR4	HLA-DR	DC-SIGN
Outer foreskin	85	126	33	12	103	17
Inner foreskin	61	108	28	2	116	11
Glans	56	104	23	20	137	18
Frenulum	41	57	16	19	89	12
Urethra	0	22	0	1	21	0
Urethral Meatus	14	47	7	11	43	2

Table 2: Mean depth of HIV-1 susceptible cell types in penile epithelia (μm)

Tissue Type	CD1a	CD4	CCR5	CXCR4	HLA-DR	DC-SIGN
Outer foreskin	43	121	103	179	176	106
Inner foreskin	19	78	71	67	94	48
Glans	67	144	89	122	147	136
Frenulum	31	108	82	100	102	108
Urethra	-	77	-	89	73	-
Urethral Meatus	40	49	102	91	129	80

Figure 1: Distribution of Langerhans cells (green) in the outer foreskin, well beneath the keratinised epithelium (200x magnification).

Figure 2: A single Langerhans cell (green) in the outer foreskin with dendritic processes extending towards the epithelial surface (630x magnification).

Figure 3a: Flaccid uncircumcised penis.

Figure 3b: Erect uncircumcised penis with the foreskin retracted showing likely sites of HIV-1 entry.

Figure 1
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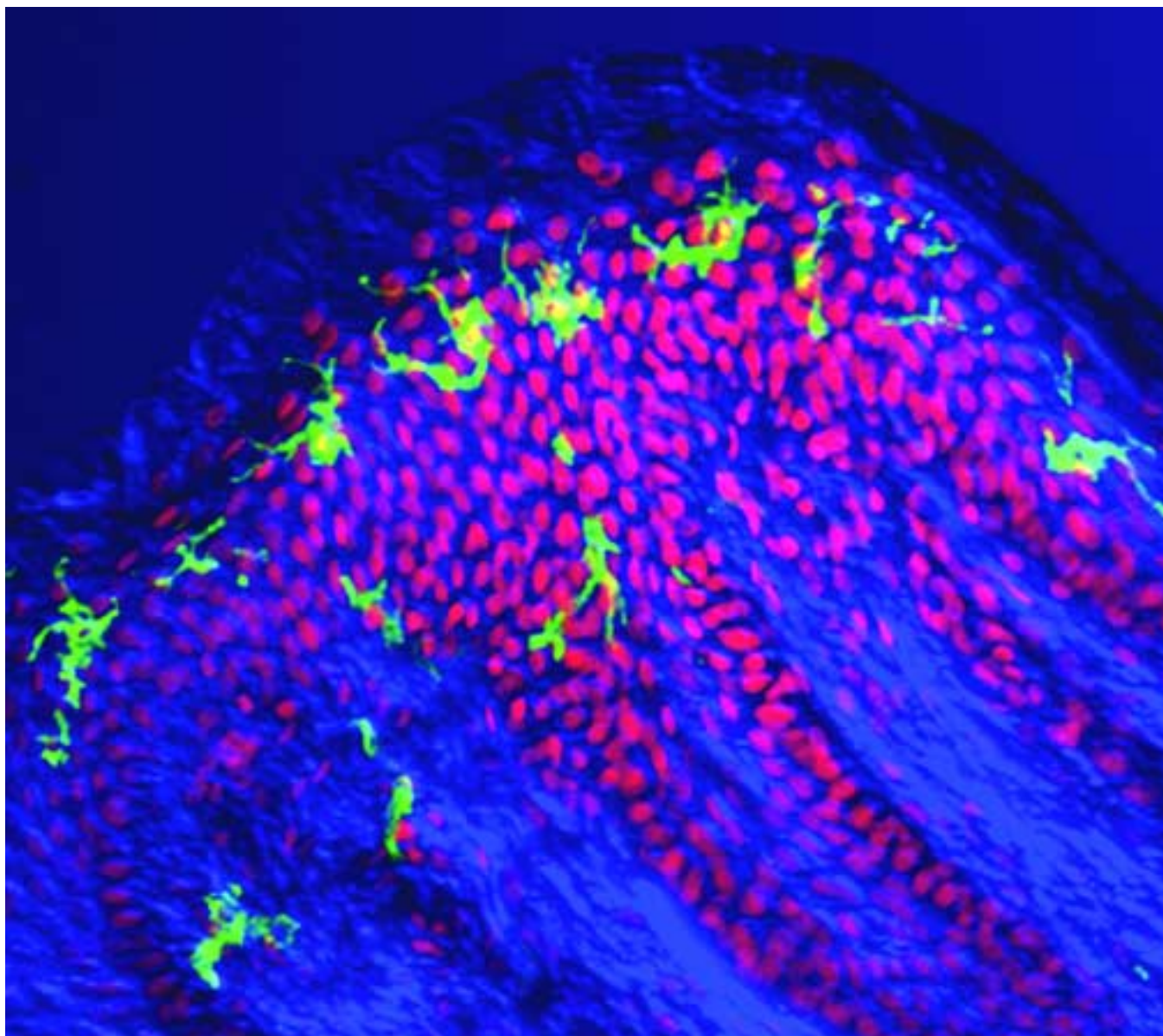


Figure 2
[Click here to download high resolution image](#)

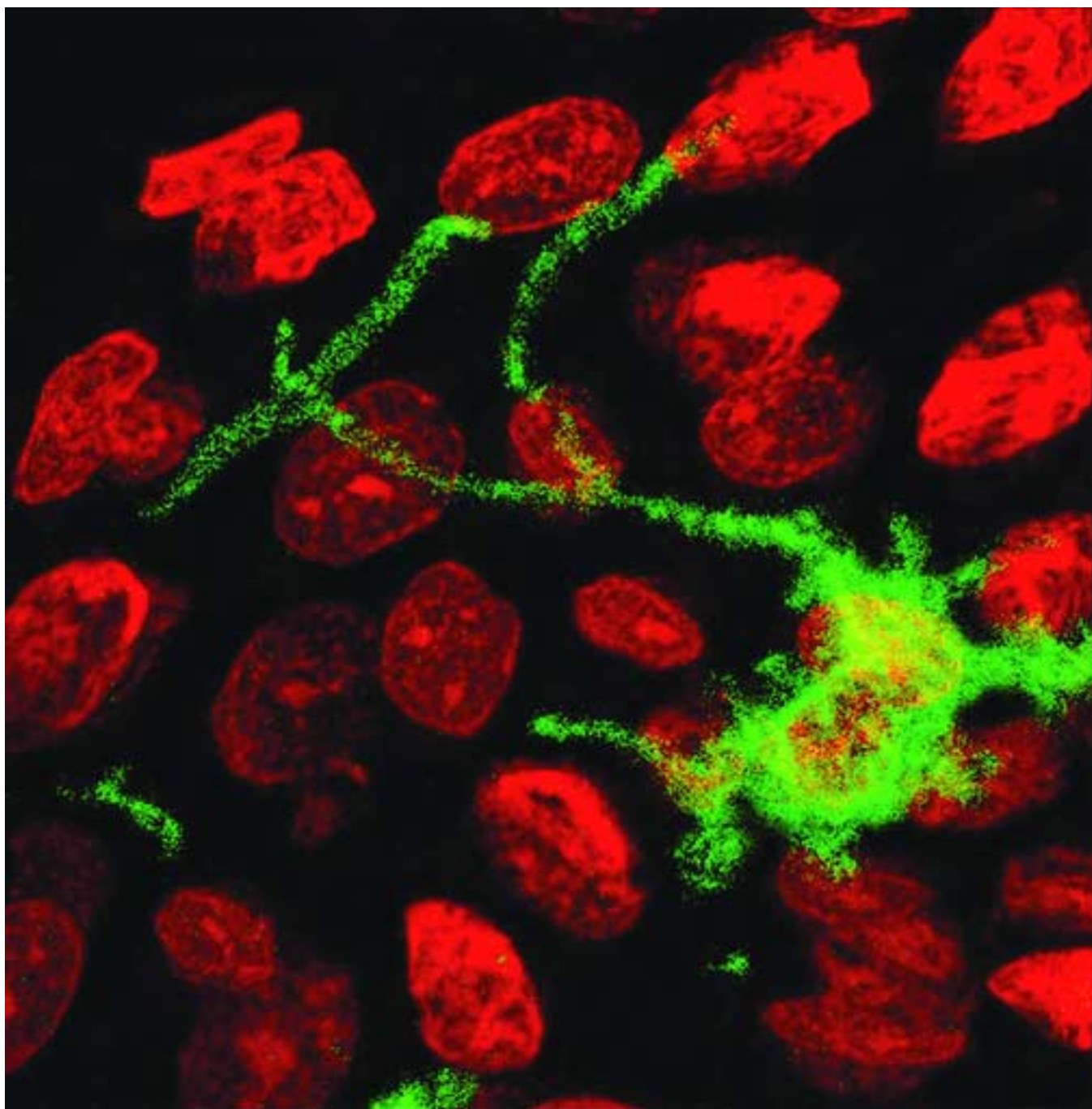


Figure 3

Figure 3a: Flaccid uncircumcised penis

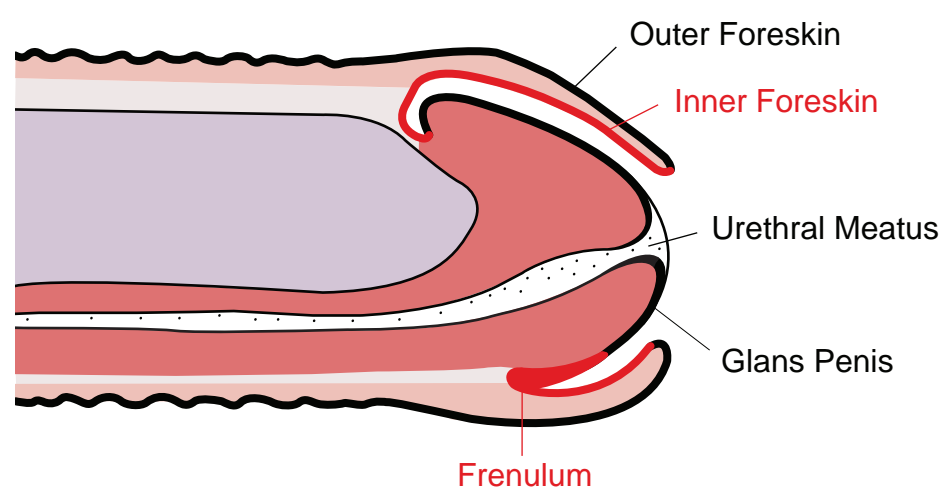


Figure 3b: Erect uncircumcised penis with the foreskin retracted showing likely sites of HIV-1 entry.

