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Chapter 6.2

Distribution of HIV-1 target cells in male and female genital tissue

Lot de Witte¹, Manja Litjens¹, Donna Fluitsma¹, Mark-Bram Bouman², Yvette van Kooyk¹, Michael A.A. van Trotsenburg³ and Teunis B.H. Geijtenbeek¹

Different cell types have been proposed to mediate HIV-1 transmission: Dendritic cell subsets, including epithelial Langerhans cells (LCs) and subepithelial DC-SIGN⁺ DCs, macrophages and CD4⁺ T cells. The aim of this study was to address whether differences between female and male genital tissues and tissues under different conditions influence HIV-1 transmission. We have characterized different genital epithelial tissues for aspects that are thought to be involved in HIV-1 transmission, including factors that might protect against HIV-1 such as thickness of the epithelial layer and density of LCs expressing Langerin; but also factors that potentially mediate HIV-1 transmission, such as the presence of DC-SIGN⁺ DCs, macrophages and CD4⁺ T cells. The mean epithelial/epidermal thickness of the glans, foreskin, cervix and vagina was not significantly different; however testosterone treatment of the females significantly affected thickness and maturation of the vaginal and cervical epithelium. The amount of LCs was significantly lower in females compared to male tissues. However, the expression of Langerin and CD4 on these LCs was not altered. Moreover, we observed a CD4⁺/HLA-DR⁻/CD1a⁻ population in both epithelium and subepithelium that was significantly more abundantly present in female tissues. In conclusion our results suggests that other mechanisms might be involved in female-to-male or male-to-female transmission of HIV-1, and this might affect the rational behind the design of a microbicide.

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(Manuscript in preparation)
Introduction

The most common route of acquiring HIV-1 worldwide is through heterosexual transmission\textsuperscript{25}. Compared to other sexual transmitted viruses, such as human papilloma virus that has an infectivity of 40%\textsuperscript{1}, sexual transmission of HIV-1 transmission is inefficient, estimated at 0.01%-1% per coital act\textsuperscript{9,25,31}. HIV-1 susceptibility is enhanced in conditions that alter the genital tissues, such as trauma, genital ulcerative disease and sex without (endogenous or exogenous) lumbrification\textsuperscript{10,25}. Furthermore, HIV-1 transmission depends on plasma viral loads of the infected partner and the mode of sexual contact (penile-anal or penile vaginal)\textsuperscript{23,25}. Resistance to HIV-1 of persons regularly practicing “unsafe sex” with HIV-positive partners is only partially explained by established factors, such as mutation in chemokine receptors, chemokine upregulation and increased cytotoxic T cell responses\textsuperscript{16,17}. Moreover, HIV-1 transmission is only slightly correlated with the number of coital acts\textsuperscript{4}. Altogether these facts strongly suggest the presence of unknown protective or transmission-mediating factors that vary between tissues, persons and conditions.

The aim of this study was to address whether differences between female and male genital tissues and tissues under different conditions influence HIV-1 transmission. Different cell types have been proposed to mediate HIV-1 transmission: Dendritic cell subsets, including epithelial Langerhans cells (LCs) and subepithelial DC-SIGN\textsuperscript{*} DCs, macrophages and CD4\textsuperscript{+} T cells. Under steady-state conditions, LCs are the only cell type that reside in the epidermis and epithelia. Due to this localization and the fact that LCs can be infected \textit{ex vivo}, these cells are often assumed to be the first target cells for HIV-1\textsuperscript{15}. In conflict with this postulation, we have demonstrated \textit{in vitro} that LCs protect against HIV-1 infection by clearing virus particles via the C-type lectin Langerin. Only high virus concentrations, saturating Langerin, or inhibition of Langerin function lead to infection of LCs (Chapter 5). Due to their localization, LCs are likely to encounter HIV-1 per coital act more often than the HIV-1 infectivity chance of 0.01%-1%. This frequent exposure to HIV-1 and the dependency of HIV-1 transmission on risk factors imply that LCs are not efficiently infected under steady state conditions. We hypothesize that either another cell type is the primary target cell for HIV-1 at the site of entry, or LCs are infected only under specific circumstances, such as with high viral loads or after activation (addressed in Chapter 6.3; 6.4) or by contact with high viral loads. It might therefore be speculated that the density and phenotype of LCs are important factors for the susceptibility to acquire HIV-1.

T cells, macrophages and DC-SIGN\textsuperscript{*} DCs are other possible target cells for HIV-1 in the genital tissue. DC-SIGN\textsuperscript{*} DCs efficiently mediate transmission of HIV-1 via interaction of the virus with the C-type lectin DC-SIGN (Section 2). CD4\textsuperscript{+} T cells and macrophages express the entry receptors for HIV-1, CD4 and CCR5/CXCR4, and are main target cells during clinical infection. DC-SIGN\textsuperscript{*} DCs, T cells and macrophages reside within the subepithelial layer. Therefore, HIV-1 is less likely to encounter these cell types in healthy genital tissues. However, loss of epithelial integrity by microtrauma caused by intercourse, ulcerations caused by sexually transmitted infections (STDs), and influx of these target cells into the epithelium during inflammation, increases the likelihood of these cells to encounter HIV-1.

In this chapter, we have characterized different genital epithelial tissues for aspects that are thought be involved in HIV-1 transmission, including factors that might protect against HIV-1 such as thickness of the epithelial layer and density of LCs expressing Langerin; but also factors that potentially mediate HIV-1 transmission, such as the presence of DC-SIGN\textsuperscript{*} DCs, macrophages and CD4\textsuperscript{+} T cells.
Results

**Subjects.**

Tissue from foreskin and glans penis were collected during male-to-female sex reassignment surgery. Tissue from vagina and cervix uteri were collected either during hysterectomies for benign reasons or during hysterectomies of female-to-male transsexuals after one year cross-sex hormonal treatment. From those women undergoing a hysterectomy for benign reasons four were pre-menopausal and three post-menopausal, none of them had a known history of genital ulcerative disease or HIV-1 infection. To investigate the role of androgens on the female genital tract we included a group of female-to-male transgender patients. These patients had received testosterone, either testosterone esters 250mg twice monthly intramuscularly (Sustanon®) or testosterone gel 5g daily transdermally (Androgel®) according the protocol of the Dutch Center for Genderdysphoria for more than 12 months until two weeks before the operation. This group represents a unique population to study the effect of steroid hormones on the female genital tissues. Due to this medication the genital tract is transformed into a status comparable to senile colpitis of postmenopausal women. However, some patients still show considerable levels of estrogens due to steroid conversion by aromatase.
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Figure 1. Thickness of the different genital epithelia/epidermis. (a-d) Cryosections of different human genital tissues were stained with haematoxylin and eosin. (a) Representative pictures are depicted. (b-d) The epithelial thickness was determined at different places in the section using a grid and the means are depicted. (b) The difference between the thickness of male tissues (glans, foreskin) and female tissues (untreated vagina and cervix) is depicted. (c) The differences between the different collected tissues are depicted (+T indicates testosterone-treated). (d) The difference between the testosterone-treated and non-treated female tissues is depicted. Statistical difference were determined using a student's t-test (b,d) or ANOVA (c); *=p<0,05; ***p=<0,001, n.s.=not significant.

**Thickness of the female genital epithelium from female origin is more variable but is certainly decreased upon testosterone treatment**

The epithelium and epidermis are thought to provide the first line of defence against invading pathogens, including HIV-1. Therefore, we analysed the thickness of the genital epithelium (vagina, ectocervix and foreskin) or epidermis (glans) on haematoxylin and eosin stained sections (Figure 1a). Epithelium and epidermis contained multiple cell layers and showed a stratified organisation, including a basal cell layer, parabasal/intermediate cell layer and superficial cell layers (Figure 1a). In women, the latter is dependent on the degree of oestrogen stimulation. Thickness of the female tissues was more variable than tissue from male origin (Figure 1b,c). As previously described, the epithelium of testosterone-treated cervix and vagina were significantly thinner consisting of only 2 to 6 cell layers and a lack of superficial cells (Figure 1a,d), indicating that testosterone impairs the organisation and maturation of the female genital lining.

**Density of Langerhans cells is lower in female than male genital tissues**

The presence of LCs has been documented in almost all genital tissues including cervix, vagina, glans penis and foreskin. In our study we compared the density of LCs by staining the different tissues with the LC marker CD1a. In male tissues CD1a+ cells were abundantly present (Figure 2a). The LCs were observed throughout the epithelia ranging from below the keratin layer until just above the basal membrane (Figure 2a). In female tissues LCs were present (Figure 2a) but the presence was less and more variable compared to male tissues (Figure 2b). In some female tissues, the presence was variable throughout the tissue, resulting in spots with high concentrations of LCs in between regions that lack LCs (data not shown). Overall, the density of LCs in female tissues, both in the epithelium and subepithelium, was significantly lower than the density in male tissues (Figure 2b). Moreover, the density of LCs varied more between female than male tissues (Figure 2b). Due to low numbers of tissues per group and multiple groups analysed by ANOVA, LC numbers were not significantly lower between the single female and male tissue groups, however the trend is clear (Figure 2c). Strikingly, testosterone treatment resulted in a lack or low density of LCs in female genital tissues (Figure 2d), indicating that androgens affect LC life cycle in the genital tissues.
6.2: Distribution of HIV-1 target cells in male and female genital tissue

A

Foreskin  Glans penis
CD1a

Vagina  Cervix

Testosterone

B

Epithelium

Density LCs (number per mm²)

Male  Female

Subepithelium

Density LCs (number per mm²)

Male  Female

C

Epithelium

Tissue

Density LCs (number per mm²)

Subepithelium

Tissue

D

Epithelium female tissue

Density LCs (number per mm²)

N.S.

Subepithelium female tissue

Density LCs (number per mm²)

N.S.
Figure 2 (previous page). The density of Langerhans cells is less in female genital tissues. (a-d) Cryosections were analysed for expression of the LC-marker CD1a by immunohistochemistry (antibody NA1/34). (a) Sections were analysed by microscopy, and representative pictures are depicted. (b-d) The density of CD1a+ cells in the epithelium and subepithelium was determined by counting the cells using a grid. (b) The difference between the thickness of male tissues (glans, foreskin) and female tissues (untreated vagina and cervix) is depicted. (c) The differences between the different tissues is depicted (+T indicates testosterone-treated). (d) The difference between the testosterone-treated and non-treated female tissues is depicted. Statistical differences were determined using a student’s t-test (b,d) or ANOVA (c); n.s.= not significant; p>0,05; ***p=<0,001.

Langerhans cells in the genital epithelia express Langerin, CD4 and contain Birbeck granules
LCs are thought to play an important role during HIV-1 transmission (Section 5). The C-type lectin Langerin also captures HIV-1 and thereby protect against infection. However, under certain conditions LCs might become infected by entry via CD4 and one of the co-receptors. Therefore, we investigated the expression of CD4 and Langerin on LCs in the different genital tissues using immunofluorescence microscopy. Sections of the different tissues were analysed for the expression of CD1a and co-localisation with Langerin or CD4 was investigated. CD1a+ cells in both the epithelium and subepithelium expressed Langerin (Figure 3a). Quantitative analyses demonstrated that more than 95% of the LCs expressed Langerin (data not shown). Moreover, a large amount of Birbeck granules was detected in both LCs of the glans penis and foreskin (Figure 3b). Next, CD4 expression was examined. Although CD4 was not highly expressed, it was detectable on all CD1a+ cells (Figure 3c). Strikingly, in some tissues a large population of CD4+/CD1a- cells was observed in the epidermis and epithelia, indicating that another subset of cells expressing CD4 is present (Figure 3d). Higher numbers of these CD4+/CD1a- cells were observed in the female tissues (Figure 3e), although due to small groups only the amount in the vagina compared to the foreskin was significant by ANOVA (Figure 3e).

HLA-DR+ cells are present in both the epithelium and subepithelium of all genital tissues
In dermis and subepithelial tissues different target cells, including macrophages and DC-SIGN+ DCs, are present for HIV-1. To investigate these populations tissues were first analysed for the presence of HLA-DR, a marker for antigen presenting cells, including B cells, macrophages and DCs. All tissues have numerous HLA-DR+ cells and the density of these cells in the subepithelium was comparable between the different tissues (Figure 4a,b). To further characterize these antigen-presenting cells we investigated the expression of DC-SIGN. DC-SIGN is expressed on a subset of DCs in the dermis and subepithelium and these cells efficiently mediate HIV-1 transmission5,8. We analysed the density of DC-SIGN+ DCs, and although the amount was variable in the different donors, there was no trend between the different tissues (Figure 4c). We further analysed the expression of CD4. The HLA-DR+ cells express CD4 both in the subepithelium and the epithelium (Figure 4d). We quantified the amount of co-localisation and more than 90% of the HLA-DR+ cells expressed CD4 (data not shown). Strikingly, a population of HLA-DR+/CD4+ cells was observed in the subepithelium and epithelium (Figure 4e, f). The amount of this population varied, and particulary in some female tissues high values were observed in the subepithelium (Figure 4f).
6.2: Distribution of HIV-1 target cells in male and female genital tissue

Figure 3. LCs express CD4 and Langerin.
(a,c-e) Cryosections were stained with antibodies against either Langerin (10E2) or CD4 (RPA-T4) and CD1a (NA1/34) and counterstained with isotype specific-Alexa antibodies (Langerin/CD4 in red; CD1a in green). (a,c) Tissues were analyzed by confocal microscopy. Representative pictures are depicted (b) Tissues were fixed and analysed by electronmicroscopy. * indicates Birbeck granule. (d,e) Nuclei were stained blue with Hoechst and sections were analyzed by fluorescence microscopy. (d) Representative pictures are depicted. (e) The density of CD1a/CD4+ cells in the epithelium and subepithelium was determined by counting the cells using a grid. Statistical differences were determined using a student’s t-test (upper panel) or ANOVA (lower panel); *p=<0.05; ***p=<0.001.
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Figure 4. The amount of HLA-DR^+ cells and DC-SIGN^+ cells is variable but not significantly different between tissues. Cryosections were analysed for expression of the antigen presenting cell marker HLA-DR using immunohistochemistry (antibody Q5/13). (a) Representative pictures are depicted. (b) The density of HLA-DR^+ cells in the subepithelium was determined by counting the cells using a grid (+T indicates testosterone-treated). (c) The sections were stained using antibodies against DC-SIGN (CSRD) and analyzed by immunofluorescence microscopy. The density of DC-SIGN^+ cells in the subepithelium was counted using a grid (+T indicates testosterone-treated). (d-f) Cryosections were stained with antibodies against HLA-DR (Q5/13) and CD4 (RPA-T4) and counterstained with isotype specific-Alexa antibodies (CD4 red; HLA-DR green). (d) Tissues were analyzed by confocal microscopy. Representative pictures are depicted. (e,f) Nuclei were stained blue with Hoechst and sections were analyzed by fluorescence microscopy. (d) Representative pictures are depicted. (e) The density of HLA-DR^+/CD4^+ cells in the epithelium and subepithelium was determined by counting the cells using a grid (+T indicates testosterone-treated).
Discussion

Here, we have characterised the different entry sites for HIV-1 during sexual transmission at a histological level. The mean epithelial/epidermal thickness of the glans, foreskin, cervix and vagina was not significantly different; however, testosterone treatment of the females significantly affected thickness and maturation of the vaginal and cervical epithelium. The amount of LCs was significantly lower in females compared to male tissues. However, the expression of Langerin and CD4 on these LCs was not altered. The amount of antigen presenting cells (APCs, HLA-DR⁺ cells) and DC-SIGN⁺ cells did not vary between the types of tissues. Moreover, we observed a CD4⁺/HLA-DR⁺/CD1a⁻ population in both epithelium and subepithelium that was significantly more abundantly present in female tissues.

LCs are thought to play a dual role during HIV-1 transmission. LCs can be infected ex vivo¹⁴,²⁴ (Chapter 6.3) and in macaques SIV-infected LCs were observed after vaginal infection¹²,¹⁵. Indeed we observed that LCs express CD4 and other studies demonstrated expression of CCR5 and maybe CXCR4²⁸,³². However, we have recently demonstrated that LCs are not susceptible to HIV-1 infection at physiological virus concentrations in vitro and these cells might protect against infection by clearing invading virus particles. HIV-1 capture and degradation are dependent on the interaction of HIV-1 with Langerin³. We here demonstrate that basically all LCs express Langerin and that LCs in foreskin and glans penis contain Birbeck granules, indicating that the LC population in general is protected against HIV-1 by the expression of Langerin. We demonstrate that the amount of LCs is highly decreased in females compared to males. Patterson et al.²⁰ also identified low numbers of LCs in cervical epithelium. Due to the lack of evidence for the in vivo role of LCs at present, low numbers of LCs could have two implications for transmission of HIV-1: women are either less protected against HIV-1 by the clearing function of Langerin or less susceptible to HIV-1 infection by LCs. We hypothesize that LCs are protective until Langerin function is breached or they are activated (further discussed in Chapter 6.3 and 6.4). Epidemiological studies indicated that disruption of the epithelial barrier enhances the susceptibility to acquire HIV-1¹⁰,²⁵. These situations allow HIV-1 to encounter subepithelial target cells. Furthermore, two studies in macaques with intact epithelial barriers demonstrated that subepithelial DCs and T cells were the first cells that were infected by SIV, in contrast to the macaque experiments discussed above²⁷,³³. However, care has to be taken into interpreting these macaque studies, since the use of primates, require an extremely high infectivity chance, to limit the numbers animals to be sacrificed. This high infectivity chance does not reflect the human steady-state situation. To address the role of these subepithelial cells in HIV-1 transmission, we analyzed the density of macrophages and DC-SIGN⁺ DCs. HLA-DR⁺/CD4⁺ cells were abundantly present throughout all tissues and we did not observe difference between the tissues. These could be divided in a population that is negative for DC-SIGN and one that is positive for DC-SIGN. These populations are thought to reflect the macrophage and subepithelial/dermal DCs respectively. However, the DC-SIGN negative cells might also include other subsets of DCs.

Strikingly, we observed a CD4⁺ population in both the epithelia/epidermis and subepithelia/dermis expressing neither CD1a nor HLA-DR. This unidentified population was more abundantly present in female epithelia and subepithelia compared to male genital tissues. The first attempts to address this population demonstrated that these cells express CD3 and CD45RO (data not shown), suggesting that these are memory T cells. The density of this population was higher in female tissues, both in the epithelium and subepithelial. This population might play a crucial role in HIV-1 transmission. Further colocalisation studies need to be performed to address the identity of this population.

Our findings are of interest for the discussion about whether or not transmission rates from male to female are higher than vice versa. Different studies indicated that female-to-male transmission is less
efficient than male-to-female\textsuperscript{4,25}, however recent studies argued these findings\textsuperscript{23,31}. However, it is evident that mechanisms underlying HIV-1 transmission depend on gender, since female-to-male transmission was highly associated with plasma viral load, in contrast to male to female transmission\textsuperscript{7}. We identified two striking differences between female and male genital tissues: female tissues contain a lower density of LCs and a higher density of CD4\textsuperscript{+}/HLA-DR\textsuperscript{-}/CD1\textsuperscript{a-} in the epithelium and subepithelium of their genital tissues. This might (partly) explain why transmission to male is more dependent on viral loads, since the function of LCs can be saturated by high virus concentrations, leading to infection of HIV-1 (Chapter 5.2). Moreover, this indicates that different mechanisms are involved in HIV-1 infection of the different sexes, which might implicate the development of female- and male-specific microbicides. The variety in density of LCs in female tissues might reflect effects in the menstrual cycle, possibly comparable to our observation that androgens influence the thickness of the epithelium. However, Patton et al., did not observe large differences in LCs between the different stages of the cycle\textsuperscript{21}. Oestrogen and progesterone have distinct functions on the thickness of the epithelium, and primate experiments indicate that oestrogen is responsible for vaginal epithelial thickening and maturation, protecting against HIV-1 transmission\textsuperscript{19,26}. Moreover, different reports have demonstrated that long-acting progesterone increases the susceptibility to HIV-1\textsuperscript{2,29} and progesterone-treatment of the vaginal epithelium of macaques resulted in thinner vaginal epithelium and increased transmission of SIV\textsuperscript{27}. As previously demonstrated\textsuperscript{30}, the maturation of the genital epithelium of testosterone-treated women is significantly impaired. The epithelial barrier provides protection against HIV-1, since HIV-1 susceptibility is enhanced in conditions that affect the epithelial tissue\textsuperscript{10,25}.

The UNAIDS report of 2006 estimated that there are currently 38.6 million people infected with HIV-1 worldwide. With approximately 4 million new infections each year, the HIV-1 pandemic is still growing\textsuperscript{13}. Since there is no curative treatment or vaccine in sight, there is a need for topical drugs (microbicides) that prevent HIV-1 transmission\textsuperscript{17}. Therefore the target cells and receptors involved in HIV-1 transmission should be identified. Here we have demonstrated distinct distribution of HIV-1 target cells in the different genital tissues. Compared to male, female tissues contain less LCs and more CD4\textsuperscript{+}/HLA-DR\textsuperscript{-} cells. This suggests that other mechanisms might be involved in female-to-male or male-to-female transmission of HIV-1, and this might affect the rational behind the design of a microbicide.
Materials and methods

Tissue collection. According to the ethical guidelines of the VU medical center, specimens were collected during surgery from tissue surplus before disposal. Tissues were collected in PBS on ice and snap-frozen en bloc in tissue-tek OCT (Miles Inc. Elkhart, IN, USA) and stored in the freezer (-80°C) until further processing. After processing and sectioning, the epithelium/epidermis of the tissues was examined. Three different tissues (including one vagina and two cervix samples) were not further analyzed because the epithelium was not clearly visible or disrupted.

Antibodies. The following antibodies were used:

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<th>Isotype</th>
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<tr>
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<td>1/400</td>
<td>Molecular Probes, Eugene, OR, USA).</td>
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Immunohistochemical analysis. Cryosections (7µ) were air-dried and fixed in 100% acetone for 10 minutes. Next, sections were washed in PBS and incubated overnight with the appropriate primary antibody (10µg/ml). After washing, the final sections were incubated with a rabbit anti-mouse-PO and the peroxidase labelling was visualized by 3,3-diaminobezidine (DAB; Sigma, ST Louis, MO, USA) in 0,05 M Tris pH 7,6 containing 0,03% H2O2 to give a brown-coloured reaction product. Finally, tissue sections were counterstained with haematoxylin. Between all incubation steps, sections were extensively washed with PBS (pH 7.4). All antibodies were diluted in PBS containing 1% bovine serum albumin, which also served as a negative control for primary antibody staining. Tissues were examined with a Nikon Eclipse E800 fluorescence microscope and recordings were made with a digital NIKON DXM1200 camera. In all presented stainings, negative controls were essentially blank. A detailed description of the antibodies used in this article is provided in Table 1.

The density of different types of immune cells was determined by counting the cells in either the epithelium or the side of the subepithelium that lines the epithelium in different fields using a grid. These analyses were done independently by two persons. The values were accepted when they differed less than 20%. The mean of the different counts was calculated. To measure the epithelial thickness, cryosections were washed in PBS and stained with haematoxylin and eosin. The thickness was determined by measuring the epithelial thickness at 10 different places throughout the section using a grid.

Immunofluorescence analysis. Cryosections (7µ) were air-dried and fixed with 100% acetone for 10 minutes, washed in PBS and stained with primary antibody combinations (Table 1; 10µg/ml) for 18 hours at 4°C. Sections were counterstained with isotype-specific Alexa488- or Alexa594-labeled anti-mouse antibodies (Table 1). Nuclei were stained with Hoechst (Molecular Probes, Eugene, OR, USA). After mounting, sections were examined with either a confocal microscope (Leica AOBS SP2 confocal lasers scanning microscope (CSLM) system containing a DMIRE2 microscope with glycerol objective lens (PL APO 63x/NA1.30)) or a fluorescence microscope (Leica DM
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6000 B using LAS AF software version 1.8.0 build 1346). Images were acquired using leica confocal software (version 2.61). The images were used to quantify the amount of co-localization in the different tissues by dividing the amount of double-stained cells by the total amount stained cells. These analyses were done independently by two persons. The values were accepted when they differed less than 10%. The mean of the different counts was calculated.

Electron-microscopy. Tissues were fixed in 2% glutaraldehyde in cacodylate buffer within 10 minutes after surgical removal. Post-fixation treatment was performed via incubation with 1% osmium tetroxide at 4°C for 1 hour. After dehydration, tissues were impregnated and embedded in a mixture of epon/araldite. Polymerization was performed at 60°C. Ultrathin sections (80 nm) were cut with an ultramicrotome, stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (model Philips CM100 Bio Twin).

Statistical analysis. The values derived from the different donors from two groups were analyzed using a two-paired student’s t-test assuming equal variances. Comparing more than one group, a one-way analysis of variance (ANOVA) was used. When the overall F test was significant, differences among the donors were further investigated with the post hoc Bonferroni test using Graphpad Prism software. In both tests a probability of $p < 0.05$ was considered statistically significant.

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