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Rapid Dissemination of SIV Follows Multisite Entry after Rectal Inoculation

Patricia Ribeiro dos Santos[1☯¤a], Magali Rancez[1☯¤b], Jean-Luc Prétet[1¤c], Alice Michel-Salzat[1], Valérie Messent[1¤d], Anna Bogdanova[1¤e], Anne Couëdel-Courteille[1¤b], Evelyne Souil[2¤f], Rémi Cheynier[3], Cécile Butor[1]*

1 Laboratoire de Transmission et Dissémination Virales, Université Paris Diderot, Sorbonne Paris Cité, Paris, France, **2** Plateforme de Morpho-Histologie, Institut Cochin, INSERM U1016, CNRS URA8104, Université Paris Descartes UMR-S1016, Paris, France, **3** Département d'Immunologie-Hématologie, Institut Cochin, INSERM U1016, CNRS URA8104, Université Paris Descartes UMR-S1016, Paris, France

Abstract

Receptive ano-rectal intercourse is a major cause of HIV infection in men having sex with men and in heterosexuals. Current knowledge of the mechanisms of entry and dissemination during HIV rectal transmission is scarce and does not allow the development of preventive strategies. We investigated the early steps of rectal infection in rhesus macaques inoculated with the pathogenic isolate SIVmac251 and necropsied four hours to nine days later. All macaques were positive for SIV. Control macaques inoculated with heat-inactivated virus were consistently negative for SIV. SIV DNA was detected in the rectum as early as four hours post infection by nested PCR for *gag* in many laser-microdissected samples of lymphoid aggregates and lamina propria but never in follicle-associated epithelium. Scarce SIV antigen positive cells were observed by immunohistofluorescence in the rectum, among intraepithelial and lamina propria cells as well as in clusters in lymphoid aggregates, four hours post infection and onwards. These cells were T cells and non-T cells that were not epithelial cells, $CD68^+$ macrophages, DC-$SIGN^+$ cells or $fascin^+$ dendritic cells. DC-$SIGN^+$ cells carried infectious virus. Detection of *Env* singly spliced mRNA in the mucosa by nested RT-PCR indicated ongoing viral replication. Strikingly, four hours post infection colic lymph nodes were also infected in all macaques as either SIV DNA or infectious virus was recovered. Rapid SIV entry and dissemination is consistent with trans-epithelial transport. Virions appear to cross the follicle-associated epithelium, and also the digestive epithelium. Viral replication could however be more efficient in lymphoid aggregates. The initial sequence of events differs from both vaginal and oral infections, which implies that prevention strategies for rectal transmission will have to be specific. Microbicides will need to protect both digestive and follicle-associated epithelia. Vaccines will need to induce immunity in lymph nodes as well as in the rectum.

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* E-mail: butor@univ-paris-diderot.fr

☯ These authors contributed equally to this work.

¤a Current address: Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal
¤b Current address: Département d'Immunologie-Hématologie, Institut Cochin, INSERM U1016, CNRS URA8104, Université Paris Descartes UMR-S1016, Paris, France
¤c Current address: EA3181, IFR133, Université Franche-Comte, and CHU Besancon, Besancon, France
¤d Current address: Laboratoire de Physiopathologie des Maladies du Système Nerveux Central INSERM UMRS 952/CNRS UMR 7224, Université Pierre et Marie Curie, Paris, France
¤e Current address: DRPH/SRBE/GSEA, Institut de Radioprotection et de Sûreté Nucléaire, Saclay, France
¤f Current address: DIP, Université Paris Descartes, Paris, France

Introduction

Receptive rectal intercourse with an HIV^+ individual carries a per act risk of transmission of 0.5% to 1.7% [1–3]. This is three to five-fold higher than receptive vaginal intercourse [3,4]. In 2009 Men having Sex with Men (MSM) represented 59.5% of all new HIV infections in the USA [5], and 39.8% in Canada [6]. In males, MSM represent 78.4% of newly acquired infections in the USA [5], and 59.5% in Canada. MSM are the population group with the highest risk of acquiring HIV worldwide, including developing countries [7]. Rectal intercourse practiced in heterosexual relationships [2,8–10] increases several fold the male to female transmission risk [11–13].

Additionally, an increase in severity of disease for MSM with respect to intravenous transmission has been noted in most but not all cohorts [14–31]. This phenomenon could be due to the

prevalence of specific opportunistic pathogens in that population [31], such as HHV-8 [29,30]. Alternatively, entry by the rectal route could modify the initial immune response and worsen the clinical course of HIV infection [14].

HIV can cross the rectal epithelium by several mechanisms: open lesion with direct access to blood leukocytes [32,33], productive infection of epithelial cells [34–37], transcytosis by epithelial cells [38,39] and/or M cells [40,41], opening of tight junctions [42–44], and capture by intraepithelial dendritic cells (DC) [45–48]. Transport of the virus out of the mucosa to lymph nodes can be due to free virions in the lymph, infected cells or virions bound to DCs [49], the C-type lectin DC-SIGN being then a prime candidate for binding the virus [50–52].

For ethical reasons, in vivo data on viral entry and dissemination can only be obtained in animal models. Experimental infection of macaques with SIV currently represents the best animal model for HIV infection [53,54]. Earlier work on rectal infection in rhesus and pigtail macaques showed that viral dissemination is rapid [55–59], proceeds in five stages with wide animal to animal variation in terms of kinetics [55,56] and differs from intravenous entry [55,56]. To our knowledge, no information was gained on the pathway followed by the virus for rectal entry. A good understanding of the mode of entry and of dissemination is important for the development of preventive strategies, whether chemical or vaccine based [44,60]. We decided to address this question in the rhesus macaque (*Macaca mulatta*) following rectal infection with the pathogenic isolate SIVmac251. We used free virions as inoculum. Current data does suggest that during sexual HIV transmission the source virus more likely originates from free viral particles than from infected cells in seminal plasma [61,62].

We show here that SIV is present in lymphoid aggregates as well as in the lamina propria of the rectum at least four hours post infection (pi). Replication appears to be initiated mostly in lymphoid aggregates. SIV disseminates away from the mucosa in less than four hours after rectal infection. The first target cells of SIV include T cells, but virus is also found associated with DC-SIGN$^+$ cells. Presence at both sites (lymphoid aggregates and lamina propria) suggests entry via digestive epithelium as well as via follicle-associated epithelium. Prevention strategies will therefore have to cover these two sites.

Results

Experimental design

Rectal infections were performed with one hundred rectal Animal Infectious Dose 50 ($rAID_{50}$). This corresponds to 7.78 log copies of viral RNA, for a final concentration of 7.31 log copies/ml of viral RNA. This is commensurate with the highest viral loads reported in human semen of 7 to 8.5 log copies/ml of viral RNA [63–70]. Macaques were necropsied four hours (R-H4.1 and R-H4.2), sixteen hours (R-H16.1, R-H16.2 and R-H16.3), twenty-four hours (R-H24.1 and R-H24.2), two, three and four days (R-D2.1, R-D2.2, R-D3.1, and R-D4.1) post infection and viral distribution was analyzed by nested PCR, nested RT-PCR, immunohistochemistry and co-culture with indicator cells. Plasma viral load was below detection for R-H16.1, R-H24.1, R-H24.2, R-D2.1, R-D2.2, R-D3.1 and R-D4.1. It was not measured for R-H4.1, R-H4.2, R-H16.2 and R-H16.3 as it was assumed to be below detection considering the early time point at which the macaques were necropsied.

The macaque rectum is 7 cm long, 6 cm circumference [71]. Most often the entire rectum was prepared for morphology (with alternate segments embedded in paraffin and frozen for cryosectionning). For some macaques alternate segments (representing one half of the rectum) were chemically and enzymatically disrupted to obtain cell suspensions enriched for epithelial cells (R-H4.1, R-H24.1, R-D2.2, R-D4.1) or for lamina propria cells (R-H16.3).

Peripheral blood mononuclear cells (PBMC) were purified by density centrifugation.

Individual colon-draining mesenteric lymph nodes were either processed for morphology (some embedded in paraffin and others frozen for cryosectionning) or were mechanically disrupted to obtain a suspension of mononuclear cells. Axillary lymph nodes were usually separated in two, which were processed for morphology or mechanically disrupted to yield mononuclear cells.

Three macaques were sacrificed five (R-D5.1), seven (R-D7.1) and nine (R-D9.1) days pi. This allowed us to determine that the 100 $rAID_{50}$ dose leads to viral dissemination pattern similar to those previously observed with ten $rAID_{50}$ (Text S1, Figure S1).

Controls included macaques infected with heat-inactivated virus and sacrificed four (M-H4.1), sixteen (M-H16.1) and twenty-four (M-H24.1) hours post inoculation, as well as two healthy rhesus macaques. Control rhesus macaques gave negative results in all assays for SIV.

SIV enters intraepithelial cells but is not amplified in rectal epithelia

Rare SIV antigen positive (SIV$^+$) intraepithelial cells were observed by immunohistofluorescence (IHF) four, sixteen and twenty-four hours pi (Figure 1, Text S1, Table S1 and Figure S2A). Some of these cells were confirmed to be T cells (Figures 1A–D). In contrast, IHF did not show SIV$^+$ epithelial cells at any time point. The presence of SIV$^+$ intraepithelial cells indicates that the virus could have crossed the digestive epithelium in less than four hours.

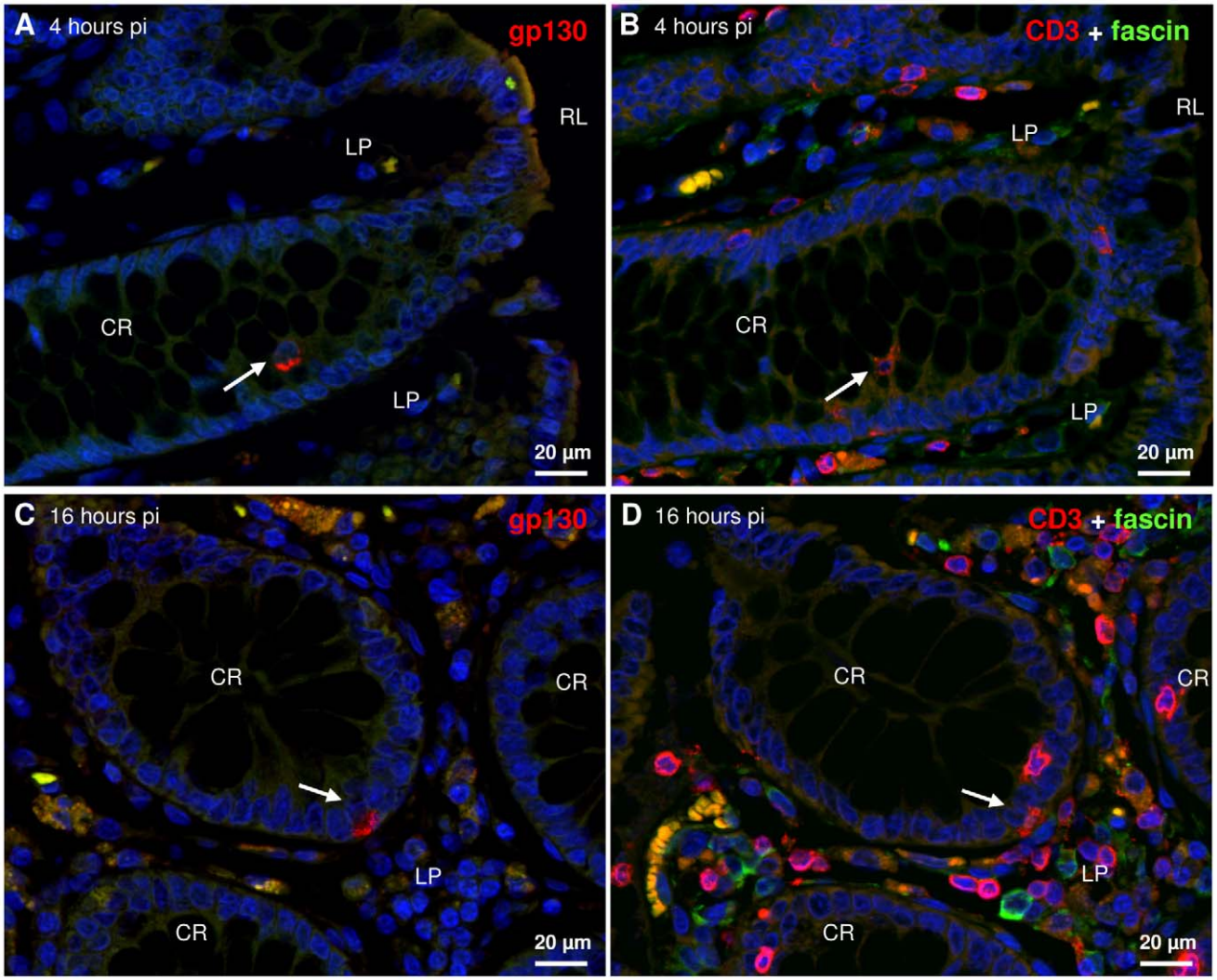
We never found SIV$^+$ cells by IHF in the follicle-associated epithelium. We also never found viral DNA amplified by PCR in laser microdissected follicle-associated epithelium (microdissection described in Text S1 and Figures S3A and S3B).

The epithelial cell fraction contains cells from the digestive epithelium, with less than 0.1‰ of cells coming from the follicle-associated epithelium [72]. Very little, if any, cell-associated virus was found in cells isolated from the rectal epithelium (Figure 2E), indicating that there was no viral amplification in cells of the epithelium. Cell-associated virus could correspond to infected intraepithelial T cells.

SIV reaches mucosal lymphoid aggregates as early as four hours post infection

Four hours pi and onwards, we detected viral DNA in microdissected lymphoid aggregates in all macaques (Figure 2A, example of microdissected area in Figures S3C and S3D). Over 60% of lymphoid aggregates were positive for SIV DNA at four and sixteen hours pi (Figure 2A). Moreover, at least one third of these positive aggregates contained more than one copy of SIV DNA (with up to 20/20 PCR positive for SIV DNA, Table S2). The number of copies of SIV DNA in microdissected samples was below the detection limit of real-time PCR, preventing more accurate quantification. The majority of lymphoid aggregates remained positive for SIV at later time points (Figure 2A). In addition, lymphoid aggregates could contain SIV DNA in several serial sections suggesting local foci of infection (Table S2). The SIV DNA load of lymphoid aggregates appeared stable from four to forty-eight hours pi (Text S1, Figure S4A).

The presence of virus in lymphoid aggregates was confirmed by IHF detection of capsid (p27) and envelope (gp130) proteins. Rare clusters of adjoining cells showed labeling for SIV proteins in



E

Animals	R-H4.1	R-H24.1	R-D2.2	R-D4.1
Epithelial fraction	6	0	+	0

Figure 1. SIV is present in the rectal epithelium as early as four hours post infection. Serial paraffin sections of R-H4.1 (sacrificed four hours pi; A and B) and R-H16.2 (sacrificed sixteen hours pi; C and D) were labeled by IHF for gp130 (Alexa-546, A and C), CD3 (TRITC, B and D) or fascin (Alexa-488, B and D) and nuclei were stained with DAPI (A–D). CR, crypt; LP, lamina propria; RL, rectal lumen. Arrows on serial sections point to intraepithelial T lymphocytes positive for SIV antigens. Cell-associated virus in the epithelial cell fraction was assayed by co-culture of cells isolated from the rectum. Results are expressed as $TCID_{50}$ per million cells. The viral load does not increase over the first four days of infection; + the $TCID_{50}$ could not be calculated due to small number of wells positive for SIV antigen (E).
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infected macaques four hours to two days pi (Figures 2C and 2E–H, Table S1, Figure S2B), the latest time point examined. The labeling was cytoplasmic, and appeared more punctate four hours pi (Figure 2C, dashed circle) than at later time points (Figures 2E–H). A control antibody of the same isotype as the antibodies to p27 and gp130 failed to bind on serial sections, demonstrating that binding is specific (Figures 2I–L). The number of SIV^+ cell clusters in the lymphoid aggregates appeared to increase slightly at days one and two pi (Text S1, Table S1 and Figure S2B).

SIV^+ cells were phenotyped. Both $CD3^+$ and $CD3^-$ cells were found positive for SIV antigens in the clusters (Figures 2B, 2D, 2F and 2H). SIV^+ $CD3^-$ cells were negative for fascin, a marker for mature DCs (Figure 2B) and for a macrophage-specific epitope of CD68 (Figure 2D). Early targets of SIV include therefore T cells as well as unidentified cells which do not appear to be mature DCs or macrophages.

The presence in lymphoid aggregates of viral DNA and cells positive for SIV antigens as early as four hours pi is a strong indication that entry can occur through follicle-associated epithelium. The slight increase over time suggests local viral replication.

SIV also reaches the lamina propria four hours post infection

The lamina propria was infected as early as four hours pi as shown by detection of viral DNA (Figures 3A and S4B) in laser-microdissected samples (Figures S3E and S3F). Lamina propria

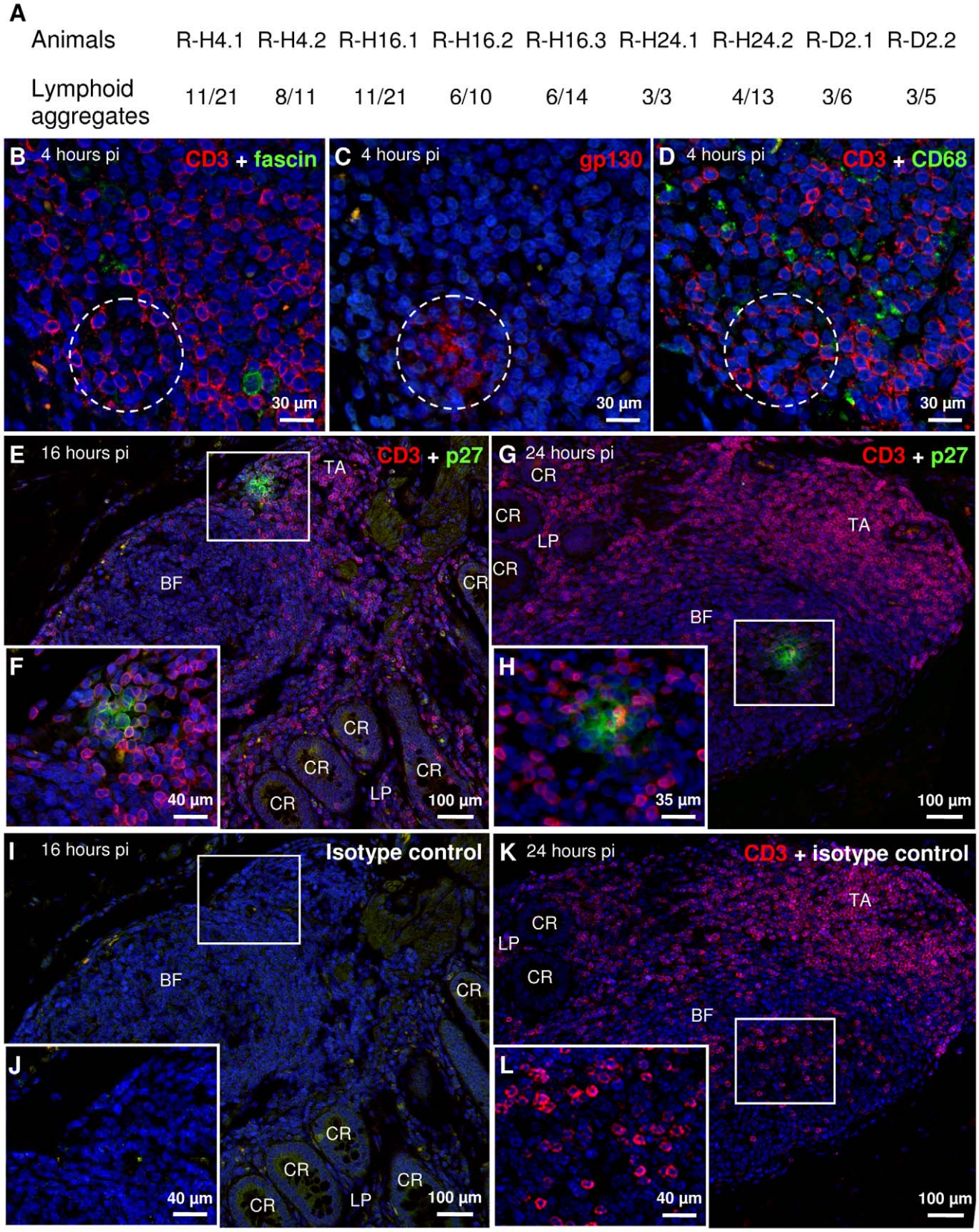

Figure 2. SIV is present in lymphoid aggregates of the colo-rectal mucosa starting from four hours post infection. SIV DNA is amplified by nested PCR for *gag* in most lymphoid aggregates microdissected from paraffin sections of macaques sacrificed four hours to two days pi (A); ratios indicate the number of lymphoid aggregates positive for SIV DNA relative to the total number of lymphoid aggregates tested for each macaque. Clusters of SIV-antigen positive cells are observed in paraffin embedded sections of the colo-rectal mucosa of R-H4.1 (sacrificed four hours pi; B, C and

D, serial sections, dashed circle indicates the same area in the three micrographs), R-H16.1 (sacrificed sixteen hours pi; E, F enlargement of area boxed in E), and R-H24.1 (sacrificed twenty-four hours pi; G, H enlargement of area boxed in G). These clusters of SIV^+ cells are not observed in serial sections of R-H16.1 (I, J enlargement of area boxed in I) or R-H24.1 (K, L enlargement of area boxed in K) labeled with an irrelevant antibody of the same isotype. These clusters contain T cells but not $CD68^+$ macrophages or $fascin^+$ DCs. Sections were labeled by IHF for gp130 (Alexa-546, C), p27 (Alexa-488, E, F, G and H), CD3 (TRITC, B, D–H, K and L), fascin (Alexa-488, B), CD68 (Alexa-488, D), irrelevant antibody (Alexa-488 I–L) and nuclei were stained with DAPI (B–L). CR, crypt; LP, lamina propria; TA, T cell area of mucosal lymphoid aggregate; BF, B cell follicle.
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samples were positive for SIV in 40% of individual microdissected sample in the first day pi (Figure 3A). In contrast with lymphoid aggregates, the ratio of positive PCRs never exceeded 3/10 in the lamina propria (data not shown).

Infection of lamina propria was confirmed by IHF. Individual SIV^+ cells were occasionally detected four hours to two days pi the latest time point examined (Figures 3B and 3C). Of note, about half of these cells were confirmed to be T cells (Figures 3B and 3C). The others were not T cells ($CD3^-$), DCs ($fascin^-$) or macrophages (negative for a macrophage-specific epitope of CD68) (Figures 3B and 3C and data not shown). The number of SIV antigen positive lamina propria cells appeared to decrease over time (Figure S2C).

The presence of virus in the lamina propria could indicate that the virus crossed the basement membrane in addition to the digestive epithelium in less than four hours.

SIV mRNA production proceeds at a low level

We assayed for the presence of SIV *Env* singly spliced mRNA in tissues of all macaques sacrificed between four hours and three days pi (except R-H24.2). As RT-PCR could not be performed on microdissected samples, the entire mucosa was used for detection of spliced mRNA. It is therefore not possible in this assay to discriminate between lymphoid aggregates and lamina propria. Singly spliced mRNA was evidenced by nested RT-PCR in the rectal mucosa of R-H4.1, R-H16.2 and R-D3.1 (Table 1). It was never detected in control macaques. Detection of SIV *Env* singly spliced mRNA in the tissues of infected macaques shows that transcription and splicing proceed during the first days of rectal SIV infection.

Heat-inactivated virus does not lead to detection of SIV in the mucosa

Free viral DNA contained in viral stocks [73] could lead to false positive PCR results at early time points. In order to ascertain that the viral DNA detected in the mucosa of SIV inoculated macaques was due to infection, we inoculated three macaques with a quantity of heat-inactivated virus corresponding to 100 $rAID_{50}$. We necropsied them four, sixteen and twenty-four hours after inoculation (macaques M-H4.1, M-H16.1 and M-H24.1 respectively), and analyzed the rectal mucosa. No SIV DNA was ever found in those samples despite performing over 220 individual PCR for M-H4.1, 250 for M-H16.1 and 280 for M-H24.1 at various dilutions to rule out PCR inhibition in the samples. This indicates that free viral DNA cannot enter the rectal mucosa. Therefore, the viral DNA observed four hours pi in the infected macaques is indeed due to ongoing viral replication in the rectal mucosa.

A

Animals	R-H4.1	R-H4.2	R-H16.1	R-H16.2	R-H16.3	R-H24.1	R-H24.2
Lamina propria	2/5	1/5	4/4	1/3	0/7	3/3	2/5

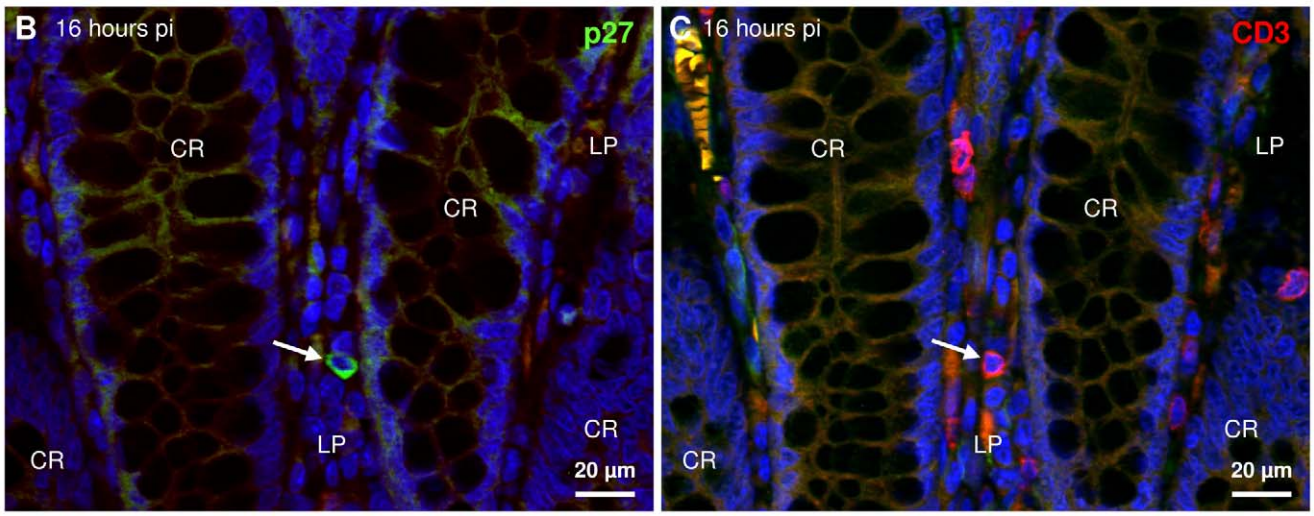

Figure 3. SIV is present in the colo-rectal lamina propria as early as four hours post infection. SIV DNA is amplified by nested PCR for *gag* in some lamina propria samples microdissected from paraffin sections four to twenty four hours pi (A); ratios indicate the number of lamina propria sites positive for SIV DNA relative to the total number of lamina propria sites tested for each macaque. Serial paraffin sections of R-H16.3 (sacrificed sixteen hours pi; B and C) were labeled for p27 (Alexa-488, B) and CD3 (TRITC, C) and nuclei were stained with DAPI (B and C). CR, crypt; LP, lamina propria. Arrows on serial sections point to a lamina propria T lymphocyte positive for SIV antigen.
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Table 1. *Env* singly spliced mRNA is detected earlier in the colo-rectal mucosa than in lymph nodes.

Macaque	Colorectal mucosa	Colic lymph nodes	Axillary lymph nodes
R-H4.1	+	−	−
R-H4.2	−	−	−
R-H16.1	−	−	−
R-H16.2	+	−	−
R-H16.3	−	−	−
R-H24.1	−	−	−
R-D2.1	−	−	−
R-D2.2	−	−	−
R-D3.1	+	−	−

The singly spliced mRNA for the *Env* gene was detected by nested RT-PCR in the indicated tissues.
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IHF for SIV antigens performed on macaques inoculated with heat-inactivated virus did not show SIV^+ cells in the rectum of these macaques (four to six different segments were extensively sampled for each macaque). This indicates that the SIV^+ cells observed in infected macaques correspond to live virions internalized by cells or to infected cells.

We also did not find evidence for the presence of spliced mRNA by nested RT-PCR in the macaques inoculated with heat-inactivated virus, indicating that the detection of singly spliced viral mRNA in infected macaques requires exposure to live virus.

Mucosal DC-SIGN$^+$ cells interact in vivo with SIV

As DC-SIGN can bind SIV, we localized and phenotyped DC-SIGN-expressing cells by IHF early in infection. DC-$SIGN^+$ cells were observed in lymphoid aggregates (Figure 4A) and in the lamina propria (Figures 4B–D), but we never could detect DC-$SIGN^+$ intraepithelial cells. Almost all DC-$SIGN^+$ cells co-expressed a macrophage-specific epitope of CD68, indicating that they are macrophages (Figure 4D). Most DC-$SIGN^+$ cells also co-express PM-2K, marking them as mature macrophages (Figure 4C). A few DC-$SIGN^+$ cells co-expressing MHC class II could carry out antigen presentation (Figures 4A and 4B).

We tested interaction of SIV with DC-$SIGN^+$ cells on the rectal collagenase fraction of macaque R-H16.3 (Figure 5). The enriched DC-$SIGN^+$ cell fraction (Figure 5B) carried a 20-fold higher viral load (Figure 5D) than the DC-$SIGN^-$ cells (Figures 5C and 5D) from the same macaque. Nevertheless, we never observed by IHF cells positive for both SIV and DC-SIGN.

SIV rapidly disseminates from the mucosa to the lymph nodes

Viral DNA was detected as early as four hours pi in colic lymph nodes but not prior to two days pi in axillary lymph nodes (Table 2). Cell-associated virus was found in colic lymph nodes in more than half of the macaques, and as early as four hours pi (Table 3). In contrast it was only transiently detected twenty-four hours pi in axillary lymph nodes (Table 3). Viral transcription was below detection in colic and axillary lymph nodes (Table 1).

Viral DNA was however detected as early as twenty-four hours pi in PBMC (Table 2). Cell-associated virus was also found in peripheral blood as early as four hours pi, albeit at a lower level (Table 3).

These data suggest that SIV disseminates in draining lymph nodes at four hours pi and to axillary lymph nodes at later time points.

Discussion

Despite the importance of rectal infection in the AIDS epidemic, current knowledge regarding HIV rectal entry is limited to in vitro work, which does not reproduce fully the complexity and specificity of the rectal milieu. An understanding of HIV mucosal entry would be of considerable help in the design of microbicides and vaccines. We addressed the question of rectal entry in the rhesus macaque infected by SIVmac251, the animal model closest to the human infection by HIV [53,54]. In order to increase our chances to detect virus during early acute infection, we chose a viral dose ten times higher than in our previous work [55,56]. This dose is in the low range of the doses used for studies of vaginal transmission [74–78]. It is commensurate with the highest viral loads reported in the semen of HIV^+ men [63–70]. This one hundred $rAID_{50}$ dose does not affect the dissemination profile.

SIV entry is massive. If we consider our sampling to be representative of the entire rectum, one can estimate the viral DNA in the rectum of each macaque four hours pi to be on the order of 55,000 copies in lymphoid aggregates and on the order of 290,000 in the lamina propria (see Text S1 for details). As we could not find SIV DNA in mock-infected macaques, this suggests that approximately 3×10^5 viral particles were able to cross the rectal epithelia and undergo reverse transcription, representing close to 1% of input viral particles. Some of these copies must be integrated as spliced mRNA could be found in the rectum as early as four hours pi. The much lower number of cells positive for SIV antigens (on the order of 10^3–10^4 per macaque, Figure S2) suggests that most copies of SIV DNA correspond to defective particles undergoing abortive infection. Cells positive for SIV antigens can be productively infected or have merely internalized viral antigens. As, in vivo, the average life cycle of SIV is under 10 hours [79], it is likely that the cells positive for SIV antigens observed sixteen hours pi and onwards are infected cells in which translation proceeds. The fraction of these cells which will go on to produce virus is currently not possible to estimate.

SIV entry is a very rapid process, as infection is established in the lamina propria, in lymphoid aggregates and in more distal sites at least at four hours pi, the earliest time point assessed. Viral entry after vaginal inoculation was shown, by in situ acidic inactivation, to be complete in less than 30 minutes [77], with reverse transcription completed as early as two hours pi [78]. The reverse transcriptase inhibitor tenofovir applied rectally two hours after rectal infection protected only one third of inoculated macaques [80], indicating that reverse transcription can be completed in less than two hours. This is faster than expected from in vitro work. However, one should note that gut lymphocytes are inflammatory but hyporesponsive [81], and that this may accelerate the viral cycle. It was not possible to narrow down further the time necessary for virus to be imported from the inoculum as, in our hands, acidic inactivation was toxic by the rectal route.

SIV entry could involve trans-epithelial transport of SIV. Early reports of HIV in rectal epithelial cells of HIV^+ patients [82–84] have not been confirmed. Many now accept that infected epithelial cells are not found in vivo [44,85]. In our previous work we had not observed infected rectocytes [56]. In the present work we could never observe infected epithelial cells either by IHF or by ISH. We did find virus at distal sites as early as four hours pi. Trans-epithelial transport is a likely explanation for this rapid

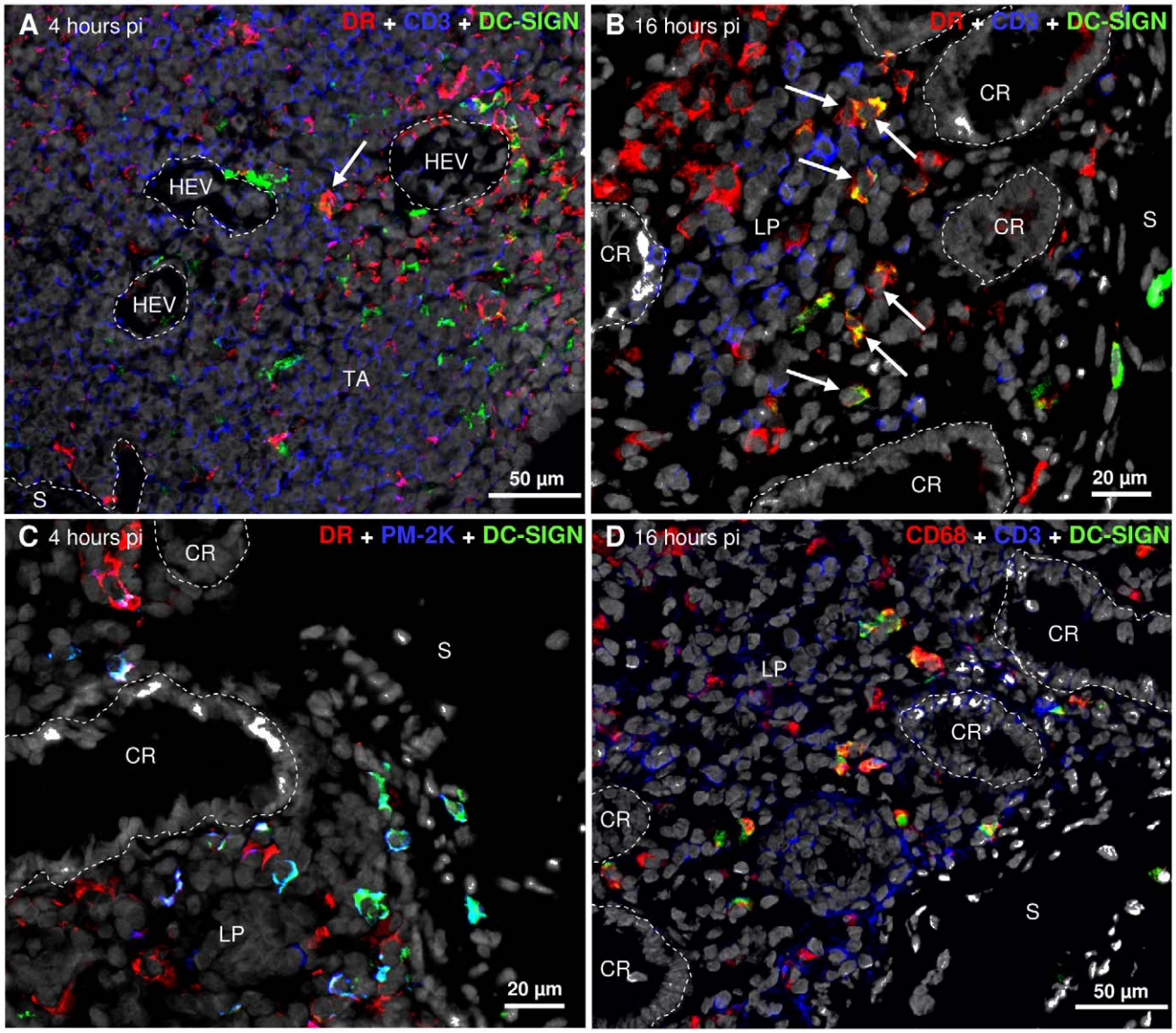

Figure 4. DC-SIGN$^+$ cells are macrophages in the colo-rectal mucosa during the first sixteen hours of infection. DC-SIGN$^+$ cells are observed in lymphoid aggregates of R-H4.2 (sacrificed four hours pi; A) and lamina propria of R-H4.1 (sacrificed four hours pi; C) and R-H16.3 (sacrificed sixteen hours pi; B and D). DC-SIGN$^+$ cells express only occasionally MHC-II molecules (A and B, arrows), but co-express PM-2K (C) and CD68 (D) marking them as macrophages. Frozen sections of colo-rectal mucosa were labeled for DC-SIGN (Alexa-488), CD3 (Alexa-350, A, B and D), MHC-II Mamula-DR (Alexa-546, A, B and C) or PM-2K (Alexa-350, C), CD68 (Alexa-546, D) and nuclei were stained with TO-PRO®-3 (A–D). The computer-generated merged images are shown. CR, crypt; LP, lamina propria; S, submucosal connective tissue; TA, T cell area of mucosal lymphoid aggregate; HEV, high endothelial venule.
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dissemination, as viral production in situ is unlikely over such a short period of time. Indeed, the estimated mean intracellular phase of the life cycle for HIV in vivo is 14.4 to 21.6 hours [86]. It is shorter for SIV, with a mean life cycle of 9.4 hours [79]. The trans-epithelial transport of SIV does not appear to involve capture by DC processes extending into the rectal lumen through tight junctions. Indeed, we never observed intraepithelial DCs at early time points of infection using classical DC markers (DC-SIGN, fascin). The presence of SIV in both lymphoid aggregates and digestive mucosa four hours pi argues in favor of transcytosis of virions by both the digestive epithelium and the follicle-associated epithelium. One should note that entry appears more efficient across the follicle-associated epithelium. However the surface of the digestive epithelium is much larger than that of the follicle-associated epithelium, and the amount of virus entering through this route could be greater.

SIV structural proteins are found intracellularly by IHF in intraepithelial cells (including T cells), in lamina propria cells (T cells and non-T cells), and in T cell-containing clusters in lymphoid aggregates. The non-T cells are neither macrophages nor mature DCs as they do not express CD68, DC-SIGN or fascin. The SIV antigen positive clusters probably correspond to the infection of a single cell by virus from the inoculum. Secondary diffusion of SIV antigen to neighboring cells could occur by infection (a possibility for macaques infected sixteen hours and onwards) or by cell-cell fusion. In favor of the former is the fact that lymphoid aggregates often contain several copies of SIV DNA (Table S2). SIV$^+$ cells are scarce and the detection of *Env* singly

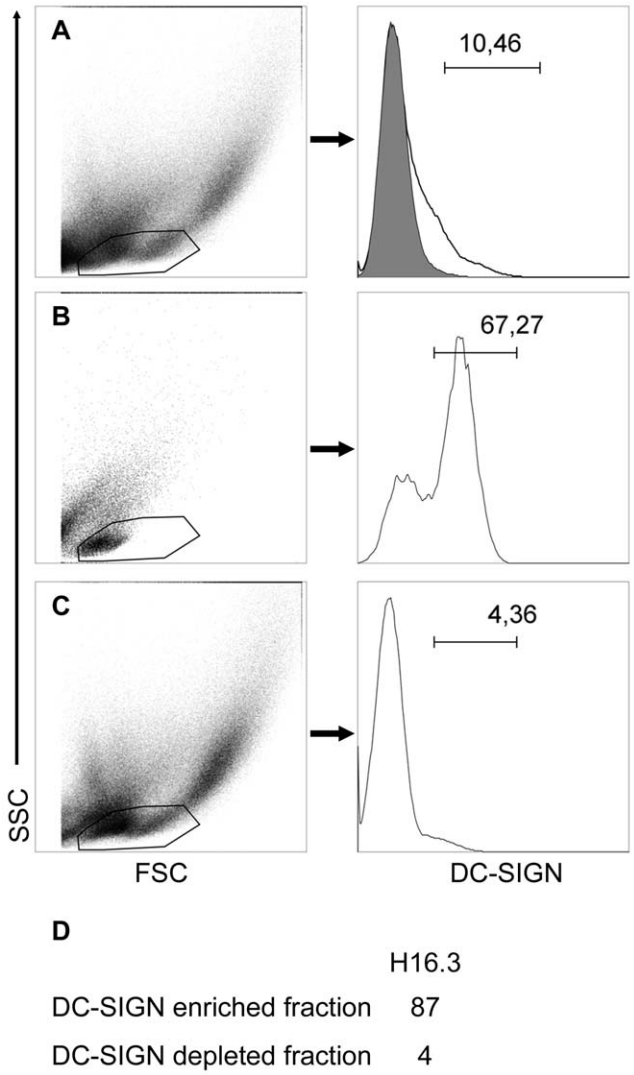

Figure 5. Rectal DC-SIGN$^+$ cells can bind SIV sixteen hours post infection. Flow cytometry analysis of cells from the rectal collagenase fraction of R-H16.3 (sacrificed sixteen hours pi) shows enrichment of DC-SIGN$^+$ cells from total cells (A) in the DC-SIGN$^+$ fraction (B) and depletion in the DC-SIGN$^-$ fraction (C); left dot plots show side scatter (SSC) versus forward scatter (FSC) and gate in which expression of DC-SIGN is analyzed; right histograms show expression of DC-SIGN and percentage of DC-SIGN$^+$ cells (bar on histogram); gray filled histogram in A corresponds to isotype control. Cell-associated viral load (expressed as $TCID_{50}$ per million cells; D) shows that over twenty-fold more cell-associated virus is found in the DC-SIGN enriched cell fraction than in the DC-SIGN depleted cell fraction (D).
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spliced mRNA is rare. These observations suggest that SIV replication is initiated in very few cells of the rectal mucosa at early stages of infection. We have no evidence for viral production in the digestive part of the mucosa. In contrast, the presence of SIV$^+$ cell clusters suggests that viral production occurs in lymphoid aggregates.

During the first day of infection at least, rectal DC-SIGN$^+$ cells have an overall distribution pattern similar to healthy human and macaque rectum [52,87]. They appear less abundant in the lamina propria, but more abundant in lymphoid aggregates than Jameson et al. observed in healthy rhesus macaque mucosa [87]. They all express macrophage markers, as was described in healthy human rectum [52]. In contrast to healthy mucosa [52,87], in early SIV infection rectal DC-SIGN$^+$ cells do not all express MHC class II. DC-SIGN$^+$ cells bind infectious virions, as infectious virus is enriched in the DC-SIGN$^+$ cell fraction. The number of virions bound by each cell is presumably small as these cells are not positive by IHF.

SIV dissemination is rapid as cell-associated virus could be recovered from draining lymph nodes and from the peripheral blood as early as four hours pi. This indicates that virus-carrying cells have left the mucosa to percolate through the colic lymph node chain and reach the circulation in less than four hours. The very early presence of viral DNA in draining lymph nodes strongly suggests that infected cells are one means of viral dissemination from the mucosa. Virus could also travel as cell-bound virions. This is one explanation of the discrepancy observed between co-culture results and nested PCR results, the other one being a

Table 2. Viral DNA is detected in colic lymph nodes prior to peripheral blood and axillary lymph nodes.

Macaque	Colic lymph nodes	Axillary lymph nodes	PBMC
R-H4.1	−	−	−
R-H4.2	+	−	−
R-H16.1	−	−	−
R-H16.2	+	−	−
R-H16.3	+	−	−
R-H24.1	+	−	+
R-H24.2	−	−	+
R-D2.1	+*	+*	+
R-D2.2	+	−	+
R-D3.1	+	+*	−
R-D4.1	+	+§	+

The presence of viral DNA was assayed on cell suspensions, on OCT-frozen samples (*) or on paraffin sections (§). +: viral DNA detected; −: no viral DNA detected. PBMC: peripheral blood mononuclear cells.
doi:10.1371/journal.pone.0019493.t002

Table 3. Cell-associated virus is detected rapidly in lymph nodes and peripheral blood.

Macaque	Colic lymph nodes	Axillary lymph nodes	PBMC
R-H4.1	2	−	−
R-H4.2	1	−	1
R-H16.1	4	−	2
R-H16.2	*	−	−
R-H16.3	−	−	*
R-H24.1	−	*	−
R-H24.2	13	*	−
R-D2.1	−	−	−
R-D2.2	9	−	*
R-D3.1	−	−	1
R-D4.1	−	−	−

Results are expressed as $TCID_{50}$ per million cells.
*at least one well was positive for SIV antigen, but the $TCID_{50}$ could not be calculated due to small number of SIV antigen positive wells. − no SIV antigen positive well in the experiment. PBMC: peripheral blood mononuclear cells.
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stochastic effect at very low viral loads. Finally, one cannot exclude transport as free virions in the lymph draining the rectum. To determine whether this occurs, one would have to cannulate lymph from the colon under prolonged anesthesia. This not only raises technical and ethical concerns, but could also modify exchanges between lymphoid tissues.

Rectal infection appears to involve rapid entry and reverse transcription, as has been noted for the vaginal [77,78] and oral routes [88] but there are clear differences with these entry pathways. Dissemination after rectal entry is possibly more rapid than after oral entry, where SIV DNA is found in many lymphoid tissues twenty-four hours pi [88]. It is also more rapid than following vaginal entry, where SIV DNA could be found at very low-levels in draining lymph nodes eighteen hours pi and in some lymphoid tissues twenty-four hours pi [77]. We found that a high proportion of virus crosses the epithelium upon rectal inoculation. This is similar to oral infection [88], but contrasts with vaginal entry where only a very small proportion of the total inoculum enters the vaginal mucosa [78]. We show that T cells, but not fascin positive DCs or macrophages, are among the first targets of SIV during rectal transmission. This is not the case during vaginal transmission where fascin positive DCs are the first cells associated with SIV [77] or during oral infection where macrophages are infected [88].

The rapid kinetics of rectal entry and dissemination has important consequences for the development of preventive strategies. Rhesus macaques can be protected from rectal infection by tenofovir applied locally prior to inoculation [80]. Protection is not observed if the plasma concentration of tenofovir is below 75 ng/ml. This is in contrast with what is observed for vaginal infection, where protection is observed with plasma concentrations of tenofovir as low as 11 ng/ml [89]. This suggests that protection from rectal infection (and possibly not from vaginal infection) requires inhibition of reverse transcription in distal sites such as lymph nodes and not exclusively in the rectum. One cannot exclude that routine use of reverse transcriptase inhibitors with such plasma concentrations will lead to side effects and present risks of selection for resistant reverse transcriptase mutants. Therefore, there is a use for microbicide preparations for rectal use aiming to prevent virion interaction with the epithelium (digestive as well as follicle-associated) and entry into cells. This would complement (or replace) preparations aiming to block reverse transcription.

In conclusion, we show that SIV crosses in less than four hours both the digestive epithelium and the follicle-associated epithelium. We propose that entry occurs by transcytosis at both sites, with rectocytes and M cells being the most likely candidates to carry out transcytosis of virions. Following entry, SIV infects T cells as well as non-T cells in the mucosa. SIV initiates replication locally in the rectal lymphoid aggregates, and to a lower extent in the lamina propria. Virus is rapidly transported to distal sites as infected cells, as virions associated with cells possibly expressing DC-SIGN or as free virions present in the lymph. Reverse transcription occurs in the rectal mucosa during the first hours of infection. Reverse transcription may also occur in draining lymph nodes. To be effective against rectal transmission of HIV, a vaccine will have to induce immunity at the rectal surface, but also in distal lymphoid sites.

Methods

Macaques and tissue collection

Macaques were housed at the L3 animal facility of the Pasteur institute (France) in accordance with the European Community guidelines (Journal Officiel des Communautés Européennes, L358, December 18, 1986). C. Butor was granted for this protocol the authorization to experiment on live non-human primates number 006322 by the Ministère de l'Agriculture et de la Pêche in 1994, then the authorization to experiment on live non-human primates number 78-76 by the Préfecture des Yvelines in 2005. A. Couëdel-Courteille was granted the authorization to experiment on live non-human primates number 007304 by the Ministère de l'Agriculture et de la Pêche in 1997, then the authorization to experiment on live non-human primates number 75-1068 in 2005. Several steps were taken to improve animal welfare according to the recommendations of the Weatherall report. Animals were housed in individual cages to prevent viral transmission, but up to twenty animals were housed in a single room allowing sight and sound contact with each other. Diet was supplemented with a variety of fresh fruit. All manipulations of animals were performed under ketamine anesthesia, according to regulations in France. Macaques were sacrificed by a lethal dose of pentothal under ketamine anesthesia. In addition, the tissues obtained after necropsies are currently used for another study in order to reduce the total number of animals used. Nineteen adult male rhesus macaques (*Macaca mulatta*) were used in this study. Fourteen macaques were inoculated atraumatically by the rectal route with 100 $rAID_{50}$ [90] and otherwise as previously described [55,56]. This corresponds to 18,000 $TCID_{50}$ and 7.78 log copies of viral RNA [91] for a final concentration of 7.31 log copies/ml of viral RNA. The SIVmac251 viral stock originally obtained from R Desrosiers was a kind gift of A-M Aubertin. Macaques were sacrificed four hours (animals R-H4.1 and R-H4.2), sixteen hours (R-H16.1, R-H16.2 and R-H16.3), twenty-four hours (R-H24.1, R-H24.2), two days (R-D2.1, R-D2.2) and three, four, five, seven and nine days (R-D3.1, R-D4.1, R-D5.1, R-D7.1 and R-D9.1) pi. Three animals were inoculated with an identical volume of the viral stock after heat inactivation (56°C for 30 min) and euthanized four, sixteen or twenty-four hours post inoculation (M-H4.1, M-H16.1 and M-H24.1 respectively). Two healthy rhesus macaques were used as controls. Peripheral blood was collected on heparin or EDTA prior to euthanasia. Lymph nodes, colon and rectum were collected separately under sterile conditions and processed as previously described [56], omitting the Percoll gradient on the epithelial and collagenase fractions.

Titration of cell-associated virus

Titrations were performed as previously described [55,56]. For macaques R-H4.1 R-H4.2, R-H16.2 and R-H16.3 we used the commercial kit ⟨⟨Innotest® HIV Antigen P24⟩⟩ (Innogenetics, Gent, Belgium) which cross-reacts with the SIV p27 capsid protein instead of our homemade ELISA. The $TCID_{50}$ was calculated according to Reed and Muench [92].

In situ hybridization

ISH was performed as previously described [56] using either INT-BCIP or NBT-BCIP as substrate for alkaline phosphatase.

Immunohistofluorescence

Five µm-thick formaldehyde-fixed paraffin-embedded sections collected on glass slides were deparaffinized and rehydrated. Antigen retrieval was performed by pressure cooking the sections for 10 min in 0.01 M buffered sodium citrate solution (pH 6). Sections were then rinsed with calcium free Dulbecco's phosphate buffered saline (PBS). Seven µm-thick cryosections were fixed for 10 min in cold acetone and rinsed in PBS.

Both types of sections were incubated for 30 min with blocking buffer (2% normal goat serum and 5% bovine serum albumin in PBS), incubated for 60 min with primary antibodies, washed in

PBS 0.5% Tween20 (Sigma, St. Louis, MO), incubated with secondary antibodies for 30 min, washed, counterstained with 4,6-diamidino-2-phenylindol (DAPI) (Molecular Probes, Cergy Pontoise, France, paraffin sections) or TO-PRO®-3 iodide (Invitrogen, Cergy Pontoise, France, cryosections) and mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL).

Primary antibodies were polyclonal rabbit anti-CD3 (DAKO, Trappes, France) mouse monoclonal antibodies to fascin (clone 55K-2, IgG1, DAKO), SIV gp130 envelope protein (clone KK46, IgG1, obtained from the NIH), SIV p27 capsid protein (IgG1, Advanced Biotechnologies Inc Columbia, MD), *Aspergillus niger* glucose oxidase (isotype control, IgG1, clone DAK-GO1, DAKO), HLA-DR (clone TÜ36, IgG2b, BD Biosciences), DC-SIGN (clone 120612, IgG2a, R&D Systems, Lille, France), DC-SIGN (clone 120507, IgG2b, R&D Systems), CD68 (clone KP1, IgG1, DAKO, Trappes, France), and tissue macrophage (clone PM-2K, IgG1, AbD Serotec, Düsseldorf, Germany). Secondary antibodies were TRITC-conjugated goat anti-rabbit antibody (Southern Biotechnology) and isotype-specific (IgG1, IgG2a and IgG2b) goat anti-mouse secondary antibodies (Molecular Probes) conjugated to Alexa 350, Alexa 488 or Alexa 546.

Image capture and analysis

IHF sections were examined under an inverted epifluorescence microscope Axiovert 200 M (Zeiss), equipped with a HBO flexible fluorescence lamp, a black and white CCD camera (Roper Scientific Coolsnap HQ) and coupled to video imaging using the Axiovision 4.4 software (Zeiss). ISH sections were examined under a DMRB (Leica) with a DC300F camera (digital module R, Leica) and the IM1000 software (Leica). Images were digitally acquired with a 20× or a 40× objective, then we used both the Axiovision software and the Photoshop software (Adobe Systems Incorporated) to analyze the different stainings.

Laser capture microdissection of rectal mucosa

Microdissection was performed on an automated system for diode ultraviolet laser cutting and infrared laser capture of tissue samples, mounted on a Nikon Eclipse TE2000 inverted microscope equipped with a color CCD camera and coupled to video imaging. Formaldehyde-fixed paraffin-embedded 5-µm-thick sections were mounted on a polyethylene foil slide (SL Microtest GmbH, Jena, Germany) and counterstained with hematoxyline. Sections were observed on a screen using a 10× or a 20× objective. An incision path was drawn on the screen and multiple overlapping laser pulses dissected the selected tissue area. The target tissue was removed from the slide with isolation caps. The efficiency of the laser capture microdissection was assessed by examining the tissue harvested under the microscope. DNA was extracted by adapting the DNeasy blood and tissue kit protocol from QIAGEN (Courtaboeuf, France) for small DNA amounts.

Detection of SIV viral DNA

DNA was extracted from 5×10^6 cells in suspension using the DNeasy blood and tissue kit (QIAGEN) and from paraffin sections using the DNeasy tissue kit (QIAGEN) according to the manufacturer's instructions. To assess the quality of the extracted DNA (presence of cellular DNA, lack of PCR inhibitor), we performed a PCR for actin using sense (5′ GGG TCA GAA GGA TTC CTA TG 3′) and antisense (5′ GGT CTC AAA CAT GAT CTG GG 3′) actin primers (Genset, Paris, France). To assess the presence of viral DNA, we used either a semi-nested PCR protocol as previously described [55,93] or a single PCR protocol with primers F-GAG-Ni-5′-CCG TCA GGA TCA GAT ATT GCA and R-GAG-Ci-5′-TTC GTA CCC AGC CCC TTC AGC in 2.5 mM $MgCl_2$. Ten to twenty individual PCRs were performed on mucosa samples. Negative PBMC and lymph node samples were tested at varying dilutions to rule out potential inhibition of the PCR due to suboptimal ratios between the DNA and the primers in the sample. The detection threshold was one to two copies as assessed with serial dilution of gag containing plasmids in different volumes of DNA extracts obtained from PBMC or from microdissected tissues of uninfected macaques.

Purification of DC-SIGN-expressing cells from the rectum

DC-SIGN-expressing cells were enriched using anti-phycoerythrin (PE) magnetic beads (Miltenyi Biotec) according to manufacturer's recommendations. Briefly lamina propria rectal cells were stained with an anti-DC-SIGN-PE antibody (clone 120507, R&D Systems), washed in MACS buffer (Miltenyi Biotec), incubated with anti-PE beads, then separated in a LS column (Miltenyi Biotec). The column was washed with MACS buffer and DC-SIGN-expressing cells were eluted. Both positive and negative fractions were washed, analyzed by flow cytometry and used to quantify cell-associated virus. Flow cytometry was performed on a FACSCalibur or a FACSCanto (BD Biosciences) and analyzed with FlowJo 8.8.6 software (TreeStar).

Detection of *Env* singly spliced mRNA

Messenger RNA was extracted with the MicroPoly(A) Purist kit (AMBION, Courtaboeuf, France) according to manufacturer's instructions from samples of colorectal mucosa, colic and axillary lymph nodes (1 mm^3) frozen in OCT. To assess the presence of *Env* singly spliced mRNA, we used a nested RT-PCR protocol. After a reverse transcription step, cDNAs were first PCR amplified with primers LTR-SIV-SD-5′-CGA CGG AGT GCT CCT ATA AA (located before the splice donor in the long terminal repeat region in 5′) and V1V2-Out3-5′-GAA GAG ACC ACC ACC TTA GAA (located before the Rev response element). A second PCR was performed on the initial PCR products with primers V1V2-In5-5′-AGG ATG TAT GGC AAC TCT TTG A and V1V2-In3-5′-CAC AAG ACT CTT GGA TAA CAG AA. All PCRs were performed in 3.5 mM $MgCl_2$ with 10 minutes initial denaturation at 95°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 5 minutes at 72°C.

Supporting Information

Figure S1 SIV dissemination after high dose rectal infection reaches colon draining lymph nodes prior to axillary lymph nodes. Cell-associated virus in tissues expressed as $TCID_{50}$ per million cells shows more than one log difference between draining lymph nodes and other lymphoid tissues; + the $TCID_{50}$ could not be calculated due to small number of wells positive for SIV antigen (A). SIV DNA amplified by nested PCR for *gag* is always found in draining lymph nodes, but not in other lymphoid tissues of R-D9.1: + viral DNA amplified, − no viral DNA amplified (B). Infected cells are detected by *in situ* hybridization for SIV in colic lymph node of R-D5.1 (C), rectal mucosa of R-D7.1 (D) and central mesenteric lymph node of R-D9.1 (E). C, INT-BCIP substrate, no counterstain; D and E NBT-BCIP substrate, eosin counterstain. Arrows point to infected cells in lymph nodes (C and E) and in the T cell area of mucosal lymphoid aggregates (D) and arrowheads to infected cells in the lamina propria (D). CR, crypt; LP, lamina propria; S, submucosal

connective tissue; TA, T cell area of mucosal lymphoid aggregate; GC, germinal center; FM, follicular mantle; BF, B cell follicle; PC, parafollicular cortex.
(TIF)

Figure S2 SIV-antigen positive elements during the first two days of infection. SIV-antigen positive intraepithelial cells (A), clusters in lymphoid aggregates (B) or lamina propria cells (C) were counted on sections labeled by IHF. Total values were computed per macaque.
(TIF)

Figure S3 Laser capture microdissection allows separate sampling of follicle-associated epithelium, lymphoid aggregates and lamina propria. Figure shows paraffin sections counterstained with hematoxylin and micrographed with an Eclipse TE2000 inverted microscope before (A, C, E) and after (B, D, F) laser capture microdissection. The red dashed line corresponds to the laser pattern. The area microdissected in A and B was follicle associated epithelium (FAE), in C and D lymphoid aggregate (LA) and in E and F lamina propria (LP).
(TIF)

Figure S4 Number of copies of SIV DNA during the first two days of infection. The presence of SIV DNA was measured by semi-quantitative PCR on microdissected lymphoid aggregates (A) and lamina propria (B). See text for details of calculations.
(TIF)

Table S1 Number of SIV antigen positive cells or cell clusters in colo-rectal segments during the first two days post infection.
(DOC)

Table S2 PCR analysis of SIV DNA on serial sections of colo-rectal lymphoid aggregates.
(DOC)

Text S1
(DOC)

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Author Contributions

Conceived and designed the experiments: PRdS MR J-LP AM-S VM AB AC-C ES RC CB. Performed the experiments: PRdS MR J-LP AM-S VM AB AC-C ES CB. Analyzed the data: PRdS MR J-LP AM-S VM AB AC-C RC CB. Wrote the paper: PRdS MR J-LP AM-S RC CB.

References

1. Varghese B, Maher JE, Peterman TA, Branson BM, Steketee RW (2002) Reducing the risk of sexual HIV transmission: quantifying the per-act risk for HIV on the basis of choice of partner, sex act, and condom use. Sex Transm Dis 29: 38–43.
2. Baggaley RF, White RG, Boily MC (2010) HIV transmission risk through anal intercourse: systematic review, meta-analysis and implications for HIV prevention. Int J Epidemiol 39: 1048–1063.
3. Boily MC, Baggaley RF, Wang L, Masse B, White RG, et al. (2009) Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. Lancet Infect Dis 9: 118–129.
4. Wawer MJ, Gray RH, Sewankambo NK, Serwadda D, Li X, et al. (2005) Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. J Infect Dis 191: 1403–1409.
5. Centers for Disease Control and Prevention (2011) *HIV Surveillance Report, 2009*; vol. 21. http://www.cdc.gov/hiv/topics/surveillance/resources/reports/. Published February 2011. Accessed 2011 April 11th.
6. Public Health Agency of Canada (2010) *HIV and AIDS in Canada. Surveillance Report to December 31, 2009*. Surveillance and Risk Assessment Division, Centre for Communicable Diseases and Infection Control, Public Health Agency of Canada.
7. UNAIDS Report on the global AIDS epidemic 2010 (2010) UNAIDS publisher, Geneva, Switzerland.
8. Leichliter JS (2008) Heterosexual anal sex: part of an expanding sexual repertoire? Sex Transm Dis 35: 910–911.
9. Maynard E, Carballo-Dieguez A, Ventuneac A, Exner T, Mayer K (2009) Women's experiences with anal sex: motivations and implications for STD prevention. Perspect Sex Reprod Health 41: 142–149.
10. Exner TM, Correale J, Carballo-Dieguez A, Salomon L, Morrow KM, et al. (2008) Women's anal sex practices: implications for formulation and promotion of a rectal microbicide. AIDS Educ Prev 20: 148–159.
11. European Study Group on Heterosexual Transmission of HIV (1992) Comparison of female to male and male to female transmission of HIV in 563 stable couples. BMJ 304: 809–813.
12. Seidlin M, Vogler M, Lee E, Lee YS, Dubin N (1993) Heterosexual transmission of HIV in a cohort of couples in New York City. AIDS 7: 1247–1254.
13. Leynaert B, Downs AM, de Vincenzi I (1998) Heterosexual transmission of human immunodeficiency virus: variability of infectivity throughout the course of infection. European Study Group on Heterosexual Transmission of HIV. Am J Epidemiol 148: 88–96.
14. Pehrson P, Lindback S, Lidman C, Gaines H, Giesecke J (1997) Longer survival after HIV infection for injecting drug users than for homosexual men: implications for immunology. AIDS 11: 1007–1012.
15. Touloumi G, Pantazis N, Babiker AG, Walker SA, Katsarou O, et al. (2004) Differences in HIV RNA levels before the initiation of antiretroviral therapy among 1864 individuals with known HIV-1 seroconversion dates. AIDS 18: 1697–1705.
16. Prins M, Veugelers PJ (1997) Comparison of progression and non-progression in injecting drug users and homosexual men with documented dates of HIV-1 seroconversion. European Seroconverter Study and the Tricontinental Seroconverter Study. AIDS 11: 621–631.
17. Prins M, Sabin CA, Lee CA, Devereux H, Coutinho RA (2000) Pre-AIDS mortality and its association with HIV disease progression in haemophilic men, injecting drug users and homosexual men. AIDS 14: 1829–1837.
18. Lyles CM, Graham NM, Astemborski J, Vlahov D, Margolick JB, et al. (1999) Cell-associated infectious HIV-1 viral load as a predictor of clinical progression and survival among HIV-1 infected injection drug users and homosexual men. Eur J Epidemiol 15: 99–108.
19. Eskild A, Magnus P, Sohlberg C, Jensen F, Kittelsen P (1994) A comparison of the progression rate to acquired immunodeficiency syndrome between intravenous drug users and homosexual men. Scand J Soc Med 22: 309–314.
20. Eskild A, Magnus P, Brekke T, Bruun JN, Heger B, et al. (1997) The impact of exposure group on the progression rate to acquired immunodeficiency syndrome. A comparison between intravenous drug users, homosexual men and heterosexually infected subjects. Scand J Infect Dis 29: 103–109.
21. Brettle RP, McNeil AJ, Gore SM, Bird AG, Leen CS, et al. (1995) The Edinburgh City Hospital cohort: analysis of enrollment, progression and mortality by baseline covariates. QJM 88: 479–491.
22. Alioum A, Leroy V, Commenges D, Dabis F, Salamon R (1998) Effect of gender, age, transmission category, and antiretroviral therapy on the progression of human immunodeficiency virus infection using multistate Markov models. Groupe d'Epidemiologie Clinique du SIDA en Aquitaine. Epidemiology 9: 605–612.
23. Rezza G (1998) Determinants of progression to AIDS in HIV-infected individuals: an update from the Italian Seroconversion Study. J Acquir Immune Defic Syndr Hum Retrovirol 17 Suppl 1: S13–16.
24. Wolfs TF, de Wolf F, Breederveld C, Sjamsjoedin-Visser LJ, Roos M, et al. (1989) Low AIDS attack rate among Dutch haemophiliacs compared to homosexual men: a correlate of HIV antigenaemia frequencies. Vox Sang 57: 127–132.
25. Multicohort Analysis Project Workshop (1994) Immunologic markers of AIDS progression: consistency across five HIV-infected cohorts. Multicohort Analysis Project Workshop. Part I AIDS 8: 911–921.
26. Galai N, Vlahov D, Margolick JB, Chen K, Graham NM, et al. (1995) Changes in markers of disease progression in HIV-1 seroconverters: a comparison between cohorts of injecting drug users and homosexual men. J Acquir Immune Defic Syndr Hum Retrovirol 8: 66–74.
27. Operskalski EA, Stram DO, Lee H, Zhou Y, Donegan E, et al. (1995) Human immunodeficiency virus type 1 infection: relationship of risk group and age to rate of progression to AIDS. Transfusion Safety Study Group. J Infect Dis 172: 648–655.
28. Pezzotti P, Galai N, Vlahov D, Rezza G, Lyles CM, et al. (1999) Direct comparison of time to AIDS and infectious disease death between HIV

seroconverter injection drug users in Italy and the United States: results from the ALIVE and ISS studies. AIDS Link to Intravenous Experiences. Italian Seroconversion Study. J Acquir Immune Defic Syndr Hum Retrovirol 20: 275–282.
29. Spijkerman IJ, Langendam MW, Veugelers PJ, van Ameijden EJ, Keet IP, et al. (1996) Differences in progression to AIDS between injection drug users and homosexual men with documented dates of seroconversion. Epidemiology 7: 571–577.
30. Vella S, Giuliano M, Floridia M, Chiesi A, Tomino C, et al. (1995) Effect of sex, age and transmission category on the progression to AIDS and survival of zidovudine-treated symptomatic patients. AIDS 9: 51–56.
31. Biggar RJ (1990) AIDS incubation in 1891 HIV seroconverters from different exposure groups. International Registry of Seroconverters. AIDS 4: 1059–1066.
32. Coates RA, Calzavara LM, Read SE, Fanning MM, Shepherd FA, et al. (1988) Risk factors for HIV infection in male sexual contacts of men with AIDS or an AIDS-related condition. Am J Epidemiol 128: 729–739.
33. Coplan PM, Gortmaker S, Hernandez-Avila M, Spiegelman D, Uribe-Zuniga P, et al. (1996) Human immunodeficiency virus infection in Mexico City. Rectal bleeding and anal warts as risk factors among men reporting sex with men. Am J Epidemiol 144: 817–827.
34. Adachi A, Koenig S, Gendelman HE, Daugherty D, Gattoni-Celli S, et al. (1987) Productive, persistent infection of human colorectal cell lines with human immunodeficiency virus. J Virol 61: 209–213.
35. Fantini J, Cook DG, Nathanson N, Spitalnik SL, Gonzalez-Scarano F (1993) Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus is associated with cell surface expression of galactosylceramide, a potential alternative gp120 receptor. Proc Natl Acad Sci U S A 90: 2700–2704.
36. Moyer MP, Gendelman HE (1991) HIV replication and persistence in human gastrointestinal cells cultured in vitro. J Leukoc Biol 49: 499–504.
37. Chenine AL, Matouskova E, Sanchez G, Reischig J, Pavlikova L, et al. (1998) Primary intestinal epithelial cells can be infected with laboratory-adapted strain HIV type 1 NDK but not with clinical primary isolates. AIDS Res Hum Retroviruses 14: 1235–1238.
38. Bomsel M (1997) Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nat Med 3: 42–47.
39. Meng G, Wei X, Wu X, Sellers MT, Decker JM, et al. (2002) Primary intestinal epithelial cells selectively transfer R5 HIV-1 to CCR5+ cells. Nat Med 8: 150–156.
40. Amerongen HM, Weltzin R, Farnet CM, Michetti P, Haseltine WA, et al. (1991) Transepithelial transport of HIV-1 by intestinal M cells: a mechanism for transmission of AIDS. J Acquir Immune Defic Syndr 4: 760–765.
41. Fotopoulos G, Harari A, Michetti P, Trono D, Pantaleo G, et al. (2002) Transepithelial transport of HIV-1 by M cells is receptor-mediated. Proc Natl Acad Sci U S A 99: 9410–9414.
42. Walters RW, Freimuth P, Moninger TO, Ganske I, Zabner J, et al. (2002) Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 110: 789–799.
43. Coyne CB, Shen L, Turner JR, Bergelson JM (2007) Coxsackievirus entry across epithelial tight junctions requires occludin and the small GTPases Rab34 and Rab5. Cell Host Microbe 2: 181–192.
44. Shacklett BL, Anton PA (2010) HIV Infection and Gut Mucosal Immune Function: Updates on Pathogenesis with Implications for Management and Intervention. Curr Infect Dis Rep 12: 19–27.
45. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, et al. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2: 361–367.
46. Niess JH, Brand S, Gu X, Landsman L, Jung S, et al. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 307: 254–258.
47. Niess JH, Adler G (2010) Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. J Immunol 184: 2026–2037.
48. Chieppa M, Rescigno M, Huang AY, Germain RN (2006) Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med 203: 2841–2852.
49. Shen R, Smythies LE, Clements RH, Novak L, Smith PD (2010) Dendritic cells transmit HIV-1 through human small intestinal mucosa. J Leukoc Biol 87: 663–670.
50. de Witte L, Nabatov A, Geijtenbeek TB (2008) Distinct roles for DC-SIGN+-dendritic cells and Langerhans cells in HIV-1 transmission. Trends Mol Med 14: 12–19.
51. Wu L, KewalRamani VN (2006) Dendritic-cell interactions with HIV: infection and viral dissemination. Nat Rev Immunol 6: 859–868.
52. Gurney KB, Elliott J, Nassanian H, Song C, Soilleux E, et al. (2005) Binding and transfer of human immunodeficiency virus by DC-SIGN+ cells in human rectal mucosa. J Virol 79: 5762–5773.
53. Hu SL (2005) Non-human primate models for AIDS vaccine research. Curr Drug Targets Infect Disord 5: 193–201.
54. Veazey RS (2008) Microbicide safety/efficacy studies in animals: macaques and small animal models. Curr Opin HIV AIDS 3: 567–573.
55. Couedel-Courteille A, Butor C, Juillard V, Guillet JG, Venet A (1999) Dissemination of SIV after rectal infection preferentially involves paracolic germinal centers. Virology 260: 277–294.
56. Couedel-Courteille A, Pretet JL, Barget N, Jacques S, Petitprez K, et al. (2003) Delayed viral replication and CD4(+) T cell depletion in the rectosigmoid mucosa of macaques during primary rectal SIV infection. Virology 316: 290–301.
57. Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, et al. (2006) Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection. J Gen Virol 87: 1311–1320.
58. Kuller L, Thompson J, Watanabe R, Iskandriati D, Alpers CE, et al. (1998) Mucosal antibody expression following rapid SIV(Mne) dissemination in intrarectally infected Macaca nemestrina. AIDS Res Hum Retroviruses 14: 1345–1356.
59. Hirsch VM, Sharkey ME, Brown CR, Brichacek B, Goldstein S, et al. (1998) Vpx is required for dissemination and pathogenesis of SIV(SM) PBj: evidence of macrophage-dependent viral amplification. Nat Med 4: 1401–1408.
60. Haase AT (2010) Targeting early infection to prevent HIV-1 mucosal transmission. Nature 464: 217–223.
61. Butler DM, Delport W, Kosakovsky Pond SL, Lakdawala MK, Cheng PM, et al. (2010) The origins of sexually transmitted HIV among men who have sex with men. Sci Transl Med 2: 18re11.
62. Sodora DL, Gettie A, Miller CJ, Marx PA (1998) Vaginal transmission of SIV: assessing infectivity and hormonal influences in macaques inoculated with cell-free and cell-associated viral stocks. AIDS Res Hum Retroviruses 14 Suppl 1: S119–123.
63. Kittikraisak W, van Griensven F, Martin M, McNicholl J, Gilbert PB, et al. (2009) Blood and seminal plasma HIV-1 RNA levels among HIV-1-infected injecting drug users participating in the AIDSVAX B/E efficacy trial in Bangkok, Thailand. J Acquir Immune Defic Syndr 51: 601–608.
64. Gupta P, Mellors J, Kingsley L, Riddler S, Singh MK, et al. (1997) High viral load in semen of human immunodeficiency virus type 1-infected men at all stages of disease and its reduction by therapy with protease and nonnucleoside reverse transcriptase inhibitors. J Virol 71: 6271–6275.
65. Zuckerman RA, Whittington WL, Celum CL, Collis TK, Lucchetti AJ, et al. (2004) Higher concentration of HIV RNA in rectal mucosa secretions than in blood and seminal plasma, among men who have sex with men, independent of antiretroviral therapy. J Infect Dis 190: 156–161.
66. Coombs RW, Speck CE, Hughes JP, Lee W, Sampoleo R, et al. (1998) Association between culturable human immunodeficiency virus type 1 (HIV-1) in semen and HIV-1 RNA levels in semen and blood: evidence for compartmentalization of HIV-1 between semen and blood. J Infect Dis 177: 320–330.
67. Chrystie IL, Mullen JE, Braude PR, Rowell P, Williams E, et al. (1998) Assisted conception in HIV discordant couples: evaluation of semen processing techniques in reducing HIV viral load. J Reprod Immunol 41: 301–306.
68. Shepard RN, Schock J, Robertson K, Shugars DC, Dyer J, et al. (2000) Quantitation of human immunodeficiency virus type 1 RNA in different biological compartments. J Clin Microbiol 38: 1414–1418.
69. Vernazza PL, Gilliam BL, Dyer J, Fiscus SA, Eron JJ, et al. (1997) Quantification of HIV in semen: correlation with antiviral treatment and immune status. AIDS 11: 987–993.
70. Borzy MS, Connell RS, Kiessling AA (1988) Detection of human immunodeficiency virus in cell-free seminal fluid. J Acquir Immune Defic Syndr 1: 419–424.
71. Golarz de Bourne MN, Bourne GH (1975) The histology and histochemistry of the rhesus monkey. In: Bourne GH, ed. The rhesus monkey volume I Anatomy and physiology. New York: Academic Press. pp 170–302.
72. Sansonetti PJ, Phalipon A (1999) M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process. Semin Immunol 11: 193–203.
73. Renoux C, Wain-Hobson S, Hurtrel B, Cheynier R (2005) Antigenic stimulation specifically reactivates the replication of archived simian immunodeficiency virus genomes in chronically infected macaques. J Virol 79: 11231–11238.
74. Spira AI, Marx PA, Patterson BK, Mahoney J, Koup RA, et al. (1996) Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. J Exp Med 183: 215–225.
75. Marthas ML, Lu D, Penedo MC, Hendrickx AG, Miller CJ (2001) Titration of an SIVmac251 stock by vaginal inoculation of Indian and Chinese origin rhesus macaques: transmission efficiency, viral loads, and antibody responses. AIDS Res Hum Retroviruses 17: 1455–1466.
76. Zhang Z, Schuler T, Zupancic M, Wietgrefe S, Staskus KA, et al. (1999) Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science 286: 1353–1357.
77. Hu J, Gardner MB, Miller CJ (2000) Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J Virol 74: 6087–6095.
78. Miller CJ, Li Q, Abel K, Kim EY, Ma ZM, et al. (2005) Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. J Virol 79: 9217–9227.
79. Pelletier E, Saurin W, Cheynier R, Letvin NL, Wain-Hobson S (1995) The tempo and mode of SIV quasispecies development in vivo calls for massive viral replication and clearance. Virology 208: 644–652.
80. Cranage M, Sharpe S, Herrera C, Cope A, Dennis M, et al. (2008) Prevention of SIV rectal transmission and priming of T cell responses in macaques after local

pre-exposure application of tenofovir gel. PLoS Med 5: e157. doi:10.1371/journal.pmed.0050157.
81. van Wijk F, Cheroutre H (2009) Intestinal T cells: facing the mucosal immune dilemma with synergy and diversity. Semin Immunol 21: 130–138.
82. Nelson JA, Wiley CA, Reynolds-Kohler C, Reese CE, Margaretten W, et al. (1988) Human immunodeficiency virus detected in bowel epithelium from patients with gastrointestinal symptoms. Lancet 1: 259–262.
83. Mathijs JM, Hing M, Grierson J, Dwyer DE, Goldschmidt C, et al. (1988) HIV infection of rectal mucosa. Lancet 1: 1111.
84. Clayton F, Reka S, Cronin WJ, Torlakovic E, Sigal SH, et al. (1992) Rectal mucosal pathology varies with human immunodeficiency virus antigen content and disease stage. Gastroenterology 103: 919–933.
85. Margolis L, Shattock R (2006) Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? Nat Rev Microbiol 4: 312–317.
86. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD (1996) HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science 271: 1582–1586.
87. Jameson B, Baribaud F, Pohlmann S, Ghavimi D, Mortari F, et al. (2002) Expression of DC-SIGN by dendritic cells of intestinal and genital mucosae in humans and rhesus macaques. J Virol 76: 1866–1875.
88. Milush JM, Kosub D, Marthas M, Schmidt K, Scott F, et al. (2004) Rapid dissemination of SIV following oral inoculation. AIDS 18: 2371–2380.
89. Parikh UM, Dobard C, Sharma S, Cong ME, Jia H, et al. (2009) Complete protection from repeated vaginal simian-human immunodeficiency virus exposures in macaques by a topical gel containing tenofovir alone or with emtricitabine. J Virol 83: 10358–10365.
90. Dormont D, Le Grand R (1993) Infection of macaques after atraumatic rectal exposure to SIVmac251. In: Huitieme Colloque des "Cent Gardes", Fondation Merieux, Lyon, France. pp 157–160.
91. Prost S, Le Dantec M, Auge S, Le Grand R, Derdouch S, et al. (2008) Human and simian immunodeficiency viruses deregulate early hematopoiesis through a Nef/PPARgamma/STAT5 signaling pathway in macaques. J Clin Invest 118: 1765–1775.
92. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Hyg 27: 493–497.
93. Le Grand R, Vogt G, Vaslin B, Roques P, Theodoro F, et al. (1992) Specific and non-specific immunity and protection of macaques against SIV infection. Vaccine 10: 873–879.