Memory CD4⁺CCR5⁺ T cells are abundantly present in the gut of newborn infants to facilitate mother-to child transmission of HIV-1

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Short title: Neonatal gut CD4⁺CCR5⁺ T cells and MTCT of HIV-1
Abstract

Despite potential clinical importance, target cells for HIV-1 in mother-to-child transmission (MTCT) have not yet been identified. Cord blood-derived CD4+ T cells are largely naïve and do not express CCR5, the mandatory co-receptor for transmitted HIV-1 R5 strains in infants. Here, we demonstrate that in the human fetal and infant gut mucosa there is already a large subset of mucosal memory CD4+CCR5+ T cells with predominantly a Th1 and Th17 phenotype. Using next-generation sequencing of the T-cell-receptor β chain, clonally expanded T cells, as a hallmark for memory development predominated in the gut mucosa (30%), while few were found in lymph node (1%) and none in cord blood (0%). The gut mucosal fetal and infant CD4+ T cells were highly susceptible to HIV-1 without any prestimulation; pol proviral DNA levels were similar to infected PHA stimulated adult PBMCs. In conclusion, we show that extensive adaptive immunity is present before birth, with the gut mucosa as the preferential site for memory CD4+ T cells. These CD4+CCR5+ T cells in the infant mucosa provide a large pool of susceptible cells for ingested HIV-1 at birth and during breastfeeding, indicating a mucosal route of MTCT, which can be targeted in prevention strategies.
Introduction

More than 300,000 children are infected annually with HIV-1 as a consequence of mother-to-child-transmission (MTCT).\(^1\) Combination anti-retroviral therapy (cART) during pregnancy succeeds in reducing the risk of MTCT from 30% to <1%.\(^1\)\(^2\) However, exposure to cART \textit{in utero} and postnatally, can have long-term adverse effects in these HIV-1 exposed, uninfected children.\(^3\)\(^-\)\(^7\) A better understanding of MTCT is crucial for the development of targeted and safe prevention strategies. However, the actual mechanism of MTCT remains unclear.

HIV-1 strains transmitted from mother to child are predominantly R5 strains, which require CCR5 as a co-receptor to infect CD4\(^+\) T cells.\(^8\) This corresponds with the protective effect of the CCR5 \textit{Δ}32 base pair genotype in MTCT of HIV-1.\(^9\) Previous data as well as our current findings show that CD4\(^+\)CCR5\(^+\) T cells are not present in cord blood.\(^10\)\(^-\)\(^11\) In accordance with this observation, studies \textit{in vitro} show that isolated CD4\(^+\) T cells from cord blood need to be activated prior to establish infection with HIV-1.\(^12\) These findings suggest that the immune system of the infant is naïve, which is supported by the general principle that substantial antigen induced memory development is initiated after birth.\(^13\)\(^-\)\(^14\) Nevertheless, memory Treg cells are described in lymph nodes in infants.\(^15\) However, this apparently does not result in CD4\(^+\)CCR5\(^+\)T cells in cord blood, leaving the question unanswered: what are the target cells for MTCT of HIV-1?

Here, we demonstrate that the CD4\(^+\)CCR5\(^+\) T cells are preferentially located in the infant gut mucosa. CD4\(^+\) T cells in the gut epithelium had distinct features of memory cells such as high HLA-DR and CD45RO expression as well as T helper subset differentiation. Importantly, the infant gut mucosal CD4\(^+\)CCR5\(^+\) T cells were highly susceptible to HIV-1 infection without prior activation with cytokines, and thus could act as prime targets for HIV-1 transmission in infants.
Methods

Subjects

The first series of immunohistochemical stainings (see below) were performed on small intestinal tissue, mesenteric lymph node and spleen of four neonates, who had died during delivery or within 24 hours after birth (Table 1). Autopsy was performed within 24 hours of death. All patients were of Caucasian descent. Three patients were born prematurely. None of the neonates had received enteral feeding prior to death.

The subsequent series were performed on neonatal tissues obtained at autopsy from patients 5–7 or obtained fresh during reconstructive surgery for small intestinal obstruction, from patients 8–11 (Table 1). None of the patients showed signs of infection or immunodeficiency. We performed studies with Medical Ethical Committee approval from our institute, AMC, and obtained informed consent from the parents in accordance with the Declaration of Helsinki.

For studies of CD4+ T cells derived from cord blood, we included four uninfected HIV-1-exposed patients. The HIV-1-exposed patients were born to HIV-1-infected mothers who had started cART at 20 weeks of gestational age and a viral load of less than 50 copies at delivery.

Tissue sampling and cell isolation

Peripheral blood mononuclear cells (PBMC) and cord blood mononuclear cells were isolated using Ficoll gradients. After autopsy or surgery the gut mucosal and submucosal layer was removed from the muscular layer. The gut tissue was cut into 5-mm² fragments and then incubated with DTT to remove mucus, and subsequently with EDTA to obtain epithelial cells. To isolate the subepithelial mononuclear cells we used a mechanical single-cell isolation method, the BD Medimachine System (BD Biosciences). This is an automated, mechanical disaggregation system of solid human tissues without enzymes. The tissue is placed into the medicon filter, with 1 ml of PBS. The medimachine spins the tissue in the medicon filter disaggregating the tissue into small fragments and single cells. A syringe placed on the outport of the medimachine aspirates the single cells in PBS through a single cell filter. This procedure is repeated until the tissue has dissolved completely. Percoll gradients were used to obtain the mononuclear cell fractions single cell suspensions. A single cell suspension was obtained from lymph node and spleen using the Falcon Cell strainer (BD Biosciences), a single-cell filter, followed by Ficoll gradient isolation.
Immunohistochemistry

Sections (5 μm) from formalin-fixed and paraffin-embedded tissue were deparaffinized in xylene, rehydrated in an alcohol series, and treated with 0.3% H₂O₂ in methanol to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed for 20 min in Tris-EDTA (pH 9.0) at 98°C. Ultra V Block (Immunologic) was used to block non-specific binding sites. Primary antibody labeling was performed with anti-CCR5 mAb kindly provided by M. Mack, Regensburg, Germany, followed by secondary labeling with an anti-mouse IgG alkaline phosphatase (AP) polymer (Immunologic). AP activity was visualized with the Vector®Blue AP Substrate Kit (Vector Laboratories). Removal of CCR5 and AP polymer was achieved by a heating step in EDTA (pH 8.0) at 98°C, leaving the blue staining untouched. This was followed by Ultra V Block, incubation with CD45RO (UCHL1, mouse IgG2a, Dako) as well as CD4 (4B12, mouse IgG1, ThermoFisher) and secondary labeling with AP-conjugated goat anti-mouse IgG2a and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (SBA, Birmingham). AP and HRP activity were visualized with Vector®RED AP Substrate Kit (Vector Laboratories) and Bright DAB (Immunologic), respectively.

The second antibody panel consisted of CCR5, followed by CD68 (PG-M1, mouse, Dako) and CD3 (SP7, rabbit, Thermo). Secondary labeling for both antibody panels involved a cocktail of anti-mouse IgG AP polymer and anti-rabbit IgG HRP polymer. AP and HRP activities were visualized with Vector Red and Bright DAB. Staining for HLA-DR (CR3/43, Dako) was performed with detection anti-mouse IgG AP polymer and Vector Red. Removal of HLA-DR and AP polymer was achieved by a 10 min heating step in EDTA (pH 8.0) at 98°C leaving the red dye untouched. The second antibody labeling involved CD45RO as described above and visualized with Vector Red.

Quantification of co-localization of staining of the lymphocyte subsets of interest was performed by spectral imaging, as described previously in detail. This technique detects different wavelengths from a particular area to identify the single-, dual- and triple-stained cells. Random digital images (1–4 per section) per tissue were taken at moderate magnification (200x) with a Leica® microscope. The Nuance 2.9 software was used to unmix and identify the spectra in the images (Cambridge Research Instrumentation) and identify co-localization of i.e. CD4 and CCR5. Observer agreement about signal co-localizations was checked for all images. After spectral unmixing, the images are presented fluorescent-like in pseudo-colors showing dual-antibody combinations within the IHC triple-staining combination.

CD4+ T helper cells subsets were identified similarly as above, using antibodies against RORγt, FoxP3 (Abcam, Cambridge, UK), T-bet, and Gata-3 (Santa Cruz). Sections were then incubated with anti-mouse (FoxP3, Gata-3) or anti-rabbit (T-bet, RORγt) IgG AP polymer (Immunologic) and visualized with Vector®Blue. CD45RO (Thermo) was incubated
with IgG1-HRP and visualized with Vector Nova Red. Owing to the absence of co-localization of a membrane (CD45RO) with nuclear markers (RORγt, FoxP3, T-bet, Gata-3), co-localization could not be determined by spectral imaging. The percentages of double-positive cells showing cell co-expression of CD45RO and a nuclear marker were assessed by conventional observation of the images by the pathologist.

Gut tissue from the tissue HIV-1 infection study was analyzed for detection of HIV-1 using the method described above, using mouse-a-p24 (Dako). Sections were then incubated with anti-mouse IgG AP polymer (Immunologic) and visualized with the Vector® Blue AP Substrate Kit. CD45RO (Thermo) to detect memory T cells was incubated with IgG-HRP polymer and visualized with Vector Nova Red.

**Flow cytometry**

The expression of surface markers on single cells from tissue or blood was analyzed by a FACS CANTO (BD Biosciences) after staining with fluorochrome-conjugated CD3, CD4, CCR5, CCR6, and CD45RO cells, obtained from BD Biosciences. Data were analyzed with FlowJo (Tree Star, Inc.)

**HIV-1 infection assay of isolated cells**

Isolated cells from gut mucosa or blood were inoculated with YU2 (R5-strain) at an MOI of 1 for 12 hours at 37°C. After removal of virus, the cells were cultured for 3 days in IMDM, supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, 50 mg/mL streptomycin and 100 IU/ml IL-2. The CD4+ cells were then isolated using Macs beads (Miltenyi) and analyzed for pol proviral DNA. The cord blood cells were inoculated directly after isolation or after initial stimulation for 4 days with 100 IU/ml IL-2. We used adult CD4+ T cells from blood, activated with phytohemagglutinin (PHA) for 3 days prior to infection as a positive control.

**HIV-1 infection assay of whole gut tissue**

The mucosal layer was obtained as described above and placed in a culture dish with culture medium, IMDM, supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, 50 mg/mL streptomycin and 100 IU/ml IL-2. HIV-1 (YU2) containing medium with 10,000 TCID was loaded on the apical side. The tissue was incubated for 12 hours, washed with IMDM, and cultured in the culture medium described above for 3 days. The tissues were fixed in formalin and then embedded in paraffin for immunohistochemical staining.
Detection of HIV-1 proviral DNA

Total DNA was isolated from CD4+ cells using the QIAamp DNA Blood kit (Qiagen). The amount of HIV-1 pol proviral DNA in the samples was determined by qPCR for the HIV-1 pol region with pol-B-02 5′-CTTCTAATGTGTACAATCTAGTTGCC-3′ and pol-E-03 5′-TGATTITAACCTGCCACCTAGTAG-3′ in conjunction with a labeled probe pol-P (FAM)-CTGTGATAATGTCAGCTAAAAGGAAGCCA-(TAMRA)-3′. A standard curve was prepared from the cell line 8E5, which contained one copy of HIV-1 DNA per cell. The reaction mix contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 µM dNTP, 250 µg/ml BSA, 500 nM primers, 250 nM probe and 1.2 U platinum Taq DNA polymerase (Invitrogen). Real-time PCR was performed using the following program on the LightCycler (Roche): (1) preincubation and denaturation: 50°C for 2 min, 95°C for 2 min; (2) amplification and quantification: 45 cycles of 95°C for 5 sec, 60°C for 30 sec. To correct for differences in the DNA input, β-actin concentrations were analyzed by SYBR green qPCR. The primers for β-actin used were: B-actin-S 5′-GGGTCAAGGATCCTATG-3′ and B-actin-AS 5′-GGTCTCAAACATGATCTGGG-3′. Specificity of the PCR products measured using the SYBR green method was confirmed by a melting curve. Real-time PCR was performed using the following program on the LightCycler (Roche): (1) preincubation and denaturation: 50°C for 2 min, 95°C for 2 min; (2) amplification and quantification: 45 cycles of 95°C for 5 sec, 55°C for 15 sec, 72°C for 15 sec; (3) melting curve: 95°C for 0 sec, 65°C for 15 min, 95°C for 0 sec with a temperature transition rate of 0.1°C/sec.

Next-generation sequencing for high-resolution screening of the T-cell receptor β-chain repertoire of clonal T cells

T cells from various tissues from patient 7, intestinal tissue and blood from patient 11 and adult PBMCs were isolated by positive selection using Macs beads (Miltenyi). Total RNA was extracted using the RNeasy Mini System (Invitrogen) cDNA was synthesized from RNA using SuperScriptIII-RT (Invitrogen). Preparation of the samples for sequencing was performed as previously described²⁷, with one modification in the linear amplification (LA) procedure; after the first round of (full repertoire) linear amplification, a generic PCR was used to amplify the TCR products for sequencing. In this step primer B was used as generic forward primer and a generic primer specific for the TCRβ-constant gene segment (5′-CTCAAACACAGCGACCTC-3′) was used as reverse primer. The reverse primer contained a multiplex identifier and primer A as described in the amplicon sequencing manual (Roche). The PCR was performed with 50% of the purified LA product in the presence of 10 pmol of each of the primers, 1x buffer B, 1 mM MgCl₂, 0.1 mM dNTPs and 3U of Hotfire (Solis Biodyne) in a volume of 40 µL (96°C (900 sec), 35× (96°C (30 sec), 60°C (60 sec), 72°C (60 sec)), 72°C (600 sec)). Samples were sequenced on a Roche Genome Sequencer FLX
(Roche Diagnostics) using the Titanium system according to the manufacturer’s protocol. The samples were adjusted for cell input. The customized bioinformatics pipeline used to obtain the TCR sequence was described previously in detail\textsuperscript{17} and contained 4 modules: (1) MID sorting, (2) identification of gene segments, (3) CDR3 detection, and (4) removal of artifacts. This then resulted in the relative frequency of individual clones. Samples were adjusted for cell input. To identify expanded clones we based our cut-off on the largest clone present in the thymus, as only few memory cells are reportedly present in thymus \textsuperscript{18}. The largest clone in the thymus had a relative contribution to the local T cell population of 0.14%. Thus, a T cell clone contributing >0.14\% to the T cell population of the specific compartment was considered to be an expanded clone. This is probably an underestimate, as it is likely that a few expanded T cell clones are present in the thymus. To validate this cut-off further, we applied it also to our control sample, adult PBMCs, and 0.14\% also nicely indentified expanded clones. The results from this adult sample were similar to published data.\textsuperscript{17} Taking into account these two observations, as well as the conservative approach we applied, a cut-off of 0.14\% best indicated an expanded T cell clone and simultaneously allowed for comparison of tissues and patients.

**Statistical Analysis**

Graphpad Software (La Jolla) was used to design the graphs as well as to perform statistical analyses. In the graphs and text mean ± SEM are shown and a double-sided Student’s \textit{t}-test was used for statistical comparison. A \textit{p}-value of less then 0.05 was considered to be significant.
Results

CD4⁺CCR5⁺ T cells reside in the fetal and infant gut mucosa

Tissues obtained from human fetuses at autopsy and newborn infants who had died within 24 hours of birth were collected (Table 1). These infants showed no signs of infection and had not received enteral feeding. The phenotype of the CD4⁺ T cells was assessed by immunohistochemical triple stainings with CD4, CCR5 and CD45RO cells. Spectral imaging on paraffin sections allowed for visualization and quantification of co-localized immunohistochemical signals in intact tissues. Individual CD4⁺ T cells were located in the mucosa of the small intestine between and just underneath the enterocytes (Figure 1A). Notably, the gut mucosa of these fetuses and infants contained a large proportion of the intra-epithelial CD4⁺CCR5⁺ T cells (29.6% ± 5.4% SEM) (Figure 2A). Moreover, we observed lymphoid aggregates of CD4⁺ T cells in gut submucosa, of which 17.3% ± 2.5% SEM also expressed CCR5 (Figure 1B and 2A). Lymphoid aggregates containing CD4⁺ T cells were also observed in mesenteric lymph nodes (Figure 1C), whereas, in the spleen, CD4⁺ T cells were relatively few and surrounded the central arterioles. Only small numbers of CD4⁺CCR5⁺ T cells were detected in spleen and mesenteric lymph nodes (Figure 2A). A similar pattern was observed for CD45RO expression on CD4⁺ T cells, with the highest frequencies observed in gut mucosa and submucosa (Figure 1 and 2B). Triple stainings for CCR5, CD3 and CD68 confirmed that the CD4⁺CCR5⁺ mononuclear cells were of T cell (CD3⁺) and not of macrophage or lymphoid tissue inducer cell origin (supplemental Figure 1). HLA-class II (HLA-DR), which is induced upon T cell activation, was specifically expressed by CD45RO⁺ cells in the gut mucosa (supplemental Figure 2). In confirmation, single cell suspensions were analyzed and mucosa CD4⁺ T cells expressed high levels of both CCR5 and CD45RO (supplemental Figure 3A). The CD3⁺ T cell population in the infant gut mucosa consisted of largely CD4⁺ T cells and less so of CD8⁺ T cells (CD4/CD8 ratio= 1.9).

Tissue dependent distribution of T helper subsets in the fetus and newborn infant

Next, we further analyzed the memory-like phenotype of the CD4⁺CCR5⁺ T cells within the human fetal and infant gut mucosa and submucosa and the other tissues, since the phenotype was markedly different from the naïve T cells found in cord blood. We characterized four main functional CD4⁺ T helper cell subsets by measuring prototypic transcription factors: T-bet for Th1, Gata-3 for Th2, RORγt for Th17 and FoxP3 for Tregs in combination with the memory T cell marker, CD45RO. RORγt co-expression was detected in approximately 50% of the CD45RO⁺ intestinal T cells (Figure 3A and B). The remaining CD45RO⁺ T cells expressed either T-bet (35%) (Figure 3D and E) or FoxP3 (15%) (data not shown), whereas the level of Gata-3 was low in the gut (<5%). In the spleen, T-bet...
expression predominated and RORγt was virtually absent (Figure 3D and F). In line with these data, analyses with flow cytometry of single cell suspensions showed that a large proportion of the CD4⁺CCR5⁺ T cells in the gut mucosa co-expressed CCR6 (supplemental Figure 4C). This chemokine receptor is predominantly expressed by Th17 cells, verifying the early presence of CD45RO⁺RORγt⁺ Th17 cells in the infant gut. This chemokine receptor is predominantly expressed by Th17 cells, verifying the early presence of CD45RO⁺RORγt⁺ Th17 cells in the infant gut. The phenotype of the Th17, CD4⁺CCR5⁺CCR6⁺ T cells in the infant gut mucosa, suggests highly susceptible qualities for HIV-1 infection.

The infant gut is the principal site for clonally expanded T cells

The identification of differentiated memory CD4⁺ T cells in the gut evoked the question whether these populations showed signs of antigen-induced activation with clonal expansion as a hallmark. Next-generation sequencing of T-cell receptor β-chain repertoire was used to quantify clonal size and obtain sequence information on the infant clonal T cells. This analysis was performed on CD3⁺ T cells derived from tissues of patient 7 and from both gut and blood from patient 11. A cut-off based on a relative contribution of a T cell clone of >0.14% to the total T cell population was used to identify expanded clones and allowed for comparison of tissues and patients. This cut-off was based on the largest clone, present in the thymus as only few memory cells are present there.

In patient 7, 30% of the T cell repertoire in the gut consisted of expanded clones, 1% in the mesenteric lymph node and 17.9% in the spleen, which identified the gut as the principal site for expanded T cell clones (Figure 4A). Remarkably, there were more expanded T cell clones in the fetal gut than in the adult peripheral blood sample, which was comparable to published data on the adult T cell repertoire of peripheral blood T cells. Only 10% of the T cell clones detected in the fetal gut could also be identified in mesenteric lymph node and 5% in spleen, illustrating compartmentalization of the T cell clones (Figure 4B). Similar results were obtained in the analyses of the term patient 11, with 28% of the T cell repertoire originating from expanded T cell clones (Figure 4A) and 0% expanded T cell clones in peripheral blood. The above analyses of the T-cell receptor β-chain repertoire reflected a similar percentage of memory T cells identified by immunohistochemistry and flow cytometry, confirming these substantial numbers of memory T cells in the infant gut and their lack in lymph node and blood.

CD4⁺ T cells from the infant gut mucosa are highly susceptible to HIV-1 infection

The phenotype of the CD4⁺ T cells in the infant gut mucosa identified them as prime targets for HIV-1. To explore this, mononuclear cells were isolated from gut tissue and
mesenteric lymph nodes obtained at autopsy or from gut tissue and peripheral blood collected during reconstructive gut surgery from patient 5-10 (Table 1). The unstimulated CD4+ T cells were incubated for 12 hours with HIV-1 and cultured for 3 days (Figure 5A) Intestinal CD4+ T cells contained significantly higher HIV-1 pol proviral DNA levels than CD4+ T cells from either lymph nodes or peripheral blood of the same patient. Notably, these cells demonstrated extremely high susceptibility to HIV-1 infection, with pol proviral DNA levels similar to the positive control; that is, adult CD4+ T cells from peripheral blood activated with phytohemagglutinin (PHA). HIV-1 infection of whole gut tissue explants revealed HIV-1 p24 in and around CD45RO+ cells in the intestinal tissue after 3 days (Figure 5B-D). These data demonstrate that, mucosal CD4+ T cells derived from the fetal and infant gut are highly susceptible to HIV-1, in contrast to infant CD4+ T cells from blood. Their memory phenotype allows for a very efficient infection by HIV-1 comparable to PHA-activated adult T cells.
Discussion

Our aim, to elucidate the mechanism of MTCT of HIV-1, led us to investigate a variety of tissues to identify the target cells for HIV-1 infection. We show that already in the fetus there is an abundant presence of CD4+CCR5+ T cells with a memory phenotype in gut epithelium and gut lymphoid aggregates. The CD4+ T cells displayed several distinctive features of previously activated T cells, specifically CCR5, HLA-DR, and CD45RO expression, T-helper differentiation and clonal expansion, which makes it likely that they are memory CD4+ T cells. These properties are markedly different from those of the naïve CD4+ T cells present in infant blood and lymph nodes. Such extensive presence of memory CD4+CCR5+T cells in the human fetal and infant gut mucosa was unknown until now. Studies in mice showed that CD4+ T cells only enter the gut mucosa after birth, leading to the assumption that CD4+ T cells in humans do not exist in the gut mucosa at birth. More recent studies in neonatal macaques had indicated the presence of a CD4+CCR5+ T cells in the lamina propria with subepithelial localization. In contrast, our study showed the abundance of memory CD4+ T cells with a specific T helper phenotype in the gut epithelium, which seems to be a unique feature of the human fetal and infant gut mucosa since epithelial lymphocytes in adults are mostly CD8+ T cells. As expected from their phenotype (CCR5+, CCR6+, HLA-DRhigh), these CD4+ T cells in fetal and infant gut mucosa were highly susceptible to infection with HIV-1 in contrast to neonatal blood CD4+ T cells. The gut with its large epithelial surface provides an extensive site for viral transfer to target cells after ingestion of HIV-1 from maternal blood and cervical mucus during delivery, or from breast milk. In the first days of life, the neonatal stomach is not yet able to inactivate HIV-1 by acidifying ingested fluids and, consequently, infectious HIV virus can be detected in gastric aspirates of newborns. A mucosal route of MTCT of HIV-1 provides a rationale for some hitherto unexplained observations, including the highly selective transmission of homogeneous R5 viruses, associated with mucosal transmission in adults, as well as the protective effect of a cesarean section, which prevents ingestion of HIV-1-containing mucus and blood at delivery. A recent study, showed an increased replicative capacity of R5 viruses compared to X4 variants in a T cell line derived from in vitro PHA activated infant T cells. This difference was not observed in a T cell line derived from adult cells. This provides further evidence of preferential infection with R5 strains in infants and the necessity of CCR5 bearing target cells in MTCT. TCR-related signaling was implicated to be partly responsible for this observation. The role of the gut in the pathogenesis of HIV infection in adults is widely recognized; our current data suggests a crucial role of the gut mucosa in transmission of HIV-1 in infants (Figure 6). Future prevention strategies could
take this specific locus into account and intervene directly at the site of HIV-1 transmission to the target cells.

The abundance of memory T cells in the gut mucosa suggests that adaptive T cell immunity is already initiated in the fetus. This is supported by the presence of clonally expanded T cells in the gut, as detected by next-generation sequencing, which we applied here to gain insight into the early T cell repertoire. T cell clones were highly compartmentalized to the specific tissues with only a small overlap between the various anatomical sites. This tissue-dependent distribution was also observed for the hallmark T helper subset nuclear transcription markers, with T-bet and RORγt together with CD45RO+ cells predominating in the gut. These cells described here are not innate cells such as lymphoid tissue-inducer cells (LTI’s) or NK-22 cells expressing RORγt, as these subsets do not express CD3 or CD45RO.19,20,35,36 The tissue-dependent distribution of T cell subsets suggests that there is not an overall naïve or tolerogenic phenotype in the fetus based on blood CD4+ T cells, but –instead– a tissue-dependent adaptive immune system that is already in place in the fetus and consists of tolerizing and inflammatory branches at specific anatomical sites. This differentiated organization of the adaptive immune system may allow the fetus and infant to execute specific responses according to local needs. Further, research is required to confirm the induction of adaptive T cell memory responses with regard to antigen-T cell interactions in the fetus, and the origin of the antigens as well as the signals that shape the tissue-dependent organization of memory T cells in the fetus. This new model of a compartmentalized neonatal adaptive immune system may help to understand hitherto unexplained pediatric diseases characterized by severe inflammation in the gut.37

In conclusion, our study identifies target cells for HIV-1 in the newborn infant in the gut mucosa. The mucosal route of MTCT can be addressed in targeted prevention strategies intervening at the anatomical site of HIV-1 transmission. Moreover, it reveals a framework of a fetal tissue-dependent immune system, which opens up new avenues of research to understand the development of the fetal and infant human adaptive immune system and its role in health and disease.
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Authorship: M.J.B and T.W.K are the principal investigators, who conceived and designed the study. M.J.B, C.M.L., P.L.K and J.L.H performed the experiments. J.C.H.W, S.T.P and K.B. assisted in acquisition of the samples. S.T.P. and R.L contributed to study design of the study. M.J.B devised and performed the analyses and wrote the first draft of the manuscript with input from all authors supervised by T.W.K. All authors approved the final manuscript revisions.

Conflict of Interest Disclosure: All authors declare there are no potential conflicts (financial, professional, or personal) that are relevant to the manuscript.

References


**Table 1. Patient characteristics.** Patients 1–4 were included in the initial immunohistochemical study using autopsy material from neonates. Patients 5–10 represent patients from whom fresh newborn tissue samples were obtained shortly after birth for the *ex vivo* HIV-1 infection studies and flow cytometry. Gut tissue from patients 7 and 11 was used in the sequencing analyses of the T-cell receptor β-chain repertoire.
Table 1

<table>
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<th>Treatment</th>
<th>Time of death after birth</th>
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</tr>
<tr>
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<td>40</td>
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N/A, not available.
Figure Legends

Figure 1. Expression of CD4, CCR5 and CD45RO cells in neonatal tissue. This was determined by triple immunohistochemical staining for CD4, CCR5 and CD45RO visualized with spectral imaging and at higher magnification. The first column shows immunohistochemistry light microscopy. The following columns show the same cubes analyzed with the Nuance software for spectral imaging. These analyses indicated more CD4⁺CCR5⁺ and CD4⁺CD45RO⁺ T cells (yellow) in (A) the gut epithelium and (B) gut lymphoid aggregates of newborn infants than in (C) lymph node or (D) spleen. Bar = 0.1 mm. These panels are representative of patients 1–10 included in our study.

Figure 2. Quantification of CD4⁺CCR5⁺ cells, CD4⁺CD45RO⁺ cells and CD4⁺CD45RO⁺CCR5⁺ cells in neonatal tissue and cord blood. The data are derived from spectral imaging analyses of triple immunohistochemical stainings and flow cytometry. The graphs show mean percentages and ± SEM of (A) CD4⁺CCR5⁺, (B) CD4⁺CD45RO⁺ and (C) CD4⁺CD45RO⁺CCR5⁺ cells. Percentages of CCR5⁺, CD45RO and triple-positive CD4⁺ T cells in various tissues all differed significantly from those of CD4⁺ T cells in cord blood (P<0.001; Student’s t-test). CD4⁺ T cells from gut epithelium and gut lymphoid aggregates (LA) expressed significantly more CCR5 and CD45RO than CD4⁺ T cells derived from lymph node (P<0.01 and P= 0.05, respectively).

Figure 3. T helper subsets in neonatal tissues. In the upper panel (A-D), RORγt⁺ (blue) and CD45RO⁺ (red) double-positive cells were detected in large numbers in (A) gut epithelium and in (B) gut lymphoid aggregates. RORγt⁺CD45RO⁺ cells were virtually absent in (C) lymph node and undetectable in (D) spleen. In (E) gut epithelium T-bet⁺CD45RO⁺ cells made up approximately 35% of the CD45RO⁺ cells and to a lesser extend these cells were also present in the (F) gut lymphoid aggregates. They were nearly absent in (G) lymph node In spleen (H), T-bet⁺CD45RO⁺ cells represented the largest CD45RO⁺ T cell subset. These panels are representative of patients 1–10 included in our study. Bar=0.1 mm.

Figure 4. TCR β-chain repertoire of clonal CD3⁺ T cells determined by next-generation sequencing. Expanded T cell clones were present in the fetal and infant gut in contrast to their absence in thymus, mesenteric lymph node (LN) and blood. (A) The scatterplot displays the percentage of recovered reads per clone (dot) for each tissue or blood sample for patient 7 (fetus), patient 11 (term infant), and the adult peripheral blood control. The clonal distribution of the adult T cells was comparable to published data. Samples were adjusted for cell input. A cut-off based on a relative contribution of a T cell clone of >0.14% to the total
T cell population was used to identify expanded clones and allowed for comparison of tissues and patients. (B) The piechart shows the distribution of the T cell clones present in the gut of patient 7 over all the compartments, including gut, LN, thymus and spleen and combinations. The T cell clones were clearly compartmentalized with a lack of distribution over the other tissues.

**Figure 5. In vitro HIV-1 infection of CD4⁺ T cells derived from neonatal tissue and blood.** (A) HIV-1 infection was determined using a quantitative PCR for pol proviral DNA in CD4⁺ T cells at day 3, expressed as the ratio pol/1000 copies of β-actin as housekeeping gene. The results from blood and lymph node were combined in the Student's t-test, because of their similar low levels. Gut-derived CD4⁺ T cells infected with HIV-1 contained significantly (P=0.02) higher levels of pol proviral DNA than cultures with CD4⁺ T cells derived from blood or lymph node. PHA-stimulated adult CD4⁺ T cells from PMBC single cell suspensions, which were used as a positive control, had comparable pol proviral DNA levels as unstimulated gut mucosa CD4⁺ T cells from fetuses and infants. (B-D) HIV-1 infection of intact infant gut explants was assessed by co-expression of viral p24 (blue) and CD45RO (brown). (B-C) p24 was co-localized with CD45RO in the newborn mucosa. Control sample (D), cultured without HIV-1. Bar = 0.1 mm.

**Figure 6. Schematic model of mucosal mother-to-child-transmission of HIV-1.** After oral ingestion at birth or breastfeeding, HIV-1 enters the intestinal lumen of the infant. In the epithelium and just underneath CD4⁺ T cells expressing CCR5 are present, which are accessible targets for HIV-1. These memory CD4⁺ T cells were further characterized by prototypic nuclear transcription factors for T helper subsets, T-bet, Gata-3, RORγt and FoxP3, indicating a predominance of Th1 and Th17 cells of activated CD4⁺ T cells in the infant gut mucosa.
Figure 1

Gut epithelium

Gut lymphoid aggregates

Lymph node

Spleen

CD4  CD45RO  CCR5
CD4  CD45RO  CCR5
CD4  CD4+CCR5+
CD4  CD4+CD45RO+
Figure 2

(A) CD4+CCR5+ T cells %
- Gut epithelium
- Gut LA
- LN
- Spleen
- Blood

(B) CD4+CD45RO+ T cells %
- Gut epithelium
- Gut LA
- LN
- Spleen
- Blood

(C) CD4+CD45RO+CCR5+ T cells %
- Gut epithelium
- Gut LA
- LN
- Spleen
- Blood
Figure 5

A

\[
\begin{align*}
pol/1000 \beta\text{-actin} \\
\text{GUT} & \quad \text{LN} & \quad \text{Blood} & \quad \text{positive control}
\end{align*}
\]

\[P=0.02\]

B

\[\text{p24 CD45RO}\]
Memory CD4+CCR5+ T cells are abundantly present in the gut of newborn infants to facilitate mother-to-child transmission of HIV-1

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