Early Sites of Virus Replication After Oral SIV_{mac251} Infection of Infant Macaques: Implications for Pathogenesis

Angela M. Amedee,¹ Bonnie Phillips,² Kara Jensen,² Spencer Robichaux,¹
Nedra Lacour,¹ Mark Burke,³ Michael Piatak, Jr.,^{4,†} Jeffrey D. Lifson,⁴
Pamela A. Kozlowski,¹ Koen K.A. Van Rompay,⁵ and Kristina De Paris²

Abstract

Despite optimization of preventative measures for vertical HIV-1 transmission, daily, roughly 400 infants become HIV infected, most of them through breastfeeding. Viral entry has been presumed to occur in the gastrointestinal tract; however, the exact entry site(s) have not been defined. Therefore, we quantified simian immunodeficiency virus (SIV) RNA and DNA in oral, intestinal, and systemic tissues of 15 infant macaques within 48–96 h after oral SIV_{mac251} exposure. SIV DNA was detected as early as 48 h, whereas SIV RNA was typically detected at later time points (72–96 h). Transmitted founder viruses were identical or very similar to a single genotype in the SIV_{mac251} challenge stock. SIV RNA and DNA were most frequently found in lymph nodes (LNs) draining the oral cavity and in the ileum. Using *in situ* hybridization, SIV-infected cells in LNs were exclusively represented by CD3⁺ T cells. SIV RNA and DNA were also detected in the lungs of 20% of the animals, and 60% of the animals had detectable SIV DNA in the cerebrum. The early detection of viral RNA or DNA in lung and brain tissues emphasizes the need for early treatment of pediatric HIV infection to prevent damage not only to the immune system but also to the respiratory tract and central nervous system.

Keywords: oral SIV infection, infant rhesus macaques, virus replication, tissue distribution

Introduction

PEDIATRIC HIV-1 INFECTIONS have been drastically reduced in the last 10 years, yet the United Nations Millennium Goal of reducing mother-to-child transmission (MTCT) by 90% by 2015 was not achieved. Although improved HIV diagnostics for pregnant women and access to antiretroviral therapy (ART) in resource-poor countries resulted in lower *in utero* and *peripartum* HIV infections,¹ these measures had little impact on reducing breast milk transmission of HIV.

In fact, HIV acquisition by breastfeeding now accounts for the majority of new pediatric HIV infections.^{2–4} HIV diagnosis of these infants generally does not occur before the onset of clinical symptoms. Prolonged virus replication and systemic dissemination before diagnosis likely increase the size of the

viral reservoir in infants. Indeed, studies of HIV-infected infants have demonstrated that early ART initiation is associated with lower proviral load.^{5–7} However, the case of the Mississippi baby highlighted that even early ART initiation (at 30 h postpartum) cannot prevent the formation of a viral reservoir.^{8,9}

In infants who acquire HIV through breastfeeding, the virus is assumed to enter through the orogastrointestinal tract. However, the exact entry site(s) have not been clearly defined. Experimental SIV/SHIV infection of rhesus macaques recapitulates many relevant features of HIV transmission in humans, including MTCT, and can thus serve as a relevant model to study early virus-host interactions.¹⁰ In this study, we evaluated how oral exposure to immunodeficiency virus drives viral infection and dissemination, and establishment of the viral reservoir in infants. The oral and esophageal mucosa, as well as the tonsils were identified as primary entry

¹Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans, Louisiana.

²Department of Microbiology and Immunology and Center for AIDS Research, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

³Howard University, Washington, District of Columbia.

⁴AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland.

⁵California National Primate Research Center, University of California, Davis, Davis, California.

[†]Deceased.

sites in a previous study.¹¹ However, the former study included only three infant macaques and the virological analysis prioritized SIV DNA; only a limited number of tissues were analyzed for viral RNA.¹¹ This study expanded upon these findings and analyzed oral, intestinal, and systemic tissues for both viral RNA and DNA from 24 to 96 h after oral challenge of neonatal ($n=2$) or infant macaques ($n=15$) with pathogenic uncloned SIV_{mac251}. In addition, we mapped viral diversity and dissemination patterns to better understand the early events associated with oral SIV infection in neonates and infants.

Materials and Methods

Animals, housing and management

Infant rhesus macaques (*Macaca mulatta*) were vaginally delivered by colony dams from the SIV-negative and type D retrovirus-free colony at the California National Primate Research Center (CNPRC, Davis, CA) and nursery reared. The study was performed in accordance with the "Guide for Care and Use of Laboratory Animals" as outlined by the American Association for Assessment and Accreditation of Laboratory Animal Care. The UC Davis Institutional Animal Care and Use Committee approved all animal procedures before study initiation. Two of the study animals (Table 1) were neonates (3 days old) at the time of SIV infection. The remaining 15 infant macaques were between 3 and 8 weeks of age when they were randomly assigned to the different study groups (Table 1).

SIV inoculations

Animals received a single oral inoculation with SIV_{mac251} by slowly and atraumatically administering the virus into the mouth by a needleless 1 cc syringe. Each 1 ml dose contained 5×10^4 TCID₅₀ SIV_{mac251} (CNPRC stock 8/12), a stock that was propagated in rhesus peripheral blood mononuclear cells and previously found to be infectious. We chose a single high-dose oral challenge model to balance practical restric-

tions of infant macaque studies with a physiologically relevant SIV challenge route to result in an 80% infection rate, and to be able to time the euthanasia relative to a single exposure event. Two neonatal rhesus macaques were orally exposed to SIV at 3 days of age under light anesthesia (10 mg/kg ketamine-HCl; Parke-Davis, Morris Plains, NC) and euthanized at 24 h ($n=1$) or 48 h ($n=1$) after challenge. To more closely simulate natural virus exposure in human breastfeeding infants, 15 infant macaques between the ages of 3 to 8 weeks were not sedated during oral SIV challenge and euthanized at 48 h ($n=5$), 72 h ($n=5$), or 96 h ($n=5$) postchallenge (Table 1).

Sample collection and processing

At the indicated times of euthanasia, blood and tissue samples were collected (without PBS perfusion) and processed as described previously.^{12,13} Briefly, plasma was isolated by centrifugation from whole blood and stored at -80°C . Single tissue cell suspensions were prepared by homogenization [lymph nodes (LNs)], gradient centrifugation (whole blood, spleen), or by collagenase digestion and Percoll gradient centrifugation (mucosal tissues) according to standard protocols.^{12,13} Tissue samples for histological analysis were formalin-fixed and paraffin-embedded.

SIV detection by polymerase chain reaction

The presence of SIV RNA or DNA in tissues was determined with a sensitive, quantitative real-time polymerase chain reaction (qPCR) assay that targets a region in SIV *gag*, using primers and a TaqMan[®] probe (ThermoFisher) as previously described.^{12,14} Briefly, RNA and DNA were prepared from tissue samples (30 mg) cryopreserved in RNeasy lysis buffer (Qiagen[®]) using the Qiagen All Prep DNA/RNA mini kit and quantified by UV spectroscopy. Samples of purified RNA were diluted to 25 ng/ μl and utilized in replicate SIV-*gag* reverse transcriptase (RT)-qPCR assays that each contained 75 ng RNA. When sufficient RNA was available, replicate reactions were performed to screen $\sim 1.5 \mu\text{g}$ of RNA for SIV. Separate reactions targeting RPS13 mRNA as a reference gene were done with 25 ng RNA to verify the presence of amplifiable RNA. The average quantity of amplifiable RNA recovered from 1 million cells was $\sim 1 \mu\text{g}$ and this value was utilized for normalization of SIV RNA copies in tissues. The virus contained in plasma or cerebrospinal fluid (CSF) samples was isolated by centrifugation at $20,000 \times g$ for 60 min, followed by RNA purification with Trizol[®] reagent (ThermoFisher). RNA from the entire sample was utilized in replicate SIV RT-qPCR assays. DNA isolated from the tissues was analyzed in replicate reactions, each containing 300 ng of DNA, to screen $\sim 1 \times 10^6$ cells ($\sim 6 \mu\text{g}$ DNA) from each sample. SIV DNA copies were normalized to cell copy number determined in separate qPCR assays that target a single copy cellular gene.

SIV RNA detection was accomplished by performing RT reactions in a 10 μl volume, using the TaqMan-RT kit (Thermo-Fisher). The reaction was overlaid with qPCR reagents (15 μl) from the BioRad iQ[™] Supermix containing SIV *gag* primers (500 nM) and probe (250 nM). For analysis of DNA samples, the RT step was omitted, with a total qPCR volume of 25 μl . This qPCR assay is capable of detecting a single copy of SIV DNA or RNA, within the limits of Poisson

TABLE 1. STUDY ANIMALS

Animal no.	Oral SIV _{mac251} infection		Time of euthanasia (h) after SIV infection
	Age (days)	Anesthesia	
43464	3	Yes	24
43452	3	Yes	48
43557	20	No	48
43695	28	No	48
44895	33	No	48
44898	33	No	48
44229	53	No	48
43723	24	No	72
44888	25	No	72
43977	24	No	72
43518	24	No	72
44228	54	No	72
43909	30	No	96
43928	25	No	96
44886	25	No	96
44031	24	No	96
44034	22	No	96

distribution, utilizing the total RNA or DNA amounts specified in each reaction. Samples in which SIV was detected in >90% of replicate samples, SIV copy numbers were determined by extrapolation of average values obtained by a standard curve included in each assay. SIV copy numbers in samples with lower levels of detection were determined by designating each positive replicate as containing one SIV copy and dividing the fraction of positive samples by the quantity of nucleic acid surveyed. SIV RNA and DNA copies were expressed per 1 million cells, while SIV RNA copies in plasma and CSF were expressed as copies/ml fluid.

Distal lymphoid tissues, systemic tissues, brain, and plasma were analyzed by RT-qPCR as described above.^{12,14} Tissues of the oral cavity, regional LNs draining the oral cavity, the gastrointestinal tract, and the lung were analyzed by a quantitative hybrid real-time/digital PCR¹⁵ in 4 of the 15 infants: 1 in the 48-h (No. 44229) and 72-h (No. 44228) groups, and 2 in the 96-h group (Nos. 44031, 44034).

Founder virus genotype and stock virus analyses

Low levels of SIV in infant tissues prohibited amplification of full-length gp160 or gp120. Therefore, the envelope V1–V2 region was selected for the characterization of transmitted/founder virus genotypes. A 488 bp region spanning the V1–V2 region of gp120 was amplified in replicate, nested PCRs using limiting dilutions of sample DNA and the Q5 polymerase (New England Biolabs) with the following conditions: 98°C for 30 s, 25 cycles of 98°C for 7 s, 66°C/72°C for 12 s, 72°C for 20 s, and a final extension hold at 72°C for 2 min. First round primers were 5′-TGGAGGAATGCGA CAATCCCCCT-3′ and 5′-TCCATCATCCTTGTGCATG AAG-3′, with annealing temperature of 66°C, while second round primers were 5′-CAGTCACAGAACAGGCAATA GA-3′ and 5′-AAGCAAAGCATAACCTGGAGGT-3′, with annealing temperature of 72°C. Single genome PCR amplification (SGA) of this region was accomplished by diluting sample DNA to yield <30% positive reactions. The resultant 488 bp SGA amplicons were directly sequenced in both forward and reverse directions, and the resulting pair end reads were manually screened, assembled, and aligned using Geneious software v8.1.8.

The envelope genotypes contained within the SIV_{mac251} stock used for infant inoculations were characterized by single genome amplification and direct sequencing, using the methodology and primer pairs previously described.¹² Full-length gp160 sequences were obtained from 25 SGA amplicons generated from stock virus RNA. Fifteen additional sequences from SGA products spanning the gp120 region were also obtained using the same PCR and sequencing protocols described, but with the substitution of 3′ PCR primers located in gp41 for the nested PCR (first round reverse primer 5′-CCTGCTGTTGCGAGAAAACCCAGG-3′; second round reverse primer 5′-GTTGCTGTTGCTGCA CTATCCC-3′). A total of 40 sequences were obtained, assembled, and compared over the V1–V2 region. Twenty-two unique V1–V2 genotypes were identified in the stock and were used for comparisons. Founder viral variants obtained from infant tissues were compared with genotypes contained in the inoculum using Geneious software. Unique genotypes within each animal were utilized for intra-animal phylogenetic comparison. For phylogenetic comparisons, unrooted

Parsimonious (M.P.) analyses treating gaps as a new state were conducted using PAUP 4.0a150.

ViewRNA™ *in situ* hybridization and fluorescent immunohistochemistry

Tonsils, retropharyngeal LNs, mesenteric LNs, and brain sections of randomly selected animals with higher (>30 copies/10⁶ cells) or very low (<1 copies/10⁶ cells) SIV RNA detection by PCR were analyzed for SIV RNA-positive cells by ViewRNA (Affymetrix) according to the manufacturer's protocol using a commercially available SIV RNA probe specific for gag, pol, and nef. The phenotype of virally infected cells was determined by staining adjacent tissue sections for CD3 (T cells), CD68 (monocytes/macrophages), and CD20 (B cells) by three-color fluorescent immunohistochemistry (F-IHC), using DAPI as nuclear marker. We analyzed 10 randomly chosen (20×) microscope fields encompassing the cortex, paracortex, medulla, and lymphoid follicles in LNs. Brain sections were stained for CD68, glial fibrillary acidic protein (GFAP, an astrocyte marker), Ki-67 (a nuclear marker for activation and proliferation), and DAPI. Images were captured digitally on a Zeiss Axio microscope and analyzed with AxioVision Software (Rel 8.6). Negative controls included tissue sections stained with an isotype control antibody, sections without the primary antibody, and SIV-negative tissues. Positive controls included ViewRNA staining for β -actin and SIV RNA *in situ* hybridization (ISH) of LN sections from an infant macaque chronically infected with SIV (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid). In addition, each fluorescent signal for a specific marker was analyzed individually to exclude false-positive SIV signals due to color overlap.

T cell activation

CD4⁺ T cell populations in blood and tissues were characterized by flow cytometry for their activation using CCR5 and Ki-67 antibodies and their differentiation status, and defined as naive (CD3⁺CD4⁺CD45RA⁺CCR7⁺), central memory (CD3⁺CD4⁺CD45RA⁺CCR7⁺), or effector memory/effector (CD3⁺CD4⁺CD45RA⁺CCR7⁺) CD4⁺ T cells applying standard staining protocols as previously described.¹² All antibodies were obtained from BD Biosciences; samples were also stained with a viability dye (Invitrogen). A total of 300,000 cells were acquired on an LSRFortessa using FACSDiva v8.0 (BD) and analyzed with FlowJo Software v10.2 (TreeStar) applying fluorescence-minus-one controls.

Statistical analysis

Correlations between SIV RNA and SIV DNA were determined by Spearman's rank correlations using GraphPad Prism, Version 7 (GraphPad Software, Inc., La Jolla, CA), and exact *p*-values are reported.

Results

Oral SIV infection of neonates

In a pilot study, two neonatal macaques were orally exposed to SIV_{mac251} at 3 days of age and euthanized at 24 or 48 h after challenge. In the neonate examined at 24 h post-

SIV challenge (No. 43464), SIV RNA and SIV DNA were confined to the gastrointestinal tract (Figs. 1 and 2). In contrast, in the neonate euthanized 48 h (No. 43452) after SIV challenge, viral RNA was found in the retropharyngeal LNs and the colon, but the highest SIV RNA levels were detected in the lung and bronchial LNs (Fig. 1). These results suggest that viral entry in the latter neonate may have occurred not only in the gastrointestinal tract but also in the trachea after swallowing, a feasible entry route considering that the epiglottis is not fully functional at this age, and these two neonates were sedated at the time of virus inoculation.¹⁶ Alternatively, the virus may have entered through a small oral lesion and spread by the hematogenous route in this animal. The latter conclusion is supported by the finding that SIV RNA was detectable in the plasma and in lymphoid tissues more distal to the oral exposure site, such as the spleen and axillary LNs (Fig. 1).

Acute infection of infant rhesus macaques after oral SIV_{mac251} challenge, T cell activation, and founder virus genotypes

To more closely simulate breast milk acquisition of HIV, we opted to infect the remaining infant macaques at 3–8 weeks of age, corresponding to human infants <6 months, an age when breastfeeding is common and highly recommended in resource-poor countries, even in infants at risk for HIV infection. Furthermore, these infant macaques were not anesthetized during virus inoculation to simulate active drinking of breast milk by human infants. In three groups with five infant macaques each, we performed a comprehensive tissue analysis at 48, 72, and 96 h after oral SIV_{mac251} challenge. We grouped the various tissues based on anatomical location into the (I) exposure site, (II) LNs draining the exposure site, (III) the upper and lower gastrointestinal tract, (IV) lung and bronchial LNs, (V) distal lymphoid tissues, (VI) other tissue sites, and (VII) brain.

Oral SIV_{mac251} exposure resulted in virus acquisition in all 15 infant macaques (Figs. 1 and 2). While SIV DNA was detected as early as 48 h in at least one tissue of all animals, SIV RNA was only detected in tissues of three of five and four of five infant macaques at 48 and 72 h postinfection, respectively. Only by 96 h, 100% of the infant macaques tested positive for SIV RNA in at least one tissue (Figs. 1 and 2). The low viral RNA/DNA levels in most animals at these early time points restricted our analysis of founder virus genotypes. We were only able to obtain sequence data for one animal at each time point (48 h: No. 44895, 72 h: 43518, 96 h: No. 44034). The sequences of virus in these three animals were identical or very similar, and they were highly homologous to the SIV_{mac251} stock genotype G12 (Table 2 and Fig. 3).¹² This G12 stock genotype comprised ~7.5% of the variants identified in our analyses of 40 endpoint diluted RT-PCR products from the stock.¹²

In addition, we were able to obtain viral sequences from the neonatal macaque No. 43452. This was the only animal in which two different viral genotypes were found. One variant was found in the spleen (variant 1) and the other in the lung (variant 2), with both variants being present in the plasma (Fig. 3). These stock genotypes, G10 and G2, comprise roughly 15% and 10% of the stock inoculum, respectively. The presence of two unique stock genotypes in separate tis-

ues at only 48 h post-SIV infection suggests that infection likely resulted from transmission of distinct genotypes at different anatomical sites. In this animal, the highest levels of SIV RNA were found in the lung, the bronchial-mediastinal LNs, and the retropharyngeal LNs, suggesting that these tissues were likely the earliest sites of replication and dissemination. However, tissue limitations precluded our ability to obtain envelope sequences from the LNs.

To determine whether the frequencies of available CD4⁺ T cell targets were associated with more frequent infection of specific tissues, we assessed T cell frequencies and activation in blood, lymphoid, and intestinal tissues. We specifically focused on the presence of CCR5⁺ CD4⁺ T cells as these serve as the main target cells for SIV_{mac251}. As expected, the highest frequencies of CCR5⁺ CD4⁺ T cells were found in the ileum and colon, whereas the lowest frequencies were observed in blood (Supplementary Fig. S2A). Frequencies of CCR5⁺ CD4⁺ T cells in spleen and LNs were comparable (median frequencies between 0.1% and 0.4% of total CD4⁺ T cells). Similarly, median frequencies of Ki-67⁺CD4⁺ T cells were highest in intestinal tissues and lowest in LNs (Supplementary Fig. S2B). Consistent with the highest median frequencies of activated CD4⁺ T cells in intestinal tissues, the ileum and colon also had higher percentages of effector/effector memory CD4⁺ T cells than the other lymphoid tissues analyzed (Supplementary Fig. S2C). Tonsillar tissue was most similar to intestinal tissues with regard to T cell activation and the relative distribution of naive and memory CD4⁺ T cell populations. However, cell numbers in tonsils of most animals were insufficient to define T cell populations using flow cytometry. Due to animal-to-animal variation and relatively small group sizes, we did not observe statistically significant differences in T cell activation or differentiation between animals euthanized at different time points post-SIV infection or between SIV RNA-negative and SIV RNA-positive animals (Supplementary Fig. S2).

Virus dissemination after oral SIV_{mac251} infection of infant macaques

In the first 48–72 h, SIV RNA was rarely detected in tissues near the exposure site, such as the cheek pouch or the sublingual mucosa; only a single animal at 72 h had detectable levels of SIV RNA in the tongue, although at low levels. Instead, the highest SIV RNA levels were observed in LNs draining the oral cavity, with measurable virus in draining nodes of 5 of 10 (50%) of the 48–72 h animals (Figs. 1 and 4A). SIV RNA in the gastrointestinal tract was only detected in a single infant macaque at 48 h (No. 44895). However, by 72 h, seeding of the gastrointestinal tract became more prevalent, with detectable virus in the ileum from three of five animals (Figs. 1 and 4A). Distal lymphoid tissues rarely tested positive for SIV RNA at these early time points (Fig. 1). In fact, within the first 72 h, no more than five tissues per animal had measurable SIV RNA. In contrast, as many as 16 tissues tested SIV RNA positive by 96 h, and 2 of 5 infants had detectable plasma viremia at this time (Figs. 1 and 4A). Despite the detection of SIV RNA in all infants by 96 h, with the exception of No. 44034, SIV RNA levels did not exceed 100 SIV RNA copies/10⁶ cell equivalents (Fig. 1). Overall, in the 15 infants tested over the period of 48 to 96 h after oral SIV infection, SIV RNA was most frequently detected in

Anatomic Site	Tissue	Neonates		Infant Macaques														
		24 h	48 h	48 h		72 h		96 h		96 h								
		43464	43452	43557	43695	44895	44898	44229*	43723	44888	43977	43518	44228	43909	43928	44886	44031	44034
Exposure Site	Cheek Pouch	<1**	<1	<0.3	<0.3	<0.6	<0.6	n.t.	<0.3	<0.8	<0.4	<0.3	n.t.	<0.3	<0.3	<1	n.t.	n.t.
	Tongue	<1	<1	<0.3	<0.3	<0.6	<0.6	n.t.	<0.5	0.9	<0.3	<0.3	n.t.	<0.3	<0.3	<0.6	3	7
	Subling. Muc.	<1	<1	<0.3	<0.3	<0.6	<0.6	<0.4	<0.3	<0.6	<0.5	<0.3	<0.5	<0.3	<0.3	52	0.7	4
	Gingiva	<1	<1	<0.3	<0.3	<0.6	<0.6	<0.3	<0.3	<0.6	<0.3	<0.3	<1.8	<0.3	<0.3	<0.6	3	2
	Parotid SG	<1	<1	<0.3	<0.3	<0.6	<0.6	<0.2	<0.5	<0.6	<0.3	<0.3	<0.3	<0.3	<0.3	<1	0.5	1
	Submand SG	<1	<1	<0.3	<0.3	<0.6	<0.6	<0.7	<0.3	<0.6	<0.3	<0.3	<0.1	<0.3	<0.3	<0.6		
Draining LNs	Tonsil	<0.8	<0.5	<0.3	<0.3	<0.6	<0.6	<0.3	<0.3	<1	<0.3	0.6	31	<0.5	<0.3	<1	0.3	11000
	Submandibular	<1	<1	<0.4	<0.3	3	0.8	<0.1	<0.3	<0.6	<0.3	<0.3	<0.1	<0.3	<0.3	<0.6	<0.1	2800
	Retropharyng.	<1	7	<0.3	<0.5	<0.6	<1	0.2	<0.3	<0.6	<0.3	100	<0.1	<0.3	0.3	1	0.9	41000
	Submental	<1	<1	<0.3	<0.5	30	<1	<0.1	<0.5	<1	<1	<0.9	<0.7	<0.3	<0.7	<0.6	0.1	12000
	Cervical	<1	<1	<0.3	<0.3	1	<0.6	<0.1	<0.3	<0.6	<0.4	<0.3	<0.1	<0.3	<0.3	<0.6	3.3	17000
GI Tract	Esophagus	<1	<1	<0.3	<0.3	<0.6	<0.6	<0.9	<0.3	<0.6	<0.5	<0.3	<0.2	<0.3	<0.3	<1	4	9
	Ileum	<0.5	<0.5	<0.3	<0.3	<0.6	<0.6	<0.1	<0.3	2	0.7	0.3	<0.2	<0.3	<0.3	<0.6	0.3	1000
	Jejunum	0.6	<0.5	<0.3	<0.3	1	<0.6	<0.1	<0.3	<0.6	<0.3	<0.3	<0.4	<0.3	0.3	<0.6	0.9	2
	Colon	<1	0.6	<0.3	<0.3	<0.6	<0.6	<0.3	<0.3	<0.6	<0.3	<0.3	<0.3	<0.3	<0.3	<0.6	1	310
	Mesenteric LNs	3	9	<0.3	<0.3	<0.6	<0.6	<0.1	<0.3	<0.6	<0.3	<0.3	<0.1	0.6	0.2	<0.6	0.6	5900
Lung	Lung	<1	10	<0.3	<0.3	<0.6	0.9	<0.1	<0.3	<0.6	<0.3	0.6	<0.5	<0.5	<0.3	<0.6	0.4	880
	Bronchial LNs	<1	8	<0.3	<0.3	<0.6	<0.6	n.t.	<0.5	0.5	<0.5	<0.3	n.t.	<0.3	<0.5	<0.6	n.t.	n.t.

Distal Lymphoid Tissues	Spleen	<1	1	<0.3	<0.3	<0.6	<0.6	<3	<0.3	<0.6	<0.3	<0.3	<5	<0.3	0.1	<0.6	2	13
	Axillary LNs	<1	1	<0.3	<0.3	0.7	<0.6	<3	<0.3	1	<0.5	<0.3	<5	<0.3	<0.3	<0.6	2	<5
Systemic Tissues	Liver	<1	<1	<0.3	<0.3	<0.6	<1	<3	<0.3	1	<0.3	<0.3	<2	<0.3	<0.3	<1	<2	<2
	Thymus	<1	<1	<0.3	<0.3	<0.6	<1	<3	<0.3	<0.6	<0.3	<0.3	<2	<0.3	<0.3	<0.6	<2	<2
	Bone Marrow	<1	<1	<0.3	<0.3	<0.6	<1	<3	<0.3	<0.6	<0.3	<0.4	<5	<0.7	<0.3	<0.6	<5	<5
Brain	Cerebrum	<1	<1	<0.3	<0.3	<0.6	<0.6	<1	<0.3	<0.6	<0.3	<0.3	<1	<0.3	<0.3	<0.6	32	<1
Plasma***		0	149	0	0	0	0	0	0	0	0	77	0	0	12	0	0	8700

FIG. 1. SIV RNA RT-qPCR results. Heatmap of the SIV RNA quantitated in different tissues of infant macaques euthanized between 24 and 96 h after oral SIV exposure. SIV RNA results are reported as copy numbers per 1 million cell equivalents; in plasma, SIV RNA copies are reported per ml of plasma.

Anatomic Site	Tissue	Neonates		Infant Macaques									
		24 h	48 h	48 h					72 h				
		43464	43452	43557	43695	44895	44898	44229*	43723	44888	43977	43518	44228
Exposure Site	Cheek Pouch	<5**	<5	<5	<5	<1	<0.7	<1	<0.8	<0.7	2	20	0.2
	Tongue	<5	<5	<3	<1	0.6	2	n.t.	<1	1	<5	<5	n.t.
	Subling. Muc.	<5	<5	<5	<5	0.7	<0.7	4	<1	<0.8	<5	<5	<0.5
	Gingiva	<5	<5	<5	<5	0.9	2	<0.3	<5	<1	<5	<5	<0.4
	Parotid SG	<5	<2	<5	<5	<0.7	0.5	0.8	<3	1	<5	<5	2
Draining LNs	Submand SG	<5	<5	<5	<5	<0.7	<0.7	0.4	<3	<1	<5	<5	0.7
	Tonsil	<5	<5	<1	<5	<0.7	<1	<0.5	<0.8	<0.7	2	20	0.2
	Submandibular	<5	<5	0.8	0.4	3	7	<0.7	<0.8	<0.7	4	<0.7	<0.6
	Retropharyng.	<2	<2	<1	<1	0.4	3	<0.3	<3	<1	2	4	<0.7
	Submental	<5	<5	<5	<5	1	3	<1	<3	8	<5	<5	<1.5
GI Tract	Cervical	<5	<2	<5	<5	4	<1	<0.6	<5	3	<0.3	<0.1	<0.4
	Esophagus	<5	<5	<5	<5	<0.7	1	<2	<3	<1	<5	<5	0.3
	Ileum	0.3	<0.5	<0.4	0.6	2	<0.7	2	<0.3	9	0.1	2	<0.4
	Jejunum	6.0	<1	<1	<0.8	2	1	2	<0.8	<0.7	<0.8	<1	0.3
	Colon	<5	0.6	<5	<5	<0.7	<0.7	<0.6	<0.8	<0.8	<5	<0.3	0.4
Lung	Mesenteric LNs	180	290	<0.7	0.5	<0.7	<0.7	<0.4	0.4	1	<0.4	<5	<0.2
	Lung	<1	100	<1	<0.8	0.5	5	0.3	<0.8	<0.7	<1	6	0.1
	Bronchial LNs	<5	80	<1	<0.8	<1	<0.7	n.t.	<0.8	<0.7	<1	<1	n.t.

Distal Lymphoid Tissues	Spleen Axillary LNs	<1	3	<1	<0.8	<1	<0.7	<2	<0.8	<0.7	3	1	<2	<1	<1	<1	90	50
		<1	1	<1	<0.8	<0.7	0.9	<2	<0.8	<0.8	<0.8	<1	<2	<1	<1	<1	2	68
Systemic Tissues	Liver	<5	<5	<5	<2	<0.7	<1	<2	<0.8	<0.8	39	<5	2	<1	<5	<1	2	2
	Thymus	<1	<5	<5	<5	<0.7	<0.7	<1	<3	<0.8	<5	<5	<2	<1	<5	<1	<1	<1
	Bone Marrow	<1	<1	<1	<0.8	<0.7	<0.7	<1	<0.8	<1	0.9	<0.7	<2	<1	<0.7	<1	2	<1
	Cerebrum	<1	<1	<1	<0.8	<1	1	3	<0.8	2	4	36	2	<0.7	<1	8	3	65

FIG. 2. SIV DNA PCR results. Heatmap of the SIV DNA quantitated in different tissues of infant macaques euthanized between 24 and 96 h after oral SIV exposure. SIV DNA results are reported as copy numbers per 1 million cell equivalents.

TABLE 2. FOUNDER VIRUS GENOTYPES

Animal	Time	No. of SIV RNA ⁺ tissues ^a	Tissues with SIV sequences ^b	Diversity among sequences, %	No. of founder genotypes	Founder virus identity with SIV stock variant
43452	48 h	7	Plasma (6)	0.3–0.8	2	Variant 1 = G10 Variant 2 = G2
			Lung (5)	0	1	Variant 2 = G2
			Spleen (2)	0	1	Variant 1 = G10
44895	48 h	5	Ileum (5)	0	1	G12
43518	72 h	5	Tonsil (6)	0	1	G12
44034	96 h	17	Tonsil (6)	0	1	G12
			Submandibular LN (2)	0	1	G12
			Retropharyngeal LN (2)	0	1	G12
			Cervical LN (4)	0	1	G12
			Lung (6)	0	1	G12
			Spleen (2)	0	1	G12
			Axillary LN (1)	0	1	G12
			Plasma (7)	0	1	G12

^aSIV RNA⁺ tissues as shown in Table 2, including plasma.

^bNumbers in parentheses represent viral sequences found/tissue. LN, lymph node.

retropharyngeal LNs (6 of 15 animals) (Figs. 1 and 4A). The ileum was the second most common tissue in which viral RNA was detected (5 of 15 animals). Thus, although the pattern of SIV RNA distribution and the levels of tissue viral RNA varied among the animals at the time points evaluated,

early virus replication occurred primarily in LNs draining the oral cavity or in the GI tract, and at later times postchallenge, the virus became more prominent in the periphery (Figs. 1 and 4A). In addition, it should be noted that virus detection within the first 96 h of SIV infection was also relatively

FIG. 3. Virus diversity. Phylogenetic tree of the 22 unique envelope V1–V2 genotypes comprising the SIV_{mac251} inoculum and the genotypes found in four orally inoculated infants (Nos. 43518, 44034, 44895, and 43452). Genotypes from the virus stock represent the unique sequences obtained from a total of 40 genotypes, which were derived by endpoint RT-PCR amplification of the RNA prepared from the SIV_{mac251} inoculum stock. The number in parentheses following the stock name indicates the number of sequences obtained with identical V1–V2 nucleotide sequences. Viral genotypes were determined in one animal from each time point (48 h: No. 44895; 72 h: No. 43518; and 96 h: No. 44034). In each of the animals, a single viral genotype was identified, which was identical or most closely related to stock genotype MAC251-G12 (outlined by blue box). In the neonatal macaque No. 43452 (48 h), two different genotypes (defined as v1 and v2) were identified, with v1 or v2 (boxes with dashed red lines) being identical to stock genotypes G10 or G2, respectively. The bar at the bottom indicates the phylogenetic distance. RT-PCR, reverse transcriptase-PCR.

FIG. 4. SIV detection in tissues between 48 and 96 h after oral SIV challenge. (A, B) Show the number of animals with detectable SIV RNA (A) or SIV (DNA) in each tissue at 48, 72, or 96 h.

common in the lung (4 of 15 infant macaques). This may have been due to partial aspiration of the viral inoculum in non-sedated infants at the time of challenge. However, we cannot exclude the possibility that SIV RNA detection in the lung might have been due to the presence of SIV-infected cells present in blood and/or lymph vessels, because the lung was not perfused at the time of euthanasia.

Tissue distribution of SIV DNA in the first 96 h after oral SIV challenge

While the number of animals with SIV RNA-positive tissues showed a more gradual increase from 48 to 96 h, the number of animals with SIV DNA in a specific tissue was more similar at all time points (Fig. 4B). Despite a wider range in the number of tissues that were SIV DNA positive by PCR at the different time points (1 to 11 tissues at 48 h; 1 to 8 tissues at 72 h; and 1–20 tissues at 96 h), the amount of SIV DNA increased over time. Thus, at 48 h, SIV DNA levels between 1 and 5 copies/ 10^6 cells were only detected in 2 animals, whereas at 72 h, SIV DNA up to 40 copies/ 10^6 cells could be detected in 4 of the 5 infants (Fig. 2). Tissues containing more than 100 copies/ 10^6 cells were not observed before 96 h (Fig. 2). Consistent with the SIV RNA tissue distribution pattern, SIV DNA was most frequently found in LNs draining the oral cavity, especially the retropharyngeal (8 of 15 infants) and submandibular LNs (9 of 15 infants), and in the ileum (8 of

15 infants). Similarly, more than 50% of the animals tested SIV DNA positive in the lung (Fig. 2). Although SIV DNA detection in tissues more distal to the oral cavity was still a relatively rare event, SIV DNA was detectable in liver and bone marrow in a few infant macaques (Fig. 2). Across all time points, there was a positive correlation between the number of animals that tested PCR positive for SIV RNA and SIV DNA ($p < .0001$; $r = 0.559$), and between the number of SIV RNA- and SIV DNA-positive tissues at specific anatomic sites ($p < .03$; $r = 0.886$). Although there was also a correlation between SIV RNA copies and SIV DNA copies ($p < .0007$; $r = 0.18$) throughout all time points, this correlation was strongest at 96 h ($p < .0001$; $r = 0.417$).

Early SIV detection in the brain

HIV entry into the brain of infants may have a detrimental impact on brain development.¹⁷ Therefore, we determined whether oral SIV infection in infant macaques resulted in rapid virus entry into the brain. Of the 15 infant macaques analyzed within 48–96 h of oral SIV exposure, only 1 animal (No. 44031) tested positive for SIV RNA in the cerebrum after 96 h (Fig. 1). In contrast, SIV DNA became detectable in the brain as early as 48 h after oral SIV challenge (Fig. 2). At 48 h post-SIV exposure, SIV DNA was detected in the cerebrum of 40% of the infants (2 of 5), and this percentage increased to 67% (4 of 6) and 60% (3 of 5) at 72 and 96 h, respectively. Thus, SIV

FIG. 5. SIV detection in LNs. (A, B) Show representative images (40 $\times$ magnification) of tonsil and mesenteric LN sections, respectively, that were stained for SIV RNA (yellow), T cells (CD3; green), B cells (CD20; red), or macrophages (CD68; red), and for the nuclear marker DAPI (blue). The dashed white lines indicate the LN border. SIV RNA-positive cells are marked by yellow circles. The larger image shows the overlay, and the smaller panels to the right show each fluorescent label alone. (A) Shows tonsil sections from the 72-h animal No. 43518 with an SIV-positive CD3⁺ T cell, which is shown enlarged in the bottom right panel. (B) Shows a mesenteric LN section of animal No. 44895 to illustrate that SIV RNA in LNs was exclusively found in T cells and not in macrophages. Note that SIV RNA-positive cells were frequently found at the border between the T cell zones and the B cell follicles. LN, lymph node.

DNA detection in the cerebrum was as frequent as in the submandibular LNs and ileum, tissues that would be expected to serve as oral entry sites (Fig. 4B). However, despite SIV DNA detection in the brain, the CSF of all animals was negative for SIV RNA (data not shown), consistent with the general lack of SIV RNA detection in the cerebrum.

Phenotype of virally infected cells in tissues

To identify the phenotype of virus-infected cells, we performed ViewRNA analysis on randomly selected brain sections and LNs. Consistent with relatively low SIV RNA copy numbers by PCR, only a few isolated virus-infected cells were found in tissue sections. However, it should be noted that SIV-positive cells could be identified, even in tissues expressing a single SIV RNA copy per 10⁶ cell equivalents (e.g., tonsil No. 43518, Fig. 5A). In LNs, SIV-infected cells were exclusively represented by CD3⁺ T cells. No SIV RNA-positive macrophages (CD68⁺) were detected (Fig. 5B). SIV-positive T cells appeared to be randomly distributed as they were not confined to a specific anatomic location within the LN.

In the only animal that tested RT-PCR positive for SIV RNA in the cerebrum (No. 44031), SIV RNA could also be detected by ISH in the brain, although the phenotype of the SIV RNA-positive cells could not be identified (Fig. 6A), because these cells did not express the macrophage marker CD68 or the astrocyte marker GFAP (Fig. 6). SIV RNA-positive cells in proximity to blood vessels, however, expressed a macrophage phenotype (Fig. 6B).

Discussion

Despite the optimization of preventative measures of MTCT of HIV, about 400 infants acquire HIV every day,¹ with the majority occurring by breastfeeding.^{2–4} HIV-

infected infants who do not receive ART show a mortality rate of about 50% by their second year of life.^{18,19} Even if infants are started on ART, suppression of viremia is generally delayed compared to adults.^{6,20,21} The relatively late diagnosis of infants who acquire HIV infection through breastfeeding further permits virus replication and virus dissemination throughout the body, and thereby the development of viral reservoirs at multiple anatomic sites. In this study, infant macaques at 3 days to 8 weeks of age were orally infected with pathogenic SIV_{mac251} to simulate breast milk transmission of HIV in human infants to determine which tissues served as early replication sites and whether systemic virus dissemination was detectable by 4 days postinfection. Our results suggest that orally administered SIV_{mac251} can enter and disseminate by different routes within days, and can be found in multiple proximal and distal tissues. Despite a variable quantity and distribution of SIV RNA among the animals at all time points evaluated, the virus appeared to replicate early on primarily in regional LNs draining the oral mucosa, spreading throughout the GI tract and disseminating systemically. These findings are consistent with an earlier report that showed SIV DNA in tissues distal to the stomach by day 1 or in regional LNs by day 2 after oral SIV challenge.¹¹ Overall, the pattern of virus dissemination after oral SIV exposure in infant macaques closely resembled the kinetics of SIV spread in juvenile macaques after oral SIV infection.¹¹ Thus, infection in the first 48 h, infection, as assessed by PCR detection of SIV RNA or DNA, is most consistently observed in tonsils and LNs draining the oral cavity, with the small and large intestine becoming prominent sites of virus replication by 72–96 h, followed by pronounced systemic dissemination to peripheral LNs and spleen.¹¹

Target cell availability was likely a key factor in determining the sites of early virus replication. Although infants

FIG. 6. SIV-positive cells in the cerebrum. Sections of the central sulcus in the areas of the primary motor and somatosensory cortices were immunostained with antibodies for Ki-67, CD68, and GFAP, and for SIV RNA (ISH). **(A)** The larger image represents an overlay of ISH and IHC images. The *dashed white line* indicates the tissue interphase. The *white diamonds* surround GFAP-positive cells that appear false positive for SIV RNA. This is demonstrated by the enlarged images in the *bottom panels* that show (from left to right) SIV RNA, CD68/SIV RNA overlays and the GFAP/SIV RNA overlay. The false-positive *yellow signal* is due to color overlay for *green* CD68 and *red* GFAP-positive cells. In contrast, *yellow circles* highlight SIV RNA-positive cells. The *yellow box* in the larger image shows five nucleated cells that did not stain positive for CD68 or GFAP, but one of the cells contained SIV RNA. The individual stains for DAPI and SIV RNA are shown enlarged in the smaller *right panels*. **(B)** In the cerebrum of the 96-h animal No. 44034, an SIV RNA-positive macrophage was detected. However, the pia mater may have been also lined by a blood vessel. GFAP, glial fibrillary acidic protein; ISH, *in situ* hybridization.

have primarily naive CD4⁺ T cells in peripheral blood, we and others have demonstrated that infant macaque tissues harbor activated CD4⁺ T cells, and many of these activated CD4⁺ T cells express a memory phenotype.^{22–25} This study confirms that the relative frequencies of naive CD4⁺ T cells are lowest, while memory CD4⁺ T cells are highest in gastrointestinal tissues and tonsils compared to LNs, spleen, and blood. A similar relationship between gastrointestinal tissues and tonsils compared to other tissues was observed for activated cells. Thus, although not statistically significant, the presented data imply that early replication of SIV in the GI tract was facilitated by higher target cell availability. In fact, the Veazey laboratory had previously reported that SIV_{mac251} preferentially replicates in activated, proliferating CD4⁺ T cells of the intestine.^{24,25}

Generally, mucosal HIV infection is established by a single transmitted founder virus.^{26,27} Transmission studies in adult macaques have confirmed that both the vaginal and rectal mucosa serve as a bottleneck and systemic infection is established by a single or few transmitted founder viruses.^{28–30} Evidence for a similar genetic bottleneck in HIV MTCT by breastfeeding was recently demonstrated by examining paired mother-infant samples.³¹ Although this study was not designed to assess viral diversity, the few viruses that could be detected in these acutely infected infant macaques exhibited very limited diversity, a finding consistent with an earlier oral SIV_{mac251} infection study in infant macaques.^{32,33} In only a single animal were two distinct viral variants transmitted. We do not know if only one of these variants might have established persistent infection in this neonate because all animals in our study were euthanized for tissue analyses.

A number of animals tested PCR positive for SIV RNA or DNA in the lung and brain. With the caveat that these tissues were not perfused before collection, the question arises as to how the virus gained rapid entry into these anatomic sites. Lymphatic or hematogenous spread of virus represents obvious potential routes for viral entry into both lung and brain tissues. Alternatively, it is possible that the orally administered virus was swallowed, and, because the epiglottis of infants is not yet fully functional,¹⁶ the virus was aspirated into the trachea and entered the lung. The rapid detection of SIV in nasopharyngeal and lung tissues has also been observed in adult macaques, but, in contrast to our study, the adult macaques were infected intravenously, thereby allowing easier systemic dissemination of the virus.³⁴ Nonetheless, these data suggest that independent of age and route of virus acquisition, mucosal sites other than the intestinal tissues might be rapidly seeded.

SIV entry into the brain could have occurred by hematogenous spread and by breaching the blood–brain barrier (BBB) or the blood-CSF-barrier, although the virus was not detected in CSF. In neonates and young infants, the BBB is still developing and susceptible to neuroinflammation, which can result in damage to tight junctions³⁵ and allow easier access for pathogens, such as HIV.³⁶ However, a previous study demonstrated the presence of SIV DNA in the brain of juvenile rhesus macaques as early as 1 day after oral SIV infection.³⁷ This latter finding argues against a more rapid and/or facilitated entry of SIV or HIV into the brain of infants compared to adults. The oral mucosa itself provides direct access for virus uptake through the rich regional network of the Waldeyer's ring that is part of the systemic lymphatic

network. In addition to the tonsils and adenoids that are an integral part of the Waldeyer's ring, the submental, submandibular, retropharyngeal, and cervical LNs also drain the oral mucosa. In close anatomic proximity, the buccal, parotid nodes, and occipital nodes drain the head and neck area.^{38–40} Together, these tissues and LNs represent an integrated and accessible network of organized lymphatic tissue that could facilitate viral dissemination. Furthermore, there is a direct lymphatic connection between the nasal mucosa and the CSF through the cribriform plate.⁴¹ In fact, the nasal route has been explored as a delivery route for drugs targeting the central nervous system.⁴²

We acknowledge and want to reiterate that SIV RNA was detected in the cerebrum of only one animal using PCR, but by ISH, SIV RNA-positive cells were detected in the cerebrum of other animals, and 9 of the 15 animals had detectable SIV DNA in the cerebrum. The discordance between the detection of SIV RNA-positive cells in tissue sections by ISH and SIV RNA detection by RT-PCR is not totally unexpected. In fact, we have made similar observations in a vaginal transmission study.⁴³ The first 96 h after mucosal SIV infection the lag phase represents during which the virus is entering and starting to replicate in a few cells, but virus production is still too low to allow spreading to multiple, more distal target cells and widespread dissemination. Therefore, the detection of virus-positive cells depends on whether or not the part of the tissue subjected to PCR analysis or ISH contained these few early infected cells. This phenomenon has been elegantly visualized by the Haase laboratory by overlaying several serial sections of vaginal tissue sections of adult macaques early after vaginal exposure.^{44,45} Of note, despite the apparent discordance in SIV RNA and SIV DNA detection in this study, across all time points and animals, the number of animals and tissues positive for SIV RNA was positively correlated to the number of SIV DNA-positive animals and tissues.

Although these results should not be overinterpreted, their potential significance warrants further exploration. It is well documented that HIV-infected infants show neurodevelopmental delays, especially in motor and cognitive function.^{46–48} Impaired neurodevelopment in HIV-infected infants has been associated with HIV viremia at the time of ART initiation.^{48,49} Similarly, we recently showed that infant rhesus macaques orally infected with SIV_{mac251} have reduced neuronal populations and regional volumes in the hippocampus.¹⁷ As SIV_{mac251} represents an uncloned virus swarm and was not specifically propagated to develop neurotropism, this result underlines the importance of potential early damage to the CNS after SIV/HIV infection. In this study, the phenotype of SIV RNA-positive cells could not be conclusively determined. There are relatively few neurohistological studies of pediatric HIV infection, but there is evidence of astrocyte infection.⁵⁰ Astrocytes represent the most abundant cells in the brain, but their ability to support active virus replication remains highly controversial.^{51–53} Indeed, other studies reported HIV infection of neurons, microglia cells, and macrophages.⁵⁴ Thus, the question remains whether SIV can quickly establish a viral reservoir in the infant brain and if so, in which cells. Addressing this issue was beyond the scope of our study; recovery of replication-competent virus was not attempted. Proof of productive HIV infection in the brain, persistence of virus replication, and the presence of a viral

reservoir should be the focus of future studies to gain deeper insights into pediatric HIV neuropathogenesis, which would then pave the way for intervention therapies.

Overall, this study provides evidence for rapid virus uptake and dissemination in infant macaques after oral challenge. Within the first 96 h of infection, the virus can be detected in local and distal lymphoid and mucosal tissues, and even the brain. The results are consistent with an earlier study that examined virus spread after oral SIV infection in three neonatal macaques.^{11,37} In the latter study, the kinetics of virus dissemination to the brain in neonatal macaques were undistinguishable from those in juvenile macaques. Thus, early CNS entry cannot be solely attributed to a more immature BBB function in infants and might be a common feature of both infant and adult HIV infection. The early detection of virus RNA or DNA in lung and brain tissues emphasizes the need for early diagnosis and treatment of pediatric HIV infection to prevent damage to not only the immune system but also the respiratory tract and central nervous system, and to reduce the magnitude of viral reservoirs that are established very early after infection⁵⁵ and are the focus of cure research.

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Author Disclosure Statement

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References

1. www.unaids.org: Global Plan 2016: On the fast-track to an AIDS-free generation. www.unaids.org/sites/default/files/media_asset/GlobalPlan2016_en.pdf, accessed September 2016.
2. Moland KM, de Paoli MM, Sellen DW, van Esterik P, Leshabari SC, Blystad A: Breastfeeding and HIV: Experiences from a decade of prevention of postnatal HIV transmission in sub-Saharan Africa. *Int Breastfeed J* 2010;5:10.
3. Rollins N, Coovadia HM: Breastfeeding and HIV transmission in the developing world: Past, present, future. *Curr Opin HIV AIDS* 2013;8:467–473.
4. Rollins NC, Ndirangu J, Bland RM, Coutoudis A, Coovadia HM, Newell ML: Exclusive breastfeeding, diarrhoeal morbidity and all-cause mortality in infants of HIV-infected and HIV uninfected mothers: An intervention cohort study in KwaZulu Natal, South Africa. *PLoS One* 2013; 8:e81307.
5. Luzuriaga K: Early combination antiretroviral therapy limits HIV-1 persistence in children. *Annu Rev Med* 2016; 67:201–213.
6. Persaud D, Palumbo PE, Ziemniak C, Hughes MD, Alvero CG, Luzuriaga K, Yogev R, Capparelli EV, Chadwick EG: Dynamics of the resting CD4(+) T-cell latent HIV reservoir in infants initiating HAART less than 6 months of age. *AIDS* 2012;26:1483–1490.
7. Persaud D, Patel K, Karalius B, Rainwater-Lovett K, Ziemniak C, Ellis A, Chen YH, Richman D, Siberry GK, Van Dyke RB, Burchett S, Seage GR, 3rd, Luzuriaga K; Pediatric HIV/AIDS Cohort Study: Influence of age at virologic control on peripheral blood human immunodeficiency virus reservoir size and serostatus in perinatally infected adolescents. *JAMA Pediatr* 2014;168:1138–1146.
8. Luzuriaga K, Gay H, Ziemniak C, Sanborn KB, Somasundaran M, Rainwater-Lovett K, Mellors JW, Rosenbloom D, Persaud D: Viremic relapse after HIV-1 remission in a perinatally infected child. *N Engl J Med* 2015;372:786–788.
9. Persaud D, Gay H, Ziemniak C, Chen YH, Piatak M, Jr., Chun TW, Strain M, Richman D, Luzuriaga K: Absence of detectable HIV-1 viremia after treatment cessation in an infant. *N Engl J Med* 2013;369:1828–1835.
10. Van Rompay KK, Jayashankar K: Animal models of HIV transmission through breastfeeding and pediatric HIV infection. *Adv Exp Med Biol* 2012;743:89–108.
11. Milush JM, Kosub D, Marthas M, Schmidt K, Scott F, Wozniakowski A, Brown C, Westmoreland S, Sodora DL: Rapid dissemination of SIV following oral inoculation. *AIDS* 2004;18:2371–2380.
12. Jensen K, Nabi R, Van Rompay KK, Robichaux S, Lifson JD, Piatak M, Jr., Jacobs WR, Jr., Fennelly G, Canfield D, Mollan KR, Hudgens MG, Larsen MH, Amedee AM, Kozlowski PA, De Paris K: Vaccine-elicited mucosal and systemic antibody responses are associated with reduced simian immunodeficiency viremia in infant rhesus macaques. *J Virol* 2016;90:7285–7302.
13. Marthas ML, Van Rompay KK, Abbott Z, Earl P, Buonocore-Buzzelli L, Moss B, Rose NF, Rose JK, Kozlowski PA, Abel K: Partial efficacy of a VSV-SIV/MVA-SIV vaccine regimen against oral SIV challenge in infant macaques. *Vaccine* 2011;29:3124–3137.
14. Robichaux S, Lacour N, Bagby GJ, Amedee AM: Validation of RPS13 as a reference gene for absolute quantification of SIV RNA in tissue of rhesus macaques. *J Virol Methods* 2016;236:245–251.
15. Hansen SG, Piatak M, Jr., Ventura AB, Hughes CM, Gilbride RM, Ford JC, Oswald K, Shoemaker R, Li Y, Lewis MS, Gilliam AN, Xu G, Whizin N, Burwitz BJ, Planer SL, Turner JM, Legasse AW, Axthelm MK, Nelson JA, Fruh K, Sacha JB, Estes JD, Keele BF, Edlefsen PT, Lifson JD, Picker LJ: Immune clearance of highly pathogenic SIV infection. *Nature* 2013;502:100–104.
16. Crompton AW, German RZ, Thexton AJ: Development of the movement of the epiglottis in infant and juvenile pigs. *Zoology (Jena)* 2008;111:339–349.
17. Carryl H, Van Rompay KK, De Paris K, Burke MW: Hippocampal neuronal loss in infant macaques orally infected with virulent simian immunodeficiency virus (SIV). *Brain Sci* 2017;7, 40.

18. De Rossi A, Masiero S, Giaquinto C, Ruga E, Comar M, Giacca M, Chieco-Bianchi L: Dynamics of viral replication in infants with vertically acquired human immunodeficiency virus type 1 infection. *J Clin Invest* 1996;97:323–330.
19. Newell ML, Coovadia H, Cortina-Borja M, Rollins N, Gaillard P, Dabis F; Ghent International AIDS Society (IAS) Working Group on HIV Infection in Women and Children. Mortality of infected and uninfected infants born to HIV-infected mothers in Africa: A pooled analysis. *Lancet* 2004;364:1236–1243.
20. Meyers TM, Yotebieng M, Kuhn L, Moultrie H: Antiretroviral therapy responses among children attending a large public clinic in Soweto, South Africa. *Pediatr Infect Dis J* 2011;30:974–979.
21. van Dijk JH, Sutcliffe CG, Munsanje B, Sinywimaanzi P, Hamangaba F, Thuma PE, Moss WJ: HIV-infected children in rural Zambia achieve good immunologic and virologic outcomes two years after initiating antiretroviral therapy. *PLoS One* 2011;6:e19006.
22. Abel K, Pahar B, Van Rompay KK, Fritts L, Sin C, Schmidt K, Colon R, McChesney M, Marthas ML: Rapid virus dissemination in infant macaques after oral simian immunodeficiency virus exposure in the presence of local innate immune responses. *J Virol* 2006;80:6357–6367.
23. Oxford KL, Dela Pena-Ponce MG, Jensen K, Eberhardt MK, Spinner A, Van Rompay KK, Rigdon J, Mollan KR, Krishnan VV, Hudgens MG, Barry PA, De Paris K: The interplay between immune maturation, age, chronic viral infection and environment. *Immun Ageing* 2015;12:3.
24. Wang X, Xu H, Pahar B, Alvarez X, Green LC, Dufour J, Moroney-Rasmussen T, Lackner AA, Veazey RS: Simian immunodeficiency virus selectively infects proliferating CD4⁺ T cells in neonatal rhesus macaques. *Blood* 2010;116:4168–4174.
25. Wang X, Rasmussen T, Pahar B, Poonia B, Alvarez X, Lackner AA, Veazey RS: Massive infection and loss of CD4⁺ T cells occurs in the intestinal tract of neonatal rhesus macaques in acute SIV infection. *Blood* 2007;109:1174–1181.
26. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM: Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 2008;105:7552–7557.
27. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH, Shaw GM: Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med* 2009;206:1273–1289.
28. Stone M, Keele BF, Ma ZM, Bailes E, Dutra J, Hahn BH, Shaw GM, Miller CJ: A limited number of simian immunodeficiency virus (SIV) env variants are transmitted to rhesus macaques vaginally inoculated with SIVmac251. *J Virol* 2010;84:7083–7095.
29. Keele BF, Li H, Learn GH, Hraber P, Giorgi EE, Grayson T, Sun C, Chen Y, Yeh WW, Letvin NL, Mascola JR, Nabel GJ, Haynes BF, Bhattacharya T, Perelson AS, Korber BT, Hahn BH, Shaw GM: Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1. *J Exp Med* 2009;206:1117–1134.
30. Joseph SB, Swanstrom R, Kashuba AD, Cohen MS: Bottlenecks in HIV-1 transmission: Insights from the study of founder viruses. *Nat Rev Microbiol* 2015;13:414–425.
31. Danaviah S, de Oliveira T, Bland R, Viljoen J, Pillay S, Tuailon E, Van de Perre P, Newell ML: Evidence of long-lived founder virus in mother-to-child HIV transmission. *PLoS One* 2015;10:e0120389.
32. Greenier JL, Van Rompay KK, Montefiori D, Earl P, Moss B, Marthas ML: Simian immunodeficiency virus (SIV) envelope quasispecies transmission and evolution in infant rhesus macaques after oral challenge with uncloned SIVmac251: Increased diversity is associated with neutralizing antibodies and improved survival in previously immunized animals. *Virol J* 2005;2:11.
33. Rychert J, Lacour N, Amedee AM: Genetic analysis of simian immunodeficiency virus expressed in milk and selectively transmitted through breastfeeding. *J Virol* 2006;80:3721–3731.
34. Santangelo PJ, Rogers KA, Zurla C, Blanchard EL, Gumber S, Strait K, Connor-Stroud F, Schuster DM, Amancha PK, Hong JJ, Byrreddy SN, Hoxie JA, Vidakovic B, Ansari AA, Hunter E, Villingier F: Whole-body immunoPET reveals active SIV dynamics in viremic and antiretroviral therapy-treated macaques. *Nat Methods* 2015;12:427–432.
35. Moretti R, Pansiot J, Bettati D, Strazielle N, Ghersi-Egea JF, Damante G, Fleiss B, Titomanlio L, Gressens P: Blood-brain barrier dysfunction in disorders of the developing brain. *Front Neurosci* 2015;9:40.
36. Saunders NR, Liddelow SA, Dziegielewska KM: Barrier mechanisms in the developing brain. *Front Pharmacol* 2012;3:46.
37. Milush JM, Chen HL, Atteberry G, Sodora DL: Early detection of simian immunodeficiency virus in the central nervous system following oral administration to rhesus macaques. *Front Immunol* 2013;4:236.
38. Weller RO, Galea I, Carare RO, Minagar A: Pathophysiology of the lymphatic drainage of the central nervous system: Implications for pathogenesis and therapy of multiple sclerosis. *Pathophysiology* 2010;17:295–306.
39. Weller RO, Djuanda E, Yow HY, Carare RO: Lymphatic drainage of the brain and the pathophysiology of neurological disease. *Acta Neuropathol* 2009;117:1–14.
40. Dissing-Olesen L, Hong S, Stevens B: New brain lymphatic vessels drain old concepts. *EBioMedicine* 2015;2:776–777.
41. Dando SJ, Mackay-Sim A, Norton R, Currie BJ, St John JA, Ekberg JA, Batzloff M, Ulett GC, Beacham IR: Pathogens penetrating the central nervous system: Infection pathways and the cellular and molecular mechanisms of invasion. *Clin Microbiol Rev* 2014;27:691–726.
42. Yi X, Manickam DS, Brynskikh A, Kabanov AV: Agile delivery of protein therapeutics to CNS. *J Control Release* 2014;190:637–663.
43. Miller CJ, Li Q, Abel K, Kim EY, Ma ZM, Wietgreffe S, La Franco-Scheuch L, Compton L, Duan L, Shore MD, Zupancic M, Busch M, Carlis J, Wolinsky S, Haase AT: Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol* 2005;79:9217–9227.

44. Li Q, Skinner PJ, Ha SJ, Duan L, Mattila TL, Hage A, White C, Barber DL, O'Mara L, Southern PJ, Reilly CS, Carlis JV, Miller CJ, Ahmed R, Haase AT: Visualizing antigen-specific and infected cells in situ predicts outcomes in early viral infection. *Science* 2009;323:1726–1729.
45. Haase AT: Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annu Rev Med* 2011;62:127–139.
46. Mitchell W: Neurological and developmental effects of HIV and AIDS in children and adolescents. *Ment Retard Dev Disabil Res Rev* 2001;7:211–216.
47. Walker SY, Pierre RB, Christie CD, Chang SM: Neurocognitive function in HIV-positive children in a developing country. *Int J Infect Dis* 2013;17:e862–e867.
48. Pollack H, Kuchuk A, Cowan L, Hacimamutoglu S, Glasberg H, David R, Krasinski K, Borkowsky W, Oberfield S: Neurodevelopment, growth, and viral load in HIV-infected infants. *Brain Behav Immun* 1996;10:298–312.
49. Lewis-de Los Angeles CP, Alpert KI, Williams PL, Malee K, Huo Y, Csernansky JG, Yogev R, Van Dyke RB, Sowell ER, Wang L; Pediatric HIV/AIDS Cohort Study: Deformed subcortical structures are related to past HIV disease severity in youth with perinatally acquired HIV infection. *J Pediatric Infect Dis Soc* 2016;5:S6–S14.
50. Tornatore C, Chandra R, Berger JR, Major EO: HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* 1994;44:481–487.
51. Li GH, Henderson L, Nath A: Astrocytes as an HIV reservoir: Mechanism of HIV Infection. *Curr HIV Res* 2016;14:373–381.
52. Churchill MJ, Wesselingh SL, Cowley D, Pardo CA, McArthur JC, Brew BJ, Gorry PR: Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. *Ann Neurol* 2009;66:253–258.
53. Gorry PR, Ong C, Thorpe J, Bannwarth S, Thompson KA, Gatignol A, Vesselingh SL, Purcell DF: Astrocyte infection by HIV-1: Mechanisms of restricted virus replication, and role in the pathogenesis of HIV-1-associated dementia. *Curr HIV Res* 2003;1:463–473.
54. Canto-Nogues C, Sanchez-Ramon S, Alvarez S, Lacruz C, Munoz-Fernandez MA: HIV-1 infection of neurons might account for progressive HIV-1-associated encephalopathy in children. *J Mol Neurosci* 2005;27:79–89.
55. Whitney JB, Hill AL, Sanisetty S, Penaloza-MacMaster P, Liu J, Shetty M, Parenteau L, Cabral C, Shields J, Blackmore S, Smith JY, Brinkman AL, Peter LE, Mathew SI, Smith KM, Borducchi EN, Rosenbloom DI, Lewis MG, Hattersley J, Li B, Hesselgesser J, Geleziunas R, Robb ML, Kim JH, Michael NL, Barouch DH: Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. *Nature* 2014;512:74–77.

Address correspondence to:

Kristina De Paris, PhD

Department of Microbiology and Immunology

and Center for AIDS Research

School of Medicine

University of North Carolina at Chapel Hill

Burnett Womack Building, Room 9029

160 Dental Circle, CB #7292

Chapel Hill, NC 27599-7292

E-mail: abelk@med.unc.edu