Coevolutionary dynamics between tribe Cercopithecini tetherins and their lentiviruses

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Human immunodeficiency virus, a primate lentivirus (PLV), causes AIDS in humans, whereas most PLVs are less or not pathogenic in monkeys. These notions suggest that the co-evolutionary process of PLVs and their hosts associates with viral pathogenicity, and therefore, that elucidating the history of virus-host co-evolution is one of the most intriguing topics in the field of virology. To address this, recent studies have focused on the interplay between intrinsic anti-viral proteins, such as tetherin, and viral antagonists. Through an experimental-phylogenetic approach, here we investigate the co-evolutionary interplay between tribe Cercopithecini tetherin and viral antagonists, Nef and Vpu. We reveal that tribe Cercopithecini tetherins are positively selected, possibly triggered by ancient Nef-like factor(s). We reconstruct the ancestral sequence of tribe Cercopithecini tetherin and demonstrate that all Nef proteins are capable of antagonizing ancestral Cercopithecini tetherin. Further, we consider the significance of evolutionary arms race between tribe Cercopithecini and their PLVs.

Based on the sequence similarity, the following two issues have been widely accepted: (i) human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome, emerged from zoonotic transmission of a simian immunodeficiency virus (SIV) in chimpanzee (SIVcpz) to humans around 100 years ago1–3; and (ii) SIVcpz appears to have emerged from the recombination of two lineages of SIVs from Old World monkeys (OWMs): SIVgsn/mon/mus lineage from greater-spot nosed monkey (Cercopithecus nictitans; GSN), mona monkey (Cercopithecus mona; MON), and mustached monkey (Cercopithecus cephus; MUS) and SIVrcm from red-capped mangabey (Cercocebus torquatus)4. Understanding the evolutionary history of primate lentiviruses (PLVs) including HIVs and SIVs is one of the most important and interesting topics in the field of retrovirology. However, because of their multiple cross-species transmissions and complicated recombination, it is difficult to elucidate

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how genetic conflicts between the ancient SIVs and their respective host species resulted in evolution and diversification.

OWMs, the family Cercopithecidae, are composed of 12 genera and a subfamily\(^7\). All SIVs identified so far encode 8 common genes: gag, pol, env, tat, rev, vpr, vif, and nef\(^8\). Among more than 40 SIVs, which have been identified in OWMs residing in Africa\(^7\), only 4 kinds of SIVs, SIVgsn in GSN, SIVmon in MON, SIVmus in MUS, and SIVden in Dent’s mona monkey (Cercopithecus denti) encode an additional accessory gene, vpu\(^6\). Importantly, the SIVs encoding vpu have been identified only in the monkeys belonging to tribe Cercopithecini including the genus Cercopithecus, strongly suggesting that the vpu gene has emerged in the evolution and transmission of SIVs in this tribe\(^5,7\).

To elucidate the co-evolutionary relationship between SIVs and their hosts, recent investigations have experimentally addressed the evolutionary conflict between viral and host proteins\(^8\) that stems from the “Red Queen hypothesis”\(^11\) or “evolutionary arms race” concept. Such an approach can be the way to explain the co-evolutionary history of SIVs and their host species. For example, Vif, a common protein encoded by all PLVs, has a robust ability to counteract a cellular anti-PLV restriction factor, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G\(^12\). In addition, another anti-PLV restriction factor, SAM domain and HD domain 1 (SAMHD1), can be antagonized by the viral accessory proteins, Vpr or Vpx\(^13,14\). The vpx gene is encoded in certain SIV lineages and HIV type 2, and it has been assumed that the vpx gene evolved from gene duplication of its ancestral gene, vpr\(^15\). Moreover, based on an experimental-phylogenetic investigation, Lim et al. recently proposed that the evolutionary interaction between Vpr/Vpx and SAMHD1 has undergone the following four steps: (i) Vpr acquired anti-SAMHD1 activity; (ii) ancestral SIV(s) created vpx by the gene duplication of vpr; (iii) Vpr transferred its anti-SAMHD1 activity to Vpx\(^16\). Namely, anti-SAMHD1 ability has been transferred from an old gene (vpr) to a new gene (vpx) during the co-evolution of SIVs and their host species.

One of the most complicated examples of the co-evolutionary relationship between OWMS and their SIVs is tetherin (also known as bone narrow stromal antigen 2, CD317 and HM1.24) and its viral antagonists\(^8,17\). Tetherin inhibits the release of nascent viral particles from virus-producing cells\(^18,19\). Similar to the case of SAMHD1 and Vpr/Vpx, SIVs encode two kinds of anti-tetherin antagonists: Nef and Vpu\(^9\). Nef is encoded in all SIVs and most SIVs antagonize tetherins of their hosts by Nef\(^8\). On the other hand, as described above, Vpu is encoded in certain SIVs in OWMS, and the Vpu proteins of these SIVs potently antagonize tetherins of their hosts\(^8,20\). However, when, why, and how the vpu gene was acquired in certain SIV lineages during their evolution is still unclear.

In this study, we particularly focus on the OWMS belonging to the tribe Cercopithecini and their SIVs, and perform investigations based on molecular phylogenetics and evolution, experimental virology, and structural biology. We reveal that the tetherins of the tribe Cercopithecini are under strong positive selection. In addition, we construct the ancestral sequences of tribe Cercopithecini tetherin and experimentally demonstrate that all Nef proteins of the SIVs isolated from the tribe Cercopithecini retain antagonistic ability to the ancestral tetherin of tribe Cercopithecini. Moreover, we estimate the time of vpu acquisition in certain SIV lineages, and further, discuss the reason why vpu has been created and/or acquired from various scientific fields of view.

Results

**Evolution of primate tetherin and CD4.** Since lentiviral Nef and Vpu proteins have the common ability to down-regulate tetherin as well as CD4\(^17,19–21\), we set out to perform molecular phylogenetic analyses on primate tetherin and CD4. In this study, we newly identified 11 tetherin sequences of 8 different OWMS belonging to the tribe Cercopithecini (1 Campbell’s mona monkey, 1 mustached monkey, 2 Sclater’s monkeys, 1 L’Hoeist’s monkey, 2 Sykes’ monkeys, 2 red-eared monkeys, 1 red-tailed monkey, and 1 sun-tailed monkey; listed in Table 1) and 3 CD4 sequences of 2 different OWMS (1 MUS and 2 sooty mangabeys; listed in Table 2). As shown in Fig. 1a,b, each family or infraorder (i.e., Hominoids, OWMs, and different OWMs belonging to the tribe Cercopithecini) listed in Table 2). As shown in Fig. 1a,b, each family or infraorder (i.e., Hominoids, OWMs, and NWMs) respectively formed a monophyletic cluster on the reconstructed trees of both tetherin and CD4. On the other hand, within the cluster of Cercopithecini, the tetherins of certain Cercopithecini monkeys, particularly mustached monkey, red-eared monkey, and Sclater’s monkey, did not form a monophyletic subcluster, respectively (Fig. 1a). This indicates that the nucleotide sequence of certain Cercopithecini tetherins, particularly mustached monkey, red-eared monkey, and Sclater’s monkey, are highly similar.

To detect positive selection in the evolution of primate tetherin and CD4, we estimated the nonsynonymous to synonymous (dN/dS) ratios. The two pairs of site models in PAML produced similar results and the results obtained from M7 (neutral model) versus M8 (selection model) comparisons are shown in Fig. 1c,d. Consistent with previous reports\(^22–24\) including ours\(^25\), the dN/dS ratio of primate tetherin was significantly greater than one for full-length (43.04), cytoplasmic tail (CT; 26.76), and transmembrane domain (TMD; 11.08) (Fig. 1c). Also, three codons, 9 (dN/dS = 5.2), 14 (dN/dS = 5.1), and 17 (dN/dS = 5.2) in primate tetherin, were identified to be positively selected sites with posterior probability greater than 0.95 (Fig. 1e). These findings on primate tetherin indicate that the functionally important regions of primate tetherin, particularly CT and TMD, have evolved under strong positive selection, which is in agreement with previous reports\(^22–25\).

On the other hand, the dN/dS ratio of primate CD4 was significantly greater than one for full-length (28.91) and the extracellular domain (ECD; 31.64), and six codons in the ECD, 48 (dN/dS = 3.6), 73 (dN/dS = 3.6), 77 (dN/dS = 3.6), 80 (dN/dS = 3.7), 113 (dN/dS = 3.7), and 265 (dN/dS = 3.7), were identified...
| Family/infraorder | Common name | Scientific name | Accession number |
|------------------|-------------|----------------|-----------------|
| Hominidae (Hominoids) | Human | Homo sapiens | AK223124 |
| | Human | Homo sapiens | NM_004335 |
| | Chimpanzee | Pan troglodytes | NM_001190480 |
| | Bonobo | Pan paniscus | HM136907 |
| | Bonobo | Pan paniscus | XM_003817802 |
| | Gorilla | Gorilla gorilla | GQ925926 |
| | Gorilla | Gorilla gorilla | HM136906 |
| | Gorilla | Gorilla gorilla | XM_004060266 |
| | Orangutan | Pongo pygmaeus | HM136908 |
| | Orangutan | Pongo abelii | NM_001172587 |
| | Gibbon | Hylobates agilis | HM136910 |
| | Gibbon | Nomascus leucogenys | HM136909 |
| | Mustached monkey | Cercopithecus cephus | GQ864267 |
| | Dent's mona monkey | Cercopithecus denti | HE880870 |
| | Red-eared monkey | Cercopithecus erythrotis | LC012317 |
| | Sclater's monkey | Cercopithecus sclateri | LC012319 |
| | Sclater's monkey | Cercopithecus sclateri | LC012320 |
| | Red-eared monkey | Cercopithecus erythrotis | LC012318 |
| | Mustached monkey | Cercopithecus cephus | LC012318 |
| | Red-tailed monkey | Cercopithecus ascanis | LC012315 |
| | Mustached monkey | Cercopithecus cephus | GQ925925 |
| | Sykes' monkey | Cercopithecus albogularis | LC012321 |
| | Sykes' monkey | Cercopithecus albogularis | LC012322 |
| | Sun-tailed monkey | Cercopithecus solatus | LC012323 |
| | L'Hoest's monkey | Cercopithecus lhoesti | LC012313 |
| | Mona monkey | Cercopithecus mona | GQ925924 |
| | Campbell's mona monkey | Cercopithecus campbelli | LC012314 |
| | Greater spot-nosed monkey | Cercopithecus nictitans | GQ925923 |
| | De Brazza's monkey | Cercopithecus neglectus | HE880871 |
| | Talapoin monkey | Miopithecus talapoin | HM136913 |
| | Patas monkey | Erythrocebus patas | HM136911 |
| | Tantalus monkey | Chlorocebus tantalus | FJ435303 |
| | Grivet monkey | Chlorocebus aethiops | FJ943430 |
| | Grivet monkey | Chlorocebus aethiops | HM136912 |
| | Rhesus macaque | Macaca mulatta | FJ943431 |
| | Rhesus macaque | Macaca mulatta | FJ943432 |
| | Rhesus macaque | Macaca mulatta | GQ304749 |
| | Rhesus macaque | Macaca mulatta | HM136914 |
| | Rhesus macaque | Macaca mulatta | HM775182 |
| | Rhesus macaque | Macaca mulatta | NM_001161666 |
| | Pig-tailed macaque | Macaca nemestrina | FJ941988 |
| | Pig-tailed macaque | Macaca nemestrina | FJ941989 |
| | Red-capped mangabeys | Cercocebus torquatus | AB907706 |
| | Red-capped mangabeys | Cercocebus torquatus | AB907707 |
| | Sooty mangabeys | Cercocebus atys | FJ864713 |
| | Sooty mangabeys | Cercocebus atys | FJ864714 |
| | Olive Baboon | Papio anubis | XM_003915138 |
| | Red-shanked douc langur | Pygathrix nemaeus | HM136916 |
| | Francois' leaf monkey | Trachypithecus francoisi | HM136917 |
| | Colobus monkey | Colobus guereza | HM136915 |
as positively selected codons by the site model analysis (Fig. 1f). These findings on primate CD4 suggest that the ECD of primate CD4 has evolved under strong positive selection. Because the ECD of CD4 molecule is homologous to immunoglobulins and plays a crucial role for immune recognition and immune responses, the evolution and diversification of the immune system may closely associate with the positive selection observed in this study (Fig. 1d,f). In contrast to primate tetherin, positive selection was detected in neither TMD nor CT of primate CD4 (Fig. 1d,f). To down-regulate these cellular proteins,

Table 1. Accession numbers of primate tetherin used in this study. aFamily (Hominidae and Cercopithecidae) and infraorder (Platyrrhini) are presented in italic. Popular name of each family/infraorder is presented in parenthesis. OWMs, old world monkeys; NWMs, new world monkeys. bThe common name of each primate is identical to that in Fig. 1a. cThe GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of tetherins are listed. dThe newly identified sequences in this study.

| Family/infraorder | Common name | Scientific name | Accession number |
|-------------------|-------------|-----------------|-----------------|
| Platyrrhini (NWMs) | Night monkey | Aotus lemurinus | FJ638414 |
|                   | Night monkey | Aotus vociferans | FJ638417 |
|                   | Night monkey | Aotus vociferans | FJ638418 |
|                   | Night monkey | Aotus vociferans | FJ638415 |
|                   | White-faced saki monkey | Pithecia pithecia | HM136920 |
|                   | White-lipped tamarin | Saguinus labiatus | HM136918 |
|                   | Common woolly monkey | Lagothrix lagotricha | HM136922 |
|                   | Bolivian red howler monkey | Alouatta sara | HM136921 |

Table 2. Accession numbers of primate CD4 used in this study. aFamily (Hominidae and Cercopithecidae) and infraorder (Platyrrhini) are presented in italic. Popular name of each family/infraorder is presented in parenthesis. bThe common name of each primate is identical to that in Fig. 1b. cThe GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of CD4s are listed. dThe newly identified sequences in this study.

| Family/infraorder | Common name | Scientific name | Accession number |
|-------------------|-------------|-----------------|-----------------|
| Hominidae (Hominoids) | Human | Homo sapiens | AK312828 |
|                   | Human | Homo sapiens | BC025782 |
|                   | Human | Homo sapiens | BT019791 |
|                   | Human | Homo sapiens | BT019811 |
|                   | Human | Homo sapiens | NM_000616 |
|                   | Chimpanzee | Pan troglodytes | EF437437 |
|                   | Chimpanzee | Pan troglodytes | EF437438 |
|                   | Chimpanzee | Pan troglodytes | EF437439 |
|                   | Chimpanzee | Pan troglodytes | EF437441 |
|                   | Chimpanzee | Pan troglodytes | EF437442 |
|                   | Chimpanzee | Pan troglodytes | NM_001009043 |
|                   | Gorilla | Gorilla gorilla | XM_004052582 |
|                   | Gibbon | Nomascus leucogenys | XM_004092147 |
|                   | Gibbon | Nomascus leucogenys | XM_004092148 |
| Cercopithecidae (OWMs) | Mustached monkey | Cercopithecus cephus | LC017837d |
|                   | Grivet monkey | Chlorocebus aethiops | D86589 |
|                   | Rhesus macaque | Macaca mulatta | D63347 |
|                   | Pig-tailed macaque | Macaca nemestrina | D63346 |
|                   | Sooty mangabeys | Cercocebus atys | KP406148d |
|                   | Sooty mangabeys | Cercocebus atys | KP406149d |
| Platyrrhini (NWMs) | Night monkey | Aotus nancymaae | FJ623078 |
|                   | Marmoset | Callithrix jacchus | NM_001267772 |
Nef targets the CTs of tetherin and CD421,27, whereas Vpu targets the TMD of tetherin and the CT of CD4, respectively8,28. Therefore, our findings suggest that primate tetherin but not CD4 has experienced the positive selection elicited by Nef and/or Vpu during evolution.

**Positive selection detected in the evolution of tribe Cercopithecini tetherin.** Among the SIVs in OWMs (identical to the family Cercopithecidae), vpu-positive SIVs have been identified only in the monkeys belonging to the tribe Cercopithecini (represented in pink in Fig. 1a,b). To elucidate the evolutionary interplay between Nef/Vpu and tetherin, we particularly focused on the tetherins of this tribe. The phylogenetic tree of 22 tetherins belonging to the tribe Cercopithecini showed that the tetherins of the hosts of vpu-positive SIVs intermingle with those of vpu-negative SIVs (Fig. 2a), suggesting that the presence of vpu did not result in the convergent evolution of Cercopithecini tetherin. Also, the site

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**Figure 1. Molecular phylogenetic analyses of primate tetherin and CD4.** (a,b) Phylogenetic trees of 58 primate tetherins (a) and 22 primate CD4s (b) reconstructed using NJ method. Both trees were rerooted with the NWM clade. The species belonging to Tribe Cercopithecini are shown in pink. The species indicated in bold are the sequences newly identified in this study. GenBank accession numbers are listed in Tables 1 and 2. In panel a, the number (8.2) indicates the age of diversification (million years ago) that is estimated in a previous study38. A phylogenetic tree of 58 primate tetherins reconstructed using ML method is shown in Supplementary Fig. 1. (c,d) The positive selection detected in different regions of tetherin gene (c) and CD4 gene (d). The regions inferred to be under positive selection with statistical significance are represented in bold. ND, not detected. (e,f) Positively selected sites identified from tetherin gene (e) and CD4 gene (f). The codons under positive selection identified by PAML with posterior probability >0.95 are shown in red. All PAML analyses were performed under two models of codon usage, F61 and F3×4, and they yield consistent results.
model analysis revealed that the dN/dS ratio of Cercopithecini tetherin was significantly greater than one for full-length (47.56) and CT (21.13) as well as ECD (12.15) (Fig. 2b). Four codons in the CT, 14 (dN/dS = 8.6), 16 (dN/dS = 8.5), 17 (dN/dS = 8.6), and 24 (dN/dS = 8.4), and two codons in the ECD, 67 (dN/dS = 8.3) and 99 (dN/dS = 8.4), were identified to be positively selected (Fig. 2c).

We then classified 22 Cercopithecini tetherins into two groups: the hosts of vpu-positive SIVs and those of vpu-negative SIVs. Because SIV has not been identified in Sclater’s monkeys 29, we excluded the tetherin sequences of 2 Sclater’s monkeys from this classification. As shown in Fig. 3a, the branch-site tests in PAML revealed that the likelihood ratio test was significant with P < 0.01 in the analysis of the tetherins of the hosts of vpu-negative SIVs as well as 22 Cercopithecini tetherins, suggesting that positive selection has most likely operated on the tetherins of the monkeys infected with vpu-negative SIVs. Also, the site model revealed that the dN/dS ratio of the tetherins of the hosts of vpu-negative SIVs was significantly greater than one for full-length (42.55), CT (25.92), and ECD (12.15) (Fig. 3b, left), and nine codons positioned at 14, 16, 17, 24, 34, 67, 99, 100, and 159 were identified to be positively selected (Fig. 3c, left). In addition, thirteen codons including the nine codons detected by the site model (indicated by asterisks in Fig. 3d) were identified as positively selected sites by the random effects likelihood (REL) analysis implemented in the HyPhy package with Bayes factor greater than 50 (Fig. 3d, left).

Furthermore, we constructed the ancestral sequence of the 22 tetherins of tribe Cercopithecini (Fig. 3e). By mapping the five positively selected sites in the ECD (positioned at 63, 67, 99, 100, and 159), we found that these amino acids were located on the same aspect of the alpha helix structure (Fig. 3e).

In contrast to the tetherins from the hosts of vpu-negative SIVs, it was notable that positive selection was not detected in the tetherins from the monkeys infected with vpu-positive SIVs by the branch-site model (Fig. 3a) and REL analyses (Fig. 3d, right). Although the site model showed that the dN/dS ratio of the tetherins of the hosts of vpu-positive SIVs was significantly greater than one for full-length (2Δl = 10.42, P < 0.01) (Fig. 3b, right), no positive selection was detected at the significant level (P < 0.05) for respective domains (Fig. 3b, right) and codons (Fig. 3c, right), which basically agreed with the result obtained from the branch-site test (Fig. 3a). To ask whether the difference in the positive selection between these two groups could be attributed to the difference in the number of tetherin sequences included, we performed genetic diversity analyses using MEGA6 30. As shown in Fig. 3f, the genetic diversity of tetherins of these two groups was comparable. Taken together, these results indicate that the much weaker selective pressure detected for the tetherins of the hosts of vpu-positive SIVs is not likely due to the smaller sample size of this group, but suggesting that Vpu did not exert a strong selective pressure on the tetherins of the hosts of vpu-positive SIVs.

Antagonism of Cercopithecini tetherin by SIV Nef. To directly evaluate the anti-viral activity of Cercopithecini tetherin and the antagonistic ability of SIV Nef proteins, we prepared an expression plasmid for the constructed ancestral Cercopithecini tetherin. Western blotting (Fig. 4a) and TZM-bl assay (Fig. 4b) revealed that increasing amounts of the ancestral Cercopithecini tetherin resulted in a...
Figure 3. Molecular phylogenetic and structural analyses of tetherins of SIV-infected monkeys.

(a) The result obtained from the three branch-site analyses for Tribe Cercopithecini (n = 22), the hosts of vpu-negative SIV (cyan, n = 14), and those of vpu-positive SIV (orange, n = 6). The clades inferred to be under positive selection with statistical significance are represented in bold. (b) The positive selection detected in different regions of tetherin gene of the hosts of vpu-negative SIV (left, n = 14) and those of vpu-positive SIV (right, n = 6). The regions inferred to be under positive selection with statistical significance are represented in bold. (c,d) Positively selected sites identified in our analyses. In panel (c), the codons under positive selection identified by PAML with posterior probability >0.95 are shown in red. In panel (d), the codons under positive selection inferred by HyPhy with Bayes factor >50 are shown in red, and the codons identified as positively selected sites by PAML are indicated with asterisks. (e) Structure modeling of the ancestral tetherin of tribe Cercopithecini. The transparent surface with the ribbon diagram of the extracellular domain (ECD) of ancestral Cercopithecini tetherin, which is generated by SWISS-MODEL server based on the ECD of human tetherin (PDB code: 3MQB)31, is shown. The two views of monomer (top) and tetramer (bottom) models, rotated by 180°, are respectively shown. The 5 positively selected sites in the ECD of tetherins of vpu-negative SIV hosts (codons 63, 67, 99, 100, and 159) are indicated in red. (f) Genetic diversity analysis. The values indicate the overall mean genetic distance, which is calculated by using Tamura-Nei model56 in MEGA6, with standard error.
dose-dependent decrease in the release of nascent virions. We confirmed that the infectious virus in the culture supernatant correlated strongly with the amount of supernatant viral p24 antigen, a physical measure of virion content (\( r = 0.947, P = 0.000031 \); Supplementary Fig. 2), which is consistent with previous reports\(^{20,22,31} \) and validates the tetherin impact on virus release.

We then prepared expression plasmids for 14 strains of SIV Nefs, which have been identified in the 12 species of \( \text{Cercopithecini} \) monkeys so far, and investigated whether these Nef proteins have the ability to antagonize the ancestral \( \text{Cercopithecini} \) tetherin. As shown in Fig. 4a, none of the Nef proteins affected expression levels of tetherin and Gag, particularly Gag precursor (Pr55 Gag; Fig. 4a), which is consistent with previous reports\(^{22,25,27} \). Moreover, we revealed that all Nef proteins enhanced viral release in the presence of the ancestral \( \text{Cercopithecini} \) tetherin (Fig. 4b). Importantly, the 6 Nef proteins of \( \text{vpu}^- \) SIVs significantly augmented viral release (Fig. 4b). These findings directly demonstrate that all SIV Nef proteins isolated from the tribe \( \text{Cercopithecini} \) potently antagonize \( \text{Cercopithecini} \) tetherin regardless of whether or not they had acquired \( \text{vpu} \).

Genetic and geographical consideration of the acquisition of \( \text{vpu} \) gene. To further assess the possibility that Vpu has exerted selective pressure on the tetherins of tribe \( \text{Cercopithecini} \), we performed a Bayesian evolutionary analysis for dating the time of \( \text{vpu} \) gene acquisition. As shown in Fig. 5a, our analysis revealed that the \( \text{vpu} \) gene has been independently acquired twice, which were estimated to have occurred in SIVgsn/mon/mus lineage (nodes 8) around 19,418 years ago and in SIVden lineage (node 13) around 19,218 years ago, respectively (Table 3). In addition, it has been reported that the gain-of-function of Vpr to degrade SAMHD1 occurred after the divergence from SIVsun/lhoest lineages\(^{16} \). Our analyses
revealed that the Vpr neofunction occurred after 35,766 years ago (Fig. 5a and Table 3). These findings suggest that vpu was acquired by the two lineages of SIVs around 20,000 years ago, which were relatively recent events in the evolutionary history of SIVs compared to the neofunction of Vpr.

Figure 5. Evolution and diversification of SIV. (a) Dating the divergence times of 34 SIV lineages. The MCC tree constructed using BEAST is shown. This analysis was conducted by using the amino acid sequences of Gag, Pol, Vif, and Env. Cyan, vpu-negative SIVs; orange, vpu-positive SIVs; black, SIVs identified in western red colobus; and grey, SIVs identified in black-and-white colobus. The orange stars (nodes 8 and 13) indicate the time of vpu gene acquisition, and the green star (node 30) indicates the time of Vpr neofunction. X-axis indicates the year before present. GenBank accession numbers of the SIV sequences used in this analysis are listed in Table 4. The estimated divergence time, posterior probability, and bootstrap value of each node of the tree are listed in Table 3. (b) Distribution of the monkeys infected with vpu-positive SIV. The data is extracted from the reference46. The image is created using Illustrator (Adobe) by overlaying the maps shown in reference46. GSN, greater spot-nosed monkey; MON, mona monkey; MUS, mustached monkey; DEN, dent's mona monkey. (c) The nucleotide length of SIV. The nucleotide length between the end of each viral gene (tat1, vpr, rev1, and vif) and the initiation codon of env are measured. Statistic differences between SIVdeb and the other vpu-negative SIV are determined by Welch's t test. PSIV, prosimian endogenous lentivirus.
We then considered the acquisition of vpu gene in geographic terms. As considered in the previous papers, the habitats of the three species of OWMs, GSN, MON, and MUS overlapped in West Africa including Cameroon, Gabon, and Nigeria, and Republic of the Congo, while the habitat of DEN was geographically separated (Fig. 5b). Therefore, it is plausible that vpu gene has been independently acquired by certain SIV(s) infecting the monkeys in these two separate geographic areas.

Furthermore, we considered the event of vpu acquisition in terms of viral genetics. Because of the restricted genome size of RNA viruses including lentiviruses, 'genome compression', which is caused by the use of overlapping genes, is a known characteristic of RNA viruses. In fact, the 3' end of all vpu genes of PLVs overlaps with the 5' end of env (Fig. 5c, top). Although a previous study has suggested that there is no preference in the direction of frameshift in newly acquired viral genes, we found that all vpu genes of OWM SIVs involved +1 (forward) frameshift compared to env (data not shown). This suggests that the manner of vpu acquisition may be common in the two lineages of vpu-positive SIVs (Fig. 5a). Moreover, we measured the nucleotide length between the 3' ends of 4 viral genes (tat1, rev1, vpr, and vif) and the 5' end of env, where vpu is encoded in certain SIVs. As expected, the nucleotide lengths of these 4 regions in vpu-positive SIVs were clearly longer than those in vpu-negative SIVs (Fig. 5c). However, it was of interest that the nucleotide lengths from the 3' ends of tat1, rev1, and vpr,

| Node  | Date (Years ago) | Date 95% HPD | Posterior Probability | ML bootstrap value |
|-------|-----------------|--------------|-----------------------|-------------------|
| 1     | 2941            | 1499         | 6932                  | 1.00              | 100 |
| 2     | 3525            | 1722         | 8098                  | 1.00              | 100 |
| 3     | 7319            | 4247         | 16908                 | 1.00              | 98  |
| 4     | 2656            | 1296         | 6073                  | 1.00              | 100 |
| 5     | 9784            | 5809         | 22301                 | 1.00              | 67  |
| 6     | 12242           | 7015         | 27055                 | 1.00              | 100 |
| 7     | 2487            | 1166         | 5889                  | 1.00              | 100 |
| 8     | 19418           | 11648        | 44898                 | 1.00              | 84  |
| 9     | 7662            | 3852         | 17731                 | 1.00              | 100 |
| 10    | 22652           | 13735        | 52774                 | 1.00              | 83  |
| 11    | 3407            | 1750         | 8020                  | 1.00              | 100 |
| 12    | 8129            | 4322         | 18855                 | 1.00              | 100 |
| 13    | 19218           | 10862        | 44407                 | 1.00              | 100 |
| 14    | 25397           | 15346        | 58954                 | 1.00              | 100 |
| 15    | 2634            | 1305         | 5968                  | 1.00              | 97  |
| 16    | 4184            | 4184         | 9774                  | 1.00              | 100 |
| 17    | 10544           | 5952         | 23873                 | 1.00              | 77  |
| 18    | 13030           | 7494         | 29962                 | 1.00              | 100 |
| 19    | 18345           | 10406        | 41995                 | 1.00              | 100 |
| 20    | 30483           | 18224        | 69491                 | 1.00              | 100 |
| 21    | 1111            | 553          | 2587                  | 1.00              | 100 |
| 22    | 3256            | 1744         | 7517                  | 1.00              | 97  |
| 23    | 4521            | 2535         | 10510                 | 1.00              | 100 |
| 24    | 3547            | 1715         | 8323                  | 1.00              | 100 |
| 25    | 10063           | 5773         | 23593                 | 1.00              | 100 |
| 26    | 21134           | 12230        | 49699                 | 1.00              | 100 |
| 27    | 2990            | 1354         | 7004                  | 1.00              | 100 |
| 28    | 7239            | 3519         | 16469                 | 1.00              | 100 |
| 29    | 28102           | 16712        | 65288                 | 1.00              | 100 |
| 30    | 35766           | 22954        | 81980                 | 0.74              | 100 |
| 31    | 5749            | 2825         | 12861                 | 1.00              | 83  |
| 32    | 9047            | 5188         | 20893                 | 1.00              | 100 |
| Root  | 40894           | 30004        | 92529                 | NA*               | NA* |

Table 3. Divergence times and node support for SIVs in Fig. 5a. Each node is correspond to that in Fig. 5a. Bootstrap value is obtained from ML tree. NA, not applicable.
but not of vif, in the 3 strains of SIVdeB were significantly longer than those of the other vpu-negative SIVs (Fig. 5c). Although the mechanism of new gene acquisition by viruses is still unknown, these findings suggest that the nucleotide length in this region can vary in vpu-negative SIVs, and that this genomic region may be adequate for SIVs to create and/or acquire new gene(s).

Discussion

In this study, we newly determined 11 tetherin sequences of the 8 species of the tribe Cercopithecini as well as 3 CD4 sequences of OWMs. In addition, we performed the in-depth molecular phylogenetic analyses and revealed that the tetherins of the tribe Cercopithecini, particularly those of the host monkeys of vpu-negative SIVs, are under strong positive selection. Furthermore, we constructed the ancestral sequence of tribe Cercopithecini tetherin and demonstrated that the ancestral Cercopithecini tetherin has a robust ability to inhibit viral release. In this regard, since a previous paper has shown that the “artificial” tetherin, which artificially forms the same topology to tetherin, sufficiently confers anti-viral activity, it might not be so surprising that the ancestral Cercopithecini tetherin estimated in this study exhibited anti-viral ability. Nevertheless, here we demonstrated that the anti-viral activity of the ancestral Cercopithecini tetherin is strongly antagonized by all SIV Nef proteins we used. Particularly noteworthy was that Nef’s antagonistic activity against Cercopithecini tetherin is not associated with the presence of vpu gene. Moreover, we estimated the time of vpu gene acquisition in certain SIVs and further considered its significance.

Among the order Primates, the domains of tetherin and CD4 under positive selection differed: primate tetherin has been under positive selection in the CT and TMD, whereas the ECD of primate CD4 was positively selected (Fig. 1). These findings suggest that the factors triggering selective pressure on these molecules differ from each other. In the case of CD4, the ECD plays critical roles in immune control such as the recognition of major histocompatibility complex class II. Moreover, CD4 is utilized for the invasion of PLV through interacting its ECD with their envelope glycoprotein (Env). Therefore, it is plausible that the ECD of CD4 has been positively selected through the immune pressures outside of the cells and the interaction with PLV Env. Moreover, positive selection was detected in neither TMD nor ECD of CT of primate CD4 (Fig. 1d, f), suggesting that these domains are evolutionary stable. It is known that both Nef and Vpu target CD4 CT for the down-regulation. On the other hand, here we revealed that the four amino acids in the TMD of human tetherin are responsible for HIV-1 Vpu-mediated antagonism and are located on the same face of its alpha-helix structure. These findings raise the possibility that four amino acids located on the same face of ECD may be positively selected from pressure caused by the other viral antagonists of tetherin. In fact, it has been revealed that the glycoprotein of Ebola virus, which sporadically causes epidemics in humans and primates residing in the central African countries such as the Democratic Republic of Congo (formerly Zaire) and Sudan, interacts with and antagonizes tetherin. Moreover, Env of certain lentiviruses potently antagonize tetherin. Therefore, these viral antagonists of tetherins and/or their ancestors could have exerted the selective pressure on the ECD of Cercopithecini tetherin. Moreover, although the main habitat of vpu-positive SIV hosts is West Africa (Fig. 5b), the monkeys infected with vpu-negative SIVs reside in a broad area of Africa including the central African countries. This further suggests that the tetherins of vpu-negative SIV hosts have had the opportunity to be exposed to different pressures compared to those of vpu-positive SIVs.

As shown in Fig. 4b, we demonstrated that the infectivity of culture supernatant was significantly suppressed by the ancestral Cercopithecini tetherin. These findings suggest that the ancestral Cercopithecini tetherin possesses a robust activity to impair viral release and that the anti-viral ability of tetherin has been maintained in its evolution. On the other hand, it was surprising that all SIV Nef proteins including those of vpu-positive SIVs (e.g., SIVgsn, SIVmon, SIVmus, and SIVden) were capable of antagonizing Cercopithecini tetherin-mediated anti-viral ability (Fig. 4). It has been demonstrated that the Vpu proteins of vpu-positive SIVs antagonize tetherins of their natural hosts. Therefore, these observations suggest that the vpu gene was not necessarily been acquired by certain SIVs to gain a novel anti-tetherin
antagonist. In the case of the evolutionary interplay between Vpr/Vpx and SAMHD1, three evolutionary steps have been proposed: (i) acquisition of anti-SAMHD1 activity by ancestral Vpr (i.e., Vpr neofunction); (ii) creation of vpx by gene duplication in certain SIV lineages; and (iii) transfer of anti-SAMHD1 activity from Vpr to Vpx5,6. In contrast to the scenario of Vpr/Vpx and SAMHD1, our findings suggest that Nef proteins of vpu-positive SIVs have not lost their anti-tetherin activity even though a new tetherin antagonist, Vpu, was acquired, and that the transfer of anti-tetherin activity from Nef to Vpu has not occurred. Moreover, here we estimated that Vpr neofunction occurred around 36,000 years ago, which is relatively older than the acquisition of the vpu gene (Fig. 5a). Therefore, these findings imply that Nef still maintains anti-tetherin activity in vpu-positive SIVs because vpu is relatively younger than vpr/vpx, and that Nefs of vpu-positive SIVs may transfer their anti-tetherin activity to Vpu in the future.

As shown in Fig. 5a, our results suggest that the vpu gene was independently acquired in two SIV lineages: SIVgsn/mon/mus and SIVden. This raises three possibilities. First, it might be possible that the vpu gene acquired in SIVgsn/mon/mus lineage has been horizontally transferred to SIVden lineage and vice versa. Because the two virus lineages, SIVgsn/mon/mus and SIVden, share strikingly similar genomic features (e.g., very similar vpu genes with nearly identical locations), the most parsimonious explanation is that the acquisition of vpu gene was not independent events, but rather descended from a single original event. However, SIVgsn/mon/mus is phylogenetically divergent from SIVden (Fig. 5a), and the habitats of the monkeys infected with these two SIV lineages are geographically separated (Fig. 5b). These two notions argue against this first hypothesis. Second, there is a possibility that the vpu gene was acquired in the common ancestor of these two SIV lineages (i.e., node 14 of Fig. 5a) and then certain SIVs (e.g., SIVval, SIVsyk, and SIVdeb) lost their own vpu. It might be possible for some viruses to lose the vpu gene because there are other routes to overcome OWM tetherin (e.g., Nef, Env)7. If something similar happened in an ancestral virus, it would relieve selective pressure to maintain a vpu gene. The third possibility is that the acquisition of vpu independently occurred twice in the two SIV lineages, perhaps the most feasible possibility. Further, it should be notified that the molecular clock analyses shown herein rely on the accessible information to date. It means that the information obtained in the future may affect the estimated age of vpu acquisition. Nevertheless, this is the first study inferring the time of accessory gene acquisition/generation by PLVs.

So, how was the vpu gene acquired and/or created? Because the genome size of RNA viruses is strictly restricted, it has been assumed that RNA viruses evolutionary repeat trial-and-error to obtain new favorable genes. Interestingly, we found that the nucleotide lengths between the 3′ end of tat1 and the 5′ end of env of prosimian endogenous lentiviruses (PSIVs), which share a common ancestor with modern SIVs47,48, were much shorter than those of SIVs (Fig. 5c). In this regard, PSIVs encode dUTPase in pol region, while PLVs including SIVs do not6. This raises a possibility that the loss of dUTPase in SIV ancestors has relaxed the restriction of viral genome space, which allowed viruses to acquire and/or create novel genes. In fact, PLVs encode multiple viral genes around the region encoding vpu more than the other lentiviruses, which encode dUTPase6,10. Therefore, it is plausible that PLVs have gained the chance to acquire and/or create new genes by losing dUTPase, and that the acquisition of vpu might be one of the consequences.

Methods

Ethic statement. To determine the sequence of tetherin, blood was collected from wild-caught monkeys: 1 Campbell's mona monkey (Cercopithecus campbelli), 1 mustached monkey (Cercopithecus cephis), 2 Scelar's monkeys (Cercopithecus sceleri), 1 L’Hoest's monkey (Cercopithecus loeesti), 2 red-eared monkeys (Cercopithecus erythrotis), 1 red-tailed monkey (Cercopithecus ascarris), and 1 sun-tailed monkey (Cercopithecus solatus) according to the Guide for the Care and Use of Laboratory Animals under a NIAID Animal Care and Use Committee-approved protocol under a NIAID Animal Care and Use Committee-approved protocol50,51. These procedures were approved by NIAID.

Sequencing PCR. Genomic DNA was extracted from cryopreserved peripheral blood mononuclear cells (PBMCs) of these 9 monkeys50,51 by using DNeasy kit (Qiagen). Also, genomic DNA was extracted from the body hair root of 2 Sykes’ monkeys (Cercopithecus albogularis), which are kept in the Japan Monkey Centre, Inuyama, Aichi, Japan) by using DNA Extractor FM kit (Wako). PCR was performed by using PhuUltra High Fidelity DNA polymerase (Agilent Technologies) and the following primers: 5′-CAG CTA GAG GGG AGA TCT GTA TG-3′; 5′-CTC ACT AGC CAG CTT CCT GGG-3′, which were used in our previous study25. The obtained PCR products were purified by gel extraction and directly sequenced by using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with the two primers described above and the following 4 primers: 5′-GGA CTT CAC CAG ACC CTG AA-3′; 5′-TTC AGG TAC TGG TGA AGT CC-3′; 5′-TCT CTC CTT TGG TCC CAA AA-3′; 5′-TTT TGG GAG CAA AGG AGA GA-3′. To determine the sequence of CD4, RNA was extracted from the cryopreserved PBMCs by using RNeasy Mini Kit (Qiagen). Reverse transcription was performed by using Thermoscript RT-PCR System (Life Technologies), and RT-PCR was performed by using Platinum Taq DNA polymerase High Fidelity (Life Technologies) and the following primers: 5′-CAG CAA GGC CAC AAT GAA C-3′ and 5′-TGC CTC AAA TGG GGC TAC-3′. The obtained RT-PCR product was purified by gel extraction and then cloned by using TOPO TA Cloning Kit (Life Technologies). The sequencing
PCR was performed by using ABI Prism 3130 xl genetic analyzer (Applied Biosystems), and the data was analyzed by Sequencher v5.1 software (Gene Codes Corporation).

Molecular phylogenetic analyses. The molecular phylogenetic analyses were performed as previously described. Briefly, the 11 tetherin sequences newly identified in this study were aligned with 47 primate tetherin sequences (listed in Table 1) by using ClustalW implemented in MEGA6. Also, the 3 CD4 sequences newly identified in this study were aligned with 19 primate CD4 sequences (listed in Table 2) as described above. The alignments were verified manually at amino acid level. Then the phylogenetic trees were reconstructed using neighbor-joining (NJ) method with MEGA6 (Fig. 1a,b) and maximum-likelihood (ML) method with PhyML (Fig. S1A). Note that the phylogenetic trees of 58 primate tetherins reconstructed by these two methods yielded similar topology with partial difference in the relationships between certain tetherins; particularly mustached monkey, Red-eared monkey, and Sclater's monkey. We assume that these minor differences are due to higher similarity among these species, because each species did not form a monophyletic cluster on the tree, and bootstrap support for most splits between the sequences were fairly low (<75%, data not shown). Moreover, both NJ and ML trees were used for further PAML analyses, and the small topological difference between these two trees did not affect the results (Fig. 1c and S1B). Furthermore, we confirmed that the nucleotide sequence of the ancestral Cercopithecini tetherin inferred by NJ tree was identical to that by ML tree (data not shown). We then conducted the analysis to detect positive selection along the tree. To infer positive selection across various primate lineages, two pairs of site models implemented in the PAML package were used to conduct the likelihood ratio tests for 58 tetherin genes (Fig. 1c,e), and 22 CD4 genes (Fig. 1d,f), respectively: M1 (neutral model) versus M2 (selection model) and M7 (neutral model) versus M8 (selectivity model). The REL method in HyPhy was also employed to detect positive selection (Fig. 3d). The ancestral Cercopithecini tetherin was inferred by using site model in the PAML analysis (Fig. 2a). Since we were particularly interested in whether the clades of tribe Cercopithecini, the hosts of vpu-negative SIVs, and those of vpu-positive SIVs have evolved under positive selection, we further focused on the 22 tetherins of this clade. First, the branch-site model in PAML was employed for the analysis. This model allows dn/ds ratio to vary both among sites and branches, which is very useful for detecting positive selection along a particular lineage or clade (pre-specified as foreground branches). In our analysis, all the 22 tetherins of tribe Cercopithecini, 14 tetherins of the hosts of vpu-negative SIVs, and 6 tetherins of the hosts of vpu-positive SIVs, were respectively specified as the foreground branches (Fig. 3a). Next, the site model in PAML and REL method in HyPhy were performed to these sequences (Fig. 3b–d). Moreover, the genetic distance (Fig. 3f) was calculated for the 14 tetherin genes of the hosts of vpu-negative SIV and the 6 genes of the hosts of vpu-positive SIV, respectively, by using MEGAn6. We computed the overall mean distance was computed by using Tamura-Nei model with 100 bootstrap replications.

Protein homology modeling. The 3D structure of the ECD of ancestral Cercopithecini tetherin (Fig. 3e) was simulated by the Swiss-Model server (http://swissmodel.expasy.org/) using the crystal structure of the ECD of human tetherin (PDB code: 3MQB) as the template.

BEAST analysis. The full-genome sequences of 34 SIV strains (listed in Table 4) were retrieved from the HIV Sequences Database (http://www.hiv.lanl.gov/content/sequence). Then, the gag, pol, env and vif genes were extracted from each viral sequence and were respectively aligned using MAFFT. The resulting alignments were manually verified at the amino acid level. We then performed the single breakpoint analysis implemented in the HyPhy package to test for the recombination in each aligned dataset. The results showed that no evidence of recombination was detected in all four analyses. We further performed Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) v 9.1b to remove poorly aligned regions from these alignments. Finally, a concatenated dataset of all four genes was created by using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) v 7.2.5. To infer the time of most recent common ancestors of these SIVs, a Bayesian approach implemented in the BEAST package (http://beast.bio.ed.ac.uk) v 1.7.5 was employed. This analysis was conducted at the amino acid level, as the 34 SIV strains were highly divergent. We used a JTT substitution model with gamma-distributed rate variation among sites. The uncorrelated lognormal relaxed molecular clock model was employed to estimate substitution rates and the Yule process of speciation was used for the tree prior. We specified a uniform distributed prior (30,000–130,000 yr, initial = 70,000 yr) for the age of the root of the tree. Five independent Markov Chain Monte Carlo (MCMC) analyses were run for 10–20 million generations with sampling every 1000 generations. We then used the program Tracer (tree.bio.ed.ac.uk/software/tracer) v 1.6 to check for the convergence and to confirm that the effective sample size (ESS) value was higher than 200 for all runs. The maximum clade credibility (MCC) tree was generated by summarizing the sample of trees produced by BEAST after a 10% burn-in using the TreeAnnotator program v 1.7.5, and the resulting MCC tree was viewed using FigTree (http://tree.bio.ed.ac.uk/) v 1.4.2.

Plasmid construction. The HA-tagged Nef expression plasmids of SIVgsn (strain 99CM166), SIVmon (strains 99CMCML1 and NG1), (SIVmus strains 01CM1085 and 01CM1239) were used in our previous study. The Nef open reading frames (ORFs) of SIVdnd (strain CD1), SIVagmSab (strain SAB1), SIVsyk
(strain KE44), SIVlhoest (strain LHO7), SIVsun (strain L14), SIVtal (strain 00CM266), and SIVdeb (strains CM5 and Kin1) were obtained from GeneArt Gene Synthesis service (Life Technologies). The Nef ORF of SIVagmVer strain TYO1 was obtained by PCR using pSA212 (an infectious molecular clone of SIVagmVer strain TYO1) as the template and the following primers: TYO1 Nef-Fwd, 5′-TTT TTT CTA GAA TGG GCT CGC AGA ACT CA-3′; TYO1 Nef-Rev, 5′-TAT ATA TAT ATA GAT ATC CTT CCT CTT CAC CAG CC-3′. The resultant DNA fragment was digested with XbaI and EcoRV and was inserted into the XbaI-EcoRV site of pCGCG vector.

Cell culture and transfection. HEK293T cells and TZM-bl cells (obtained through NIH AIDS Research and Reference Reagent Program) were maintained in Dulbecco’s modified Eagle medium (Sigma) containing 10% heat-inactivated FCS and antibiotics. Transfection was performed by using PEI Max (GE Healthcare) according to the manufacturer’s protocol. Various amounts of KGC-tagged ancestral Cercocebusi tetherin expression plasmids (0, 10, 40 ng) and pNL4-3ΔvpuΔnef (1,200 ng) were cointroduced with or without respective SIV Nef expression plasmid (400 ng) into HEK293T cells.
At 48 hours post-transfection, the culture supernatants and transfected cells were harvested and were respectively used for TZM-bl assay and Western blotting as described below.

**Western blotting and TZM-bl assay.** The culture supernatant harvested at 48 hours post-transfection was centrifuged to remove cells and produce virus suspensions. The infectivity of virus suspensions was measured by TZM-bl assay as previously described. Briefly, 100 μl of the virus solution was inoculated into TZM-bl cells in 96-well plate (Nunc), and the β-galactosidase activity was measured by using the Galacto-Star mammalian reporter gene assay system (Applied Biosystems) and a 2030 ARVO X multilabel counter instrument (PerkinElmer) according to the manufacturers’ procedure. Western blotting was performed as previously described by using the following antibodies: anti-p24 polyclonal antibody (ViroStat), anti-KGC antibody (clone 21B10; Medical and Biological Laboratories, Inc.), anti-HA antibody (3F10; Roche), and anti-alpha-Tubulin (TUBA) antibody (DM1A; Sigma).

**Statistical analyses.** The data expressed as average with standard error (Fig. 3f) or standard deviation (Fig. 4b), and significant differences were determined by Student’s t test (Fig. 4b) or Welch’s t test (Fig. 5c).

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F.R., Y.K. and K.S. wrote the main manuscript text; J.S.T., F.R. and H.T. performed molecular phylogenetic analyses and prepared Figures 1, 2, 3d,f, and 5a; T.I. performed homology modeling and prepared Figure 3e; J.S.T., R.Y., E.Y., Y.N., T.K. and N.M. performed the experiments and prepared Figure 4; K.S. performed in silico analyses and prepared Figures 5b,c; K.M., Y.S., K.S.W., R.G.C. and V.M.H. contributed to the monkey samples; K.S. conceived and designed the experiments. All authors reviewed the manuscript.

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