Replication-Competent Simian Immunodeficiency Virus (SIV) Gag Escape Mutations Archived in Latent Reservoirs during Antiretroviral Treatment of SIV-Infected Macaques

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In response to pressure exerted by major histocompatibility complex (MHC) class I-mediated CD8+ T cell control, human immunodeficiency virus (HIV) escape mutations often arise in immunodominant epitopes recognized by MHC class I alleles. While the current standard of care for HIV-infected patients is treatment with highly active antiretroviral therapy (HAART), suppression of viral replication in these patients is not absolute and latently infected cells persist as lifelong reservoirs. To determine whether HIV escape from MHC class I-restricted CD8+ T cell control develops during HAART treatment and then enters latent reservoirs in the periphery and central nervous system (CNS), we tracked the longitudinal development of the simian immunodeficiency virus (SIV) Gag escape mutation K165R in HAART-treated SIV-infected pigtailed macaques. Key findings of these studies included: (i) SIV Gag K165R escape mutations emerged in both plasma and cerebrospinal fluid (CSF) during the decaying phase of viremia after HAART initiation before suppression of viral replication, (ii) SIV K165R Gag escape mutations were archived in latent proviral DNA reservoirs, including the brain in animals receiving HAART that suppressed viral replication, and (iii) replication-competent SIV Gag K165R escape mutations were present in the resting CD4+ T cell reservoir in HAART-treated SIV-infected macaques. Despite early administration of aggressive antiretroviral treatment, HIV immune escape from CD8+ T cell control can still develop during the decaying phases of viremia and then persist in latent reservoirs, including the brain, with the potential to emerge if HAART therapy is interrupted.

Major histocompatibility complex (MHC) class I-restricted CD8+ T cells regulate critical host adaptive immune responses to viral infections, including human immunodeficiency virus type 1 (HIV-1) (4, 5). HIV-infected individuals expressing the human leukocyte antigen (HLA) HLA-B*27, HLA-B*5701, and HLA-B*5801 MHC class I alleles have delayed progression to AIDS (3, 11, 25). In contrast, individuals expressing HLA-B*3503 or HLA-B*5802 progress more rapidly to AIDS. The current standard of care for HIV-infected patients is treatment with highly active antiretroviral therapy (HAART) to control HIV replication. While HAART limits HIV replication, latently infected resting memory CD4+ T cells persist as lifelong viral reservoirs in HIV-infected individuals and in simian immunodeficiency virus (SIV)-infected macaques (6, 14, 14, 32, 37). Furthermore, suppression of viral replication in many HAART-treated HIV-infected patients is not absolute, with low-level, intermittent spikes in plasma viremia recognized even in well-managed HIV patients (2). It is likely that both latently infected resting memory CD4+ T cells and additional latent reservoirs such as the central nervous system (CNS) contribute to residual viremia during HAART treatment (2). It is crucial to determine whether HIV CTL escape mutations arise early during infection even with prompt HAART. In addition, if CTL escape mutations are laid down in the latent HIV reservoir despite adequate treatment, it is then possible that escape mutations may emerge from the latent reservoir in the brain or other sites during the course of infection. Because studies addressing these issues would be difficult to pursue in HIV-infected individuals, we have developed an SIV/macaque model of HIV latency (9).
To determine whether HIV escape from MHC class I-restricted CD8\(^+\) T cell control develops during HAART treatment and then enters peripheral and/or CNS latent reservoirs with the potential to emerge as replication-competent virus, we tracked the longitudinal development of SIV Gag escape in plasma and cerebrospinal fluid (CSF) from HAART-treated SIV-infected pigtailed macaques. As there are strong parallels between macaque Mane-A*10 and human HLA-B*5701, we focused studies on Mane-A*10 (recently renamed Mane-A1*08401), a pigtailed macaque MHC class I allele that restricts the SIV Gag KP9 epitope (24, 27, 34). Both Mane-A1*08401 and HLA-B*5701 recognize overlapping immunodominant epitopes in the capsid region of Gag, sharing 62.5% sequence homology at the amino acid level (16). The SIV Gag KP9 epitope typically undergoes canonical escape in the key anchoring second amino acid (K165R escape) early in the course of SIV infection (24, 34). The SIV model of HAART therapy in HIV-infected individuals used in this study combined four antiretroviral agents to reduce viral load in both blood and CSF to extremely low levels at the majority of time points (9). With this drug regimen, the number of long-term latentently infected resting CD4\(^+\) cells in the blood and lymphoid tissues of SIV-infected macaques is comparable to that of HIV-infected patients on HAART, providing an excellent model to study latent HIV/SIV reservoirs, including the CNS. Although viral replication in the brain was dramatically reduced by HAART in this model, viral DNA levels in the brain were unchanged compared to untreated SIV-infected macaques, thus demonstrating that the brain also is a significant viral reservoir (39).

This study addresses three key questions: (i) whether K165R SIV Gag escape mutations emerge in plasma or CSF during the primary phase of decline in viremia despite initiating HAART treatment during acute infection, (ii) whether SIV K165R Gag escape mutations are archived in latent proviral DNA reservoirs in the periphery or in the brain of HAART-treated animals with suppressed viral replication, and (iii) whether replication competent SIV K165R Gag escape mutations could emerge from the resting CD4\(^+\) T cell reservoir present in HAART-treated SIV-infected macaques.

**MATERIALS AND METHODS**

**Animal studies.** Seven juvenile Mane-A*10-positive pigtailed macaques (Macaca nemestrina) were intravenously inoculated with SIV/DeltaB670 (50AID50) and SIV/17E-Fr (AID50 10,000) as previously described (38). Macaques were MHC typed using sequence-specific-primer (SSP)-PCR (24). Beginning 12 days after inoculation, three of the animals were treated daily with a 4-drug combination (HAART) until necropsy, approximately 160 days after beginning treatment. Treatment included nucleotide reverse transcriptase inhibitor (NRTI) PMPA, an analog of tenofovir (Gilead; 30 mg/kg body weight once daily), zidovudine (AZT; 400 mg/kg orally twice daily), and abacavir (30 mg/kg orally twice daily), and the integrase inhibitor L-780712 (Merck; 10 mg/kg orally twice daily) as previously described (9). This antiretroviral drug regimen had a low combined CNS penetration-effectiveness (CPE) score of 0.5 (21). Blood and CSF samples were collected postinoculation (p.i.) at 7, 10, 14, 21, and 28 days postinoculation (p.i.), and every 2 weeks thereafter until sacrifice. Animals were perfused with sterile saline at euthanasia to remove blood and circulating virus from the brain. These studies were performed in compliance with the recommendations in the National Research Council’s Guide for the Care and Use of Laboratory Animals of the National Institutes of Health according to a protocol approved by the Johns Hopkins University Institutional Animal Care and Use Committee (IHU IACUC: PR09M296). Procedures were performed on animals chemically restrained with the anesthetic ketamine hydrochloride.

**Quantitation of SIV RNA.** Viral RNA was measured in the plasma, CSF, and tissues by real-time reverse transcription (RT)-PCR using primers in the SIV gag region on an Applied Biosystems Prism 5700 sequence detection system. The primers to detect unspliced viral RNA included the following: forward primer, SAOG03, 5'-CAGGGAAGAAACGACAGTGAATTG-3' and reverse primer, SAGA04, 5'-GTTCGCGCTCCTCTCCTGGC-3'. and probe, pSUS05, 5'-[6-carboxyfluorescein [FAM]]ATTGGGATTACG AAAGGCCTGTTGAGG (6-carboxytetramethylrhodamine [TAMRA])-3', using the Quantitect Probe RT-PCR kit (Qiagen) and cycle conditions of 50°C for 30 min, 94°C for 15 min to reverse transcribe RNA, which was followed by 45 cycles of PCR at 94°C for 15 s, 55°C for 1 s, and 60°C for 30 s. If viral load levels in plasma were below the level of detection for our standard real-time RT-PCR assay (100 copies/ml detection limit beginning with a 1:40-ml volume of plasma or CSF), we then used a more sensitive assay in which we isolated and purified SIV RNA from 1 ml of plasma and then performed quantitative RT-PCR (qRT-PCR) with the same primers, probes, and cycling conditions to obtain a detection limit of 15 copies/ml, consistent with previous SIV assay reports (7). RNA samples were run in quintuplicate.

**Cloning and sequencing of the KP9 Gag epitope.** PCR was performed on cDNA generated from the plasma, CSF, basal ganglia, and leukocytes isolated from the lymph nodes and spleen of Mane-A*10-positive and -negative macaques with the SIV Gag-specific primers forward 5'--CACGCAGAAGAGAAA--3' and reverse 5'--GTTCCTCTCCTGAAT(AG)GC(T)GTGAATCC--3', using Platinum PCR supermix (Invitrogen) and specific cycle conditions (94°C for 2 min; 30 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for 1 min; and a final extension of 72°C for 8 min). PCR products from 2 to 4 independently prepared samples were then cloned into pCR2.1 vector (Invitrogen) using the TOPO TA cloning kit (Invitrogen). Colonies were plated on LB kanamycin (Kan) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and grown overnight, and 50 to 100 colonies per sample were picked and grown on LB Kan 100 μg/ml plates for 12 h statically. Cloned DNA was then sequenced by Genewiz (Rockville, MD). Sequences were aligned and analyzed using Geneious 5.0.2 software as previously described (24).

**Isolation of resting memory CD4\(^+\) T cells from SIV-infected HAART-treated macaques.** Resting memory CD4\(^+\) T cells from macaques were isolated as previously described (9). In brief, lymph node and spleen samples were mechanically agitated to obtain single-cell suspensions, and peripheral blood mononuclear cells (PBMCs) were isolated from blood by discontinuous density gradient centrifugation using 25% Percoll (GE Healthcare). Cell populations were then enriched for resting (HLA-DR) CD4\(^+\) T cells using magnetic bead separation (Miltenyi Biotec). CD4\(^+\) T cells were first isolated from lymph node and spleen suspensions and from PBMC using antibody-conjugated microbeads to deplete cells expressing CD8, CD11b, CD16, CD20, CD56, or CD66abce, thus yielding CD4\(^+\) T cells. Nonadherent cells were labeled with PE-conjugated anti-CD4 and PBMCs were cultured, which raised the limit of quantification from 10\(^2\) cells per well. In the event that the eluted CD4\(^+\) T cells did not yield enough cells for culturing 10\(^5\) cells to 3.2 × 10\(^5\) cells per well. In the event that the isolation of resting CD4\(^+\) T cells did not yield enough cells for culturing 10\(^6\) cells, 2 × 10\(^6\) cells were cultured, which raised the limit of quantification from 0.32 infectious units per million (IUPM) resting CD4\(^+\) T cells to 1.6 IUPM. The CEMs 174 cell line serves both to activate resting SIV Gag positive T cells through CD2-CD58 interactions and to express viruses released from latently infected cells that become activated (30). The presence of replication-competent SIV was determined by a p27 enzyme-linked immunosorbent assay (ELISA) of culture supernatants at 3 weeks of culture.

**Detection of replication-competent SIV Gag KP9 escape in the resting memory CD4\(^+\) T cell latent reservoir.** To detect replicating SIV Gag KP9 escape in isolated resting CD4\(^+\) T cells, we used a coculture assay in which isolated resting CD4\(^+\) T cells were cultured with the CEMs174 cell line as previously described (31). Resting CD4\(^+\) T cells were cultured in duplicate in a 5-fold dilution series ranging from 1 × 10\(^6\) cells to 3.2 × 10\(^5\) cells per well. In the event that the isolation of resting CD4\(^+\) T cells did not yield enough cells for culturing 10\(^6\) cells, 2 × 10\(^6\) cells were cultured, which raised the limit of quantification from 0.32 infectious units per million (IUPM) resting CD4\(^+\) T cells to 1.6 IUPM. The CEMs 174 cell line serves both to activate resting macro T cells through CD2-CD58 interactions and to express viruses released from latently infected cells that become activated (30). The presence of replication-competent SIV was determined by a p27 enzyme-linked immunosorbent assay (ELISA) of culture supernatants at 3 weeks of culture.

**Tetramer staining of PBMCs.** Macaque PBMCs were isolated using a standard Ficoll protocol. Cells then were resuspended in staining buffer (1% bovine serum albumin [BSA] in phosphate buffered saline [PBS]) at a concentration of 3 × 10\(^6\)/ml. Six staining conditions included (i) unstained control, (ii) CD3-PerCP (clone SP3), (iii) CD4-PE (clone SK3), (iv) CD8-FITC (clone SK1), (v) Mane-A*10/KP9 Tetramer-APC, and (vi) all antibodies in combination (34). After incubating with antibodies at room temperature for 45 min, cells were washed once with PBS and resuspended in 0.4 ml of staining buffer followed by FACs analysis.
RESULTS

Kinetics of viral replication with HAART treatment. To verify that HAART treatment of SIV-infected macaques expressing Mane-A*10 resulted in lower viral loads in plasma and CSF, SIV RNA levels in plasma and CSF were measured by qRT-PCR at serial time points post-inoculation. Following the initiation of HAART treatment at 12 days post-SIV inoculation, both plasma and CSF RNA levels were dramatically lower in the three treated animals compared to those in the four untreated animals. In treated animals, after 63 days p.i., plasma and CSF viral loads fell below the level of detection of our standard SIV RNA assay (100 copies/ml) and then varied until sacrifice (175 days p.i.) (9). We performed a more sensitive assay for SIV RNA, with a detection limit of 15 copies/ml to measure the SIV plasma load at time points when SIV RNA was less than 100 copies/ml as measured by our standard qRT-PCR assay. After initial suppression of plasma viremia to <15 copies/ml, two of the three animals (POy1 and PFy1) had intermittent blips of detectable virus in plasma at subsequent time points. The assay detection limit for CSF was 100 copies of SIV/ml (dashed line), as an insufficient CSF volume was available to perform a more sensitive SIV assay.

FIG. 1. Kinetics of viral replication with HAART treatment. Longitudinal SIV RNA levels in the plasma (individual animal data [A] and group medians [C]) and CSF (individual animal data [B] and group medians [D]) of Mane-A*10-positive macaques showed a marked decrease in viral load in animals treated with HAART beginning 12 days p.i. (n = 3 animals, PFy1, POy1, and PYd2) compared with those who did not receive treatment (n = 4 animals, 11U, PBm2, PJk2, and PRk2). Plasma viral loads in HAART-treated animals were <15 copies/ml (assay detection limit for plasma indicated by dashed line) by day 105 p.i. for all treated animals but then varied until the study end point at approximately day 175 p.i. After initial suppression of plasma viremia to <15 copies/ml, two of the three animals (POy1 and PFy1) had intermittent blips of detectable virus in plasma at subsequent time points. The assay detection limit for CSF was 100 copies of SIV/ml (dashed line), as an insufficient CSF volume was available to perform a more sensitive SIV assay.

Kinetics of viral escape in plasma and CSF. To determine whether administration of HAART alters development of SIV escape from MHC class I-restricted T cell control in the periphery and CNS during the decaying phase of plasma viremia, we first examined longitudinal plasma and CSF samples from untreated SIV-infected pigtailed macaques, from day 28 p.i. until terminal time points (24). In contrast, Mane-A*10 expression did not influence plasma viral load at any time point post-infection, as we have previously shown (24).

VOL. 85, 2011 LATENT REPLICATION-COMPETENT SIV ESCAPE IN HAART 9169
wild-type KP9 throughout infection in these different compartments. In two of the four untreated SIV-infected animals, the K165R escape mutation outcompeted the wild-type KP9 by the terminal time point. One macaque, animal PBm2, had no K165R escape mutation in the plasma at any time point postinoculation. Remarkably, however, the K165R escape mutation was detected in the CSF after 42 days p.i.

FIG. 2. Kinetics of viral escape in SIV infection. Untreated SIV-infected MHC-A*01-positive pigtailed macaques had various levels of wild-type KP9 (blue) versus K165R escape mutation (red) in both plasma and CSF. K165R escape mutation appeared as early as day 21 p.i. in plasma and persisted throughout infection. One pigtailed macaque, PBm2, did not have any detectable K165R escape mutation in plasma, though it was present in CSF at both days 56 and 77 p.i. A novel SIV Gag KP9 mutation, V171A (yellow), was also present as a minor variant in PRk2 day 70 p.i. CSF and PBm2 day 56 p.i. CSF samples.
Evolving CTL responses select for SIV Gag K165R escape. To examine the relationship between the emergence of SIV Gag K165R escape and the development of anti-KP9 CTL responses, we measured circulating KP9-specific CTLs from untreated Mane-A*10-positive pigtailed macaques using a Mane-A*10/KP9-specific tetramer by fluorescence-activated cell sorter (FACS) analysis (Fig. 3). In the three untreated macaques that developed a K165R escape mutation in plasma, we observed an increase in the number of tetramer-positive CD3⁺/CD8⁺ lymphocytes over time corresponding to the evolution of viral escape, consistent with CTL-driven selective pressure driving K165R escape at the SIV Gag KP9 epitope. Macaque PBm2, who had no escape mutation in the plasma at any time point postinoculation, had the lowest tetramer-positive CD3⁺/CD8⁺ lymphocyte response over time. Absolute levels of wild-type KP9 remained above 10⁶ copies/ml in plasma throughout infection in these untreated SIV-infected animals, providing a sustained antigenic drive to maintain KP9-specific CTLs despite evolution of the K165R escape mutation (Fig. 1).

Impact of HAART on kinetics of viral escape in plasma and CSF. In HAART-treated macaques, cloning and sequencing of the KP9 epitope in replicating SIV unexpectedly showed a pattern of escape similar to that seen with untreated macaques, even though plasma and CSF levels of SIV RNA were rapidly decaying (Fig. 4). The consensus escape mutation K165R arose as early as 21 days p.i., 9 days after HAART initiation. K165R was present in both plasma and CSF, and it arose at different times and in different proportions to wild-type KP9 in each compartment in individual animals. After 63 days p.i., viral loads in plasma and CSF were often below the level of detection. Antiretroviral treatment did not appear to alter the kinetics of viral escape from MHC class I control during the decay phase of viremia.

Identification of a novel escape mutation in SIV Gag KP9. In addition to the consensus escape mutation SIV Gag K165R, a second mutation in the SIV Gag KP9 epitope, SIV Gag V171A, was detected in both plasma and CSF samples from all three HAART-treated macaques beginning at day 42 p.i., with prevalence ranging from 5% to 37% of sequenced clones (Fig. 4). This novel mutation in SIV Gag has not been previously reported. The SIV Gag V171A mutation was rarely present in the untreated macaques, with only a single clone identified in the CSF from macaque PRk2 at day 70 p.i. and a single clone from macaque PBm2 CSF at day 56 p.i. (Fig. 2). Both plasma and CSF samples from additional SIV-infected HAART-treated Mane-A*10-negative animals were analyzed for the presence of V171A to exclude the possibility that this mutation might be a consequence of the drug regimen. The V171A Gag mutation was not present in any of these samples (data not shown).

Archiving of escape in proviral DNA. To determine whether the SIV Gag K165R escape mutation present in the plasma and CSF could be archived in latent reservoirs as proviral DNA, we cloned and sequenced the KP9 epitope present in SIV DNA in leukocytes isolated from the spleen, from submandibular and cervical lymph nodes that drained the head (head lymph nodes), and from basal ganglia in the brain (Fig. 5). The consensus escape mutation K165R was detected in all three of these compartments in all three HAART-treated macaques and in three of the four untreated macaques. PBm2, which had no escape in plasma, also had no escape in the spleen or basal ganglia. In contrast, SIV Gag K165R was found in the CSF at days 56 and 77 p.i.; K165R was also present in the DNA isolated from the head lymph nodes. The novel V171A mutation was also present in proviral DNA isolated from the cervical lymph nodes from HAART-treated macaques PYd2 and PFy1, and from untreated animal PBm2. V171A was also present in the proviral DNA isolated from the spleen of all three HAART-treated macaques. Absolute viral DNA levels...
from these three sites were equivalent in the untreated and HAART-treated animals, suggesting that latent virus reservoirs are of comparable sizes.

Isolation of replication-competent SIV Gag escape variants from a latent reservoir. To determine whether SIV bearing the K165R escape mutation can emerge from latency as replication-competent virus, we isolated resting memory CD4+ T cells from lymph nodes and spleen, as resting memory CD4+ T cells are well described for HIV/SIV latent reservoirs (9, 13, 32). Isolated CD4+ T cells were then cocultured with indicator CEMX174 cells, an SIV-susceptible cell line. Replication-competent SIV that emerged from the latent reservoir present in culture supernatants was detected by a p27 ELISA. Culture supernatants that were SIV p27 positive were then analyzed by cloning and sequencing SIV KP9 to determine if wild-type KP9 and/or the consensus escape mutation K165R were both present in replicating SIV (Table 1). In all three HAART-treated macaques, at least one clone of K165R was detected in the CD4+ spleen cell supernatants. In one of three HAART-treated macaques, K165R escape predominated in the CD4+ lymphocytes isolated from the head lymph nodes. The novel SIV Gag V171A mutation also reemerged from the CD4+ cells isolated from the spleen of HAART-treated macaque PYd2, demonstrating that V171A in the latent reservoir was replication competent. PYd2 also had the V171A mutation in plasma (Fig. 4) and proviral DNA from the spleen (Fig. 5). Parallel real-time RT-PCR of these latency sites in the HAART-treated animals demonstrated that there was not ongoing viral escape.

FIG. 4. Kinetics of viral escape during HAART treatment. The K165R escape mutation (red) was present in plasma and CSF of SIV-infected, HAART-treated Mane-A*10-positive macaques in various amounts beginning at day 21 p.i., which corresponded to 9 days of HAART therapy. The K165R escape mutation then persisted until 49 days p.i., the last time point at which SIV was still detectable in both plasma and CSF. The novel mutation V171A (yellow) was present in both the plasma and CSF in all treated macaques. The proportion of this mutation ranged from 5% to 37% of all clones beginning at day 42 p.i. A CSF sample was not obtained for PFy1 at day 28 p.i.
replication in these sites terminally. As the untreated macaques had high levels of replicating virus in these tissues, it was impossible to differentiate between viral RNA from productively infected cells and viral RNA reemerging from latently infected cells.

**DISCUSSION**

This study characterized the early emergence of SIV Gag K165R escape mutations within the Mane-A*10-restricted KP9 Gag epitope in both the plasma and CSF of SIV-infected macaques treated with combination antiretroviral therapy to suppress virus replication. Interestingly, K165R SIV Gag escape mutations developed in both plasma and CSF during the decaying phase of viremia after initiating HAART treatment before suppression of viral replication. After HAART suppression of SIV replication, SIV K165R Gag escape mutations persisted, archived in latent proviral DNA reservoirs, including resting CD4\(^+\) T cells isolated from lymph nodes and the spleen. The presence of SIV K165R Gag escape mutations in the resting CD4\(^+\) T cell reservoir isolated from HAART-treated SIV-infected Mane-A*10-positive pigtail macaques demonstrated that archived SIV K165R Gag escape mutations present in proviral DNA were replication competent. It remains to be determined whether SIV persists in CNS microglia, macrophages, or astrocytes either as latent replication-competent DNA genomes or via chronic low-level expression (1). Together, these findings revealed that replication-competent HIV/SIV escape mutations capable of evading MHC class I-mediated CTL control are archived in latent SIV reservoirs despite suppressive HAART therapy. These findings are highly relevant to understanding the consequences of HIV escape from MHC class I control in the face of suppressive HAART.

Virus escape from Mane-A*10 restriction is a consequence of the selective pressure from host CTL responses and is seen as early as 21 days p.i. with SIV, a time when host CTL responses have expanded to contain the exponential phase of virus replication during acute infection. Immediately prior to HAART suppression of SIV replication in plasma and CSF, we found approximately equal amounts of the SIV Gag KP9 wild type and the SIV Gag K165R escape mutation circulating in plasma. In contrast, wild-type SIV Gag KP9 predominated in proviral DNA after SIV suppression. This finding suggests that the latent reservoir is constantly replenished over time as plasma viremia declines with HAART; archived virus in latent reservoirs does not simply reflect circulating SIV genotypes present immediately prior to HAART control. This study also demonstrated that SIV escape mutations may be present in distinct compartments, including resting CD4\(^+\) T cells and the CNS independent of virus escape mutations present in the circulation.

Once escape develops at the Gag KP9 epitope, it can be archived in the host as proviral DNA. Our previous studies of the SIV/macaque model have demonstrated that as early as 4 days p.i., a latent reservoir has been established in the brain and the amount of proviral SIV DNA in the CNS reservoir remains stable throughout infection regardless of HAART (1, 36, 39). However, the presence of the SIV Gag K165R escape mutations in the CNS reservoir suggests that there may be

| Animal | Tissue source | % of clones of SIV Gag KP9 WT | K165R | V171A |
|--------|---------------|-----------------------------|-------|-------|
| PYy2   | Head lymph nodes | 48                          | 52    |       |
|        | Spleen        | 56                          | 42    | 2     |
| PFy1   | Head lymph nodes | 100                         |       |       |
|        | Spleen        | 99                          | 1     |       |
| POy1   | Head lymph nodes | 100                         |       |       |
|        | Spleen        | 99                          | 1     |       |

\(^a\) The replicating SIV K165R escape mutation emerged from the latent reservoir of CD4\(^+\) T cells isolated from the spleen of all three SIV-infected HAART-treated Mane-A*10-positive pigtail macaques. The replicating SIV K165R escape mutation also was recovered from the CD4\(^+\) T cells isolated from the lymph nodes that drained the head in one of the three macaques (PYy2). Replication-competent SIV V171A, a novel mutation frequently detected before HAART control, also emerged from the resting CD4\(^+\) T cells isolated from the spleen of the same animal. Genotype frequency was determined by sequencing 100 clones/tissue.
continual turnover and reseeding of the CNS reservoir throughout infection. The timings of initial escape in plasma and CSF compartments are similar and reflect the proportion of SIV escape mutations present in latent reservoirs. In the plasma of pigtailed macaque 11U, the K165R escape mutation was first detected at day 21 p.i. This animal had 85% SIV Gag K165R escape mutation archived at the terminal time point in the proviral DNA of the spleen, representing SIV circulating in the plasma. In the CSF of macaque 11U, the K165R escape mutation was first found at day 28 p.i., with 96% K165R escape mutation at the terminal time point in the proviral DNA of the head-draining lymph nodes, representative of SIV circulating in the CSF. In contrast, in macaque PBm2, the K165R escape mutation was not detected in plasma at any time point or in the proviral DNA of the spleen. However, in the CSF, the K165R escape mutation was present at day 56 p.i. Terminally, PBm2 also had 4% K165R escape mutation in proviral DNA of the head-draining lymph nodes. These animals illustrate that the SIV Gag K165R escape mutation may be confined largely to either peripheral or CNS compartments in different individuals.

In HAART-treated animals, virus replication is lowered by initiation of HAART at day 12 p.i., potentially dampening early CTL responses. However, KP9 escape still develops at early time points and in a proportion comparable to that of untreated SIV-infected macaques. In HIV-positive individuals, HAART is typically started later, during asymptomatic infection, when anti-HIV CTL responses are likely even stronger than acute stages of infection. For this reason, HIV escape mutations may be more prevalent in latent reservoirs in HIV-infected patients than is seen in these SIV studies.

It is important to verify that archived viral genotypes, including escape variants present during HAART suppression are replication competent. To determine whether archived SIV Gag K165R can emerge from latency leading to productive infection, we looked for the presence of replicating K165R emerging from resting CD4+ lymphocytes isolated from lymph nodes draining the CNS and spleen of HAART-treated macaques. Replicating virus emerging from cultured CD4+ lymphocytes contained both wild-type SIV Gag KP9 and the SIV Gag K165R escape mutation. The presence of both SIV Gag wild-type and escape variants in long-term cultures of isolated resting CD4+ T cells suggests that there is not a high fitness cost associated with the SIV Gag K165R mutation. However, studies have shown that naïve Mane-A*10-negative animals inoculated with SIV Gag K165R inserted in the SIVmac239 background have rapid reversion to SIV Gag KP9, suggesting that wild-type KP9 is more fit in vivo (12, 23). In vivo studies are needed to improve our understanding of the fitness costs of viral escape mutations in specific compartments, including the CNS.

In this study, we identified a novel SIV Gag mutation in KP9, SIV Gag V171A, prevalent in the plasma and CSF of HAART-treated macaques prior to viral suppression. SIV Gag V171A was also identified in proviral DNA in tissues and in replicating virus emerging from the isolated resting CD4+ T cell latent reservoir. This Gag escape mutation was (i) rarely found in untreated SIV-infected animals (<0.01% of all sequenced clones) and (ii) not present in SIV-infected HAART-treated animals that did not express Mane-A*10. It remains to be determined why the combination of HAART and Mane-A*10 expression contributes to the evolution of the V171A mutation. Unlike the canonical lysine-to-arginine mutation in the key second anchor position of the SIV Gag KP9 motif recognized by Mane-A*10 (K165R), the V171A mutation is at the eighth position, a site less likely to be critical for MHC class I peptide recognition. To examine whether KP9-specific CTLs recognize these two KP9 mutations, we evaluated PBMC responses to KP9, K165R, and V171A peptides using gamma interferon intracellular cytokine assays. These studies showed that there is no cross-reactivity between KP9-specific T cells and SIV Gag K165R and very limited cross-reactivity (~10%) with SIV Gag V171A in comparison with KP9, demonstrating that both of these KP9 mutations evade MHC class I presentation of HIV Gag peptides by the antiviral nucleoside analogue ribavirin (35).

The possibility that additional mutations in Gag epitopes recognized by MHC class I alleles arise with administration of HAART illustrates the need to study alterations in MHC class I-restricted HIV epitopes in HAART-treated patients. Despite early administration of aggressive antiretroviral treatment, HIV immune escape from CD8+ T cell control can still develop during decaying phases of viremia and then persist in latent reservoirs, including the brain, with the potential to emerge if HAART therapy is interrupted. Given that low-level viral replication may continue despite aggressive HAART treatment, the opportunity for HIV immune escape to evolve is more persistent than previously realized and underscores the need to improve our understanding of the relationship between CTL and HIV escape mutations in the setting of antiviral therapy.

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