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An SIV/Macaque Model Targeted To Study HIV-associated Neurocognitive Disorders

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Abstract

SIV infection of pigtailed macaques is a highly representative and well-characterized animal model for HIV neuropathogenesis studies that provides an excellent opportunity to study and develop prognostic markers of HAND for HIV-infected individuals. SIV studies can be performed in a controlled setting that enhances reproducibility and offers high translational value. Similar to observations in HIV-infected patients receiving anti-retroviral therapy (ART), ongoing neurodegeneration and inflammation are present in SIV-infected pigtailed macaques treated with suppressive ART. By developing quantitative viral outgrowth assays that measure both CD4+ T cells and macrophages harboring replication competent SIV as well as a highly sensitive mouse-based viral outgrowth assay, we have positioned the SIV/pigtailed macaque model to advance our understanding of latent cellular reservoirs, including potential CNS reservoirs, to promote HIV cure. In addition to contributing to our understanding of the pathogenesis of HAND, the SIV/pigtailed macaque model also provides an excellent opportunity to test innovative approaches to eliminate the latent HIV reservoir in the brain.

Although antiretroviral treatment (ART) has reduced the incidence of HIV dementia, a high prevalence of HIV-associated neurocognitive disorders (HAND) persists in the ART era

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Conflict of Interest

All of the authors declare that they have no conflict of interest.

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ranging from asymptomatic neurocognitive impairment (ANI) to severe HIV-associated dementia (HAD)(1). Prior to ART, HAD developed frequently in HIV-infected individuals with low CD4+ T-cell counts and high HIV plasma viral loads. With effective ART, the incidence of HAD and AIDS decreased (1, 2). However, mild to moderate forms of HAND still occur despite long-term suppression with ART (2). Estimates of HAND in HIV+ people on ART range from 15–55% in various studies (3). Despite persistent HAND during ART, our understanding of the pathogenesis of ongoing CNS damage underlying HAND remains incomplete. Possible mechanisms include legacy effects (viral or inflammatory-mediated damage present pre-ART), sustained CNS inflammation, various host genetic factors, and neurotoxicity of ART compounds (4, 5).

The SIV/Macaque Model of HAND

SIV/macaque models have been of great value in elucidating the pathogenesis of HIV-induced nervous system damage. A particularly informative SIV CNS model has been developed and optimized in the Retrovirus Biology Laboratory at Johns Hopkins University over the last 25 years. To develop a reliable SIV model of HIV CNS disease, we mapped the viral determinants of both macrophage-tropism and neurovirulence, thereby generating the neurovirulent molecular clone SIV/17E-Fr. Studies with recombinant SIV indicated that while changes in *env* sequence were sufficient to confer macrophage tropism, *env*, *nef*, and 3'LTR sequences all were necessary for virus replication in the CNS and neurovirulence (6–8). Subsequent studies discovered that inoculating pigtailed macaques (*Macaca nemestrina*) intravenously with both SIV/17E-Fr and the immunosuppressive CD4+ T cell-depleting SIV swarm SIV/DeltaB670 led to consistent development of AIDS and prototypic SIV encephalitis in the majority of animals within a three-month period in contrast to inoculating rhesus macaques (*Macaca mulatta*) with the identical combination of SIV (9, 10). We recently detailed the differences between these two macaque species with respect to induction of SIV CNS disease with a focus on the neuroprotective roles of MHC class I alleles in each species (11). This comparison is of note because, in an alternate SIV/rhesus macaque model, SIV encephalitis develops in the majority SIVmac251-infected rhesus macaques only after transient depletion of CD8+ cells (12–14). Although the SIV CD8+ depletion model illustrates the key role of CD8+ cells in neuropathogenesis and has been informative regarding CNS macrophage biology, study of the specific role of cell-mediated immunity becomes limited when both CD8+ T cells and NK cells are depleted.

For SIV studies, it is crucial to use specific-pathogen-free macaques to avoid confounding co-infections. All pigtailed macaques in our studies are tested negative for SIV, STLV, SRV, and *Cercopithecine herpesvirus 1* prior to study entry. Macaques are also screened twice yearly for *Mycobacterium tuberculosis*. While SIV macaque studies ideally would include balanced numbers of males and females, most SIV studies use primarily male macaques. Female macaques are highly valuable for breeding colonies and hence are difficult to obtain for most SIV studies.

Nervous System Alterations in the SIV/Pigtailed Macaque Model

The SIV/pigtailed macaque model has served as the platform to characterize the successive immunologic and viral parameters developed throughout progressive infection during acute, asymptomatic (an ~8 week interval), and terminal stages of disease (15). The SIV/pigtailed macaque model offers many parallels to HIV infection including development of characteristic CNS inflammation that correlates with high viral load in the brain, cognitive and motor deficits typical of HAND, and classic lesions of HIV/SIV encephalitis (9, 16–20). SIV-induced neuropathology is characterized by numerous perivascular inflammatory infiltrates in brain and spinal cord predominantly composed of macrophages and multinucleate giant cells that frequently contain replicating SIV, recapitulating classic HIV CNS pathology (10, 21). Neuroinflammatory changes are accompanied by metabolic alterations including decreased glucose transport across the blood-brain barrier (22). Neuronal dysfunction manifested by accumulation of amyloid precursor protein (APP) in axons correlated with behavioral outcome measures including decreased performance on bimanual motor tasks (19, 20). Synaptodendritic alterations including increased synaptophysin expression in the brain of untreated infected macaques compared to ART-suppressed macaques also have been characterized (23, 24). In addition, the potential value of PET neuroimaging studies employing the ligand PK1195 to measure peripheral benzodiazepine receptor expression in the brain reflecting microglial activation present in SIV and HIV was first shown in this SIV model (25).

Peripheral nervous system (PNS) alterations resembling HIV lesions include inflammation in the trigeminal and dorsal root ganglia (DRG; ganglionitis) and loss of epidermal nerve fibers (ENF) (26, 27). DRG inflammation corresponds with loss of epidermal nerve fiber density as well as slowed conduction velocity in small unmyelinated sensory C-fibers in the sural nerve, a feature of various painful neuropathic conditions (27). SIV-induced sensory fiber loss extends to the dense sub-basal sensory nerve plexus of the cornea, suggesting that *in vivo* corneal confocal microscopy, a technique that has shown promise for the assessment of sensory nerve fibers in patients with diabetes mellitus, may be used to track progressive PNS damage caused by HIV as well as neurotoxic antiretroviral treatments (28). To determine whether suppressive ART altered SIV PNS outcomes, we examined DRG and skin samples from SIV-infected pigtailed macaques receiving ART (ART1, Table 1). Although ART suppressed SIV replication and reduced macrophage activation in DRG, epidermal nerve fiber measurements remained significantly lower as compared to uninfected, untreated pigtailed macaques. These findings demonstrate that significant peripheral nervous system damage persists in SIV-infected macaques despite ART, providing the basis for studying HIV-induced PNS damage that persists in the ART era (29).

Role of MHC Class I Alleles in SIV CNS Disease

A valuable finding uncovered in the SIV/pigtailed macaque model was that a subset of SIV-infected pigtailed macaques inoculated with both SIV/Delta B670 and SIV/17E-Fr (approximately one third of animals) did not develop SIV encephalitis despite progressing to AIDS. To compensate for this variability in CNS disease outcomes, especially for experimental design of intervention studies testing novel neuroprotective or latency

reactivating strategies, group sizes would need to be doubled for most outcome measures, greatly increasing costs and effort. By studying animals with and without SIV-induced encephalitis, our lab discovered a neuroprotective MHC class I allele that accounted for the majority of the variation in CNS disease outcomes; animals that expressed the MHC class I allele *Mane-A1*084* were much less likely to develop SIV encephalitis (30, 31). This finding substantially refined the SIV model by allowing us to select for or against inclusion of animals expressing this MHC class I allele based on study design. The MHC class I allele *Mane-A1*084:01* (formerly *Mane-A*10*) presents an immunodominant SIV Gag capsid epitope termed KP9 (KKFGAEVVP₁₆₄₋₁₇₂), which is a critical capsid region homologous to HIV Gag KF11, an immunodominant epitope that is recognized by *HLA-B*5701* in humans (32, 33). In SIV-infected pigtailed macaques expressing *Mane-A1*084*, immune pressure on the KP9 epitope drives the canonical lysine to arginine escape mutation, K165R (33). Interestingly, in our model, *Mane-A1*084* influence was specific to CNS disease outcomes and did not impact either plasma viral loads or progression to AIDS (30).

Identification of *Mane-A1*084* as a neuroprotective allele led to additional insights into the role that cytotoxic T cells play in establishing the latent HIV reservoir in the CNS. SIV K165R Gag escape mutations were archived in latent proviral DNA reservoirs including the CNS in animals receiving ART that suppressed viral replication (31). Replication-competent SIV Gag K165R escape mutations also were identified in the resting CD4⁺ T cell reservoir, demonstrating that escape from MHC class I-mediated control occurs during decaying phases of viremia prior to suppression and then persists as replication-competent provirus in tissue sites including the brain (31).

We also demonstrated a CNS compartment-specific fitness cost to viral escape from MHC class I-mediated immunologic pressure. After inserting the canonical SIV Gag escape mutation K165R into the neurovirulent molecular clone SIV/17E-Fr, we inoculated *Mane-A1*084*-positive pigtailed macaques with the cloned escape mutant virus and showed decreased viral load in CSF but not plasma. Viral sequencing revealed transient reversion to wild-type Gag KP9 only in the CSF, consistent with decreased CNS fitness of SIV with K165R. In reciprocal experiments, we vaccinated pigtailed macaques with a virus-like particle-based Gag KP9 construct to focus CTL responses on Gag KP9. Subsequent challenge with SIV/17E-Fr demonstrated lower viral load in CSF but not plasma. Combined, these findings demonstrate a CNS-specific loss in viral fitness attributed to a single Gag mutation that permits escape from *Mane-A1*084* control (26, 27). Given that a single-epitope vaccine is not likely to prevent SIV or HIV infection because of potential development of escape mutations, studies in which macaques are vaccinated with multiple SIV Gag epitopes (as well as epitopes beyond Gag) are needed. In addition, our data support development of a therapeutic vaccine approach to control CNS reservoirs given that stimulating *Mane-A1*084*-mediated CTL responses by virus-like particle (VLP) KP9 vaccination prior to SIV infection had a CNS-specific protective effect with lower CSF viral loads (34). Ongoing efforts aim to identify whether therapeutic vaccination protocols employing a similar strategy to stimulate CNS-specific MHC class I mediated responses may be pivotal in HIV cure efforts, especially for targeting the CNS compartment.

The SIV/Pigtailed Macaque Model as a Biomarker Discovery Platform

SIV/macaque models are especially valuable for studying the serial events in the neuropathogenesis of HIV as paired samples of plasma and CSF can be obtained from multiple longitudinal time points throughout the course of infection. To discover biomarkers in blood and CSF that preceded and predicted development of SIV encephalitis, host immune response mediators and viral RNA levels have been characterized. In brief, predictive CSF markers including CCL2, IL-6, neopterin, YKL-40, and SIV RNA load were elevated beginning in the asymptomatic phase of infection and sustained until terminal stages of disease. In blood, the macrophage activation marker sCD163 as well as hemoglobin level and platelet count were all predictive biomarkers for development of CNS disease (15, 35).

One of the most surprising circulating hematologic markers of retroviral-associated CNS disease is decline in platelet count. Platelet decline during asymptomatic infection has been associated with significantly increased risk for the development of HIV-associated dementia (36, 37). In our cohort of SIV-inoculated pigtailed macaques, we observed pronounced declines in platelet count during the acute (d7–14 p.i.) phase of infection and again in asymptomatic and terminal infection (38). After day 28 post-inoculation, animals that later developed SIV encephalitis during terminal infection had a greater decrease in platelet count than animals that did not develop SIV encephalitis, though the magnitude of platelet loss during acute infection was similar between SIV-infected animals that progressed to SIV encephalitis and those that did not (37). These data emphasize the importance of considering absolute change from baseline platelet count as a prognostic indicator of increased risk for the development of HIV-associated CNS disease. This association was further supported by studying HIV-infected patients and additional SIV/macaque model data (15, 36). This predictive platelet loss represents the combined effect of decreased platelet production, increased platelet destruction, and increased association of activated platelets with other cells including CD16+ monocytes. The last two associations may reflect a fundamental role for platelet activation in the pathogenesis of HAND (39, 40).

Anti-Retroviral Therapy (ART) in SIV-infected Pigtailed Macaques

To study HAND in the context of ART, we have tested a number of ART regimens in the SIV-infected pigtailed macaque model beginning at day 12 post-SIV inoculation for all regimens (41). Initially, SIV-infected pigtailed macaques were treated with PMPA, saquinavir, atazanavir, and the Merck integrase inhibitor L-870812, successfully reducing viral load in plasma and in CSF to below the limit of detection in this accelerated model of HIV (ART1, Table 1 and Figure 1) (42). As reported for HAND, sustained CNS inflammation persisted despite efficacy of ART, with elevated TNF α and CCL2 in the brain. Furthermore, by comparing blood and CSF samples obtained pre-infection with samples obtained at terminal study time points representing longest duration of SIV replication control, we found strong parallels between our SIV model and reports of HAND biomarkers identified in HIV-infected groups receiving ART (Figure 2A and B). In CSF, levels of both the inflammation marker neopterin and the neuronal damage marker neurofilament light were elevated despite long-term ART. In the plasma, sCD163, a monocyte/macrophage

activation marker, and CCL2 were also higher than pre-infection time points. These findings show that SIV-infected pigtailed macaques receiving suppressive ART nonetheless develop persistent inflammation and neuronal damage that closely corresponds with multiple HIV HAND reports on these biomarkers.

With availability of newer ART options, access to saquinavir for SIV studies has become limited. Given this constraint, we shifted to an ART regimen consisting of PMPA, darunavir with ritonavir boost, and the integrase inhibitor (ART2, Figure 1 and Table 1). This regimen proved similar to the original ART regimen (ART1) with respect to SIV suppression in plasma and CSF and served as the basis for performing studies aimed at reactivating latent SIV including from potential CNS reservoirs (43). A limitation of this regimen was the requirement of twice/day oral dosing of large amounts of three compounds, a treatment scheme that was defined by pharmacokinetic studies prior to use in SIV-infected macaques. To further refine the use of ART in SIV-infected macaques, we next tested the efficacy of a newer regime consisting of a single once/day subcutaneous injection of PMPA, FTC, and dolutegravir (ART3; Table 1). Previous studies had shown that this combination successfully suppressed SIVmac251 replication in the plasma of rhesus macaques (44). Our studies showed that the ART3 combination also rapidly suppresses SIV/17EFr and SIV/DeltaB670 in both plasma and CSF of pigtailed macaques (Figure 1). Suppression is sustained in both plasma and CSF over time, providing a long-term suppression model to study both persistent CNS immune activation and neuronal damage that may underlie HAND, as well as the adverse sequelae that follow cessation of ART (45). Furthermore, this ART regimen optimizes the SIV-infected pigtailed macaque model to study HIV latency in the CNS and test novel HIV cure strategies.

Measuring Latent Reservoirs in the SIV/Pigtailed Macaque Model

There is an extensive literature that details the frequency of HIV infection and latency in resting CD4⁺T cells in HIV-infected ART-suppressed humans. To demonstrate that a comparable resting CD4⁺ T cell reservoir also is established in SIV-infected pigtailed macaques on ART, we developed the rCD4⁺ QVOA for the SIV model. We used this assay to measure the frequency of rCD4⁺ cells in blood as well as in both lymph nodes and spleen. This led to the important insight that the frequency of rCD4⁺ cells that harbor replication competent SIV is very similar in blood, lymph nodes and spleen. In addition the frequency of resting CD4⁺ T cells in blood that contained replication competent virus in our SIV macaque model was equivalent (1 cell in a million) to that observed in HIV patients on ART (42).

To evaluate the SIV model in depth as a platform for studying SIV and HIV latency with a focus on macrophage reservoirs including the CNS compartment, we developed a macrophage (MΦ) quantitative outgrowth assay (49). The assay measures levels of SIV-infected CD11b⁺ cells isolated from the brain as well as other tissues. Serial dilutions of purified MΦs are plated in poly-L-lysine coated wells and then stimulated with TNF-α, which is known to stimulate HIV-1 gene expression in the U1 MΦ cell line (50) and which, in preliminary experiments, enhanced the recovery of infectious SIV. Potential contamination with infected CD4⁺ T cells is assessed by both flow cytometry and a

calibrated RT-qPCR assay for rearranged T cell receptor (TCR) β chain RNA, which is only found in T cells. Replication-competent SIV released from stimulated M Φ s is amplified in culture through the addition of the CEMx174 cells (42, 51, 52) and levels of SIV RNA in the supernatant are quantitated by RT-qPCR for 2 weeks. With this approach, infected M Φ s have been detected in brain, lungs, and spleen isolated from viremic SIV-infected macaques (53). The frequency of productively infected M Φ s that can release replication-competent SIV in this assay varies from animal to animal in the range of 100–10,000/10⁶ M Φ s and is generally higher in animals with high levels of viremia (53). This striking level of M Φ infection cannot be explained by T cell contamination.

The use of M Φ -QVOAs will be a critical tool for defining myeloid cell reservoirs. Indeed, preliminary studies indicate that the frequency of infected M Φ s is dramatically lower in animals on ART. In ART-suppressed macaques, an average of 0.23 circulating monocytes per million harbored replication-competent latent SIV genomes (Figure 3). Monocytes are released from the bone marrow and have a lifespan of 48–72 hours. Some reports demonstrate that monocytes can traffic through tissues (spleen, skin) without differentiating into tissue M Φ s (54–56). Although monocytes do not usually fit the definition of “latent viral reservoirs”, it is possible that SIV-infected bone marrow promonocytes, which do not express CD34 but express high levels of CD4, proliferate into latently infected blood monocytes that could traffic into tissues and maintain M Φ reservoirs. Levels of infected macrophages in tissues from ART-suppressed macaques varied from 0.23 (brain) to 1 (spleen and lung) per site, suggesting that tissues in suppressed macaques harbor a significant number of latently infected tissue macrophages that have the potential for reactivation after ART interruption (Figure 3).

A Murine Viral Outgrowth Assay (MVOA) for HIV and SIV

A limitation of CD4⁺ T cell QVOAs is that they underrepresent the actual replication competent reservoir; the macrophage QVOA may have the same limitation (57). To overcome this restriction, the Metcalf Pate and Blankson labs at JHU collaboratively developed a novel ultrasensitive murine viral outgrowth assay (MVOA) for HIV and SIV (58). In the MVOA, the xenoreactivity of human or macaque T cells towards murine antigens induces a high level of T cell activation that reverses latency. Viruses released from infected cells replicate in the xenografted activated CD4⁺ T cells, producing viremia. Because large numbers of patient or macaque cells can be evaluated (up to 50 million per mouse) and multiple mice can easily be xenografted at once, this assay has a greater potential dynamic range than the QVOA and is anticipated to be especially advantageous in measuring large reservoir reductions induced by curative strategies. An additional important advantage of the MVOA is that the probability that a latently infected CD4⁺ T cell becomes activated is greatly enhanced by the prolonged and profound immune activation of the xenografted T cells by xenoantigens. Xenografted CD4⁺ T cells can be further stimulated *in vivo* in the mouse with anti-CD3 and anti-CD28 antibodies to induce an even higher level of activation. This assay has been validated and refined by two independent groups to date to detect intact, non-induced proviruses (INP) that are missed by the QVOA and thus may provide a more accurate estimate of reservoir size (59, 60). We have successfully used this assay to detect SIV in pigtailed macaque peripheral blood mononuclear cells and purified

CD4⁺ T cells (58), and are in the process of further refining the assay for the detection of SIV in lymphoid and non-lymphoid organs to identify latent viral reservoirs in sanctuary organs.

Conclusion

SIV infection of pigtailed macaques is a highly representative and well-characterized animal model for HIV neuropathogenesis studies including SIV-infected animals on ART. The pigtailed macaque model of accelerated SIV-associated CNS disease provides an excellent opportunity to study and develop prognostic markers of HAND for HIV-infected individuals in a controlled setting that offers high translational value. In addition, similar to observations in ART-treated HIV-infected patients, we have reported evidence of ongoing neurodegenerative and inflammatory changes in pigtailed macaques treated with suppressive ART. Biomarkers of HAND provide us with the capability of tracking the CNS status of SIV-infected macaques serially throughout studies, thereby optimizing a biomarker panel that may be valuable for monitoring the development of HAND in clinical settings. In addition to contributing to our understanding of the pathogenesis of HAND, the SIV/pigtailed macaque model also provides an excellent opportunity to test innovative approaches to eliminate the latent HIV CNS reservoir. Finally, our SIV ART studies also have identified potential adjunctive therapies for HAND including maraviroc, minocycline, and a combination of fluconazole and paroxetine (46–48); the SIV ART model we have established will facilitate testing additional therapies for HAND.

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Figure 1.

SIV-infected pigtailed macaques receiving ART beginning day 12 post-inoculation had sustained viral suppression in both **plasma** (left) and **CSF** (right) although time to suppression varied with ART regimen. For comparison, untreated SIV-infected plasma and CSF viral loads are shown in red (circles). ART1 regimen (purple down triangles) consisted of PMPA, saquinavir, atazanavir, and a Merck integrase inhibitor. ART2 (green up triangles) contained darunavir, ritonavir, and the integrase inhibitor. ART3 (blue squares), the most efficacious with shortest interval to suppression, consisted of a single daily subcutaneous injection of PMPA, FTC, and dolutegravir. All anti-retroviral compounds were graciously donated (detailed in acknowledgements).

CSF

Plasma

Figure 2.

HAND biomarkers in CSF (top panel) and plasma (bottom panel) samples from SIV-infected pigtailed macaques receiving ART demonstrate parallels between HIV cohort studies and the SIV model.

Figure 3.

Number of SIV-infected blood monocytes, tissue macrophages, and blood CD4⁺ T cells in SIV-infected pigtailed macaques untreated or treated with antiretroviral therapy (ART). Values are shown as infectious units per million (IUPM) cells. Lines represent medians.

Table 1**ART Regimens in the SIV/Pigtailed Macaque Model**

Regimen	Compound	Class	Dosage	Route	Frequency	References
ART1	PMPA	NRTI	10 mg/kg *	Subcutaneous	Once/day	
	Saquinavir	Protease Inhibitor	205 mg/kg	Oral	Twice/day	Dinso (42)
	Atazanavir	Protease Inhibitor	270 mg/kg	Oral	Twice/day	
	L-870812	Integrase Inhibitor	10 mg/kg	Oral	Twice/day	
ART 2	PMPA	NRTI	10 mg/kg	Subcutaneous	Once/day	
	Darunavir	Protease Inhibitor	480 mg/kg	Oral	Twice/day	
	Ritonavir	PI Boost	24 mg/kg	Oral	Twice/day	Gama (43)
	L-870812	Integrase Inhibitor	10 mg/kg	Oral	Twice/day	
ART 3	PMPA	NRTI	20 mg/kg	Subcutaneous		
	Emtricitabine (FTC)	NRTI	40 mg/kg	Single Combined	Once/day	Whitney (44)
	Dolutegravir	Integrase Inhibitor	2.5 mg/kg	Injection		

* PMPA dose was initially 30 mg/kg for the first two weeks of treatment then reduced to 10 mg/kg.