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SIV Latency in Macrophages in the CNS

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Abstract

Lentiviruses infect myeloid cells, leading to acute infection followed by persistent/latent infections not cleared by the host immune system. HIV and SIV are lentiviruses that infect CD4⁺ lymphocytes in addition to myeloid cells in blood and tissues. HIV infection of myeloid cells in brain, lung and heart cause tissue specific diseases that are mostly observed during severe immunosuppression, when the number of circulating CD4⁺ T cells declines to exceedingly low levels. Antiretroviral therapy (ART) controls viral replication but does not successfully eliminate latent virus, which leads to viral rebound once ART is interrupted. HIV latency in CD4⁺ lymphocytes is the main focus of research and concern when HIV eradication efforts are considered. However, myeloid cells in tissues are long-lived and have not been routinely examined as a potential reservoir. Based on a quantitative viral outgrowth assay (QVOA) designed to evaluate latently infected CD4⁺ lymphocytes, a similar protocol was developed for the assessment of latently infected myeloid cells in blood and tissues. Using an SIV ART model, it was demonstrated that myeloid cells in blood and brain harbor latent SIV that can be reactivated and produce infectious virus in vitro, demonstrating that myeloid cells have the potential to be an additional latent reservoir of HIV that should be considered during HIV eradication strategies.

Introduction

AIDS emerged as a new disease in 1980 and was shown to be caused by a retrovirus, the human T-cell lymphotropic virus III (HTLV-III), thought to be similar to human viruses HTLV-I and II (1–4). AIDS pathogenesis included not only immunosuppression but also infection of tissues; in particular the brain, causing encephalitis and dementia in adults and children (5, 6). Soon after the AIDS virus was isolated and molecular clones were constructed to further characterize the virus molecular hybridization studies demonstrated that HTLV-III was actually more closely related to the ungulate lentiviruses than to the human deltaretroviruses HTLV-I and II, and the virus was renamed human immunodeficiency virus (HIV) (7, 8). HIV infection in vivo has many parallels to lentivirus pathogenesis causing not only primary immunodeficiency but also CNS- and lung-specific diseases. In contrast to most lentiviruses, the cellular tropism of HIV included not only macrophage lineage cells but also CD4⁺ lymphocytes (9). While classic lentiviruses like

visna virus do not infect lymphocytes, infection of macrophages does cause lymphocyte activation and lymphocytic proliferation in infected tissues such as brain, lung, and joints (10, 11). Lentivirus infections are characterized by an acute phase followed by suppression of virus replication in blood and tissues that led to a state of undetectable virus in most animals (12). Despite lack of detectable viral RNA, cells from infected, suppressed animals can be activated to produce virus *in vitro*. This was also an early observation in studies of the ovine (visna virus) and caprine (caprine arthritis-encephalitis virus, CAEV) viruses in which monocytes from infected animals without detectable viral RNA mature *in vitro* into macrophages with subsequent reactivation and detection of viral cellular RNA and virus in the culture supernatant (13). Viral latency in myeloid lineage cells in lentiviruses *in vivo* is also a feature of non-primate lentiviruses shared by SIV and HIV.

The pathogenesis of HIV during the early AIDS epidemic and before the development of antiretroviral therapy was characterized by immunodeficiency disease as a result of loss of circulating CD4⁺ T cells and by subsequent opportunistic infections. CNS neurologic disease accompanies these infections and infectious virus is detected in the cerebrospinal fluid (CSF) (5, 14, 15). HIV infection in brain of infected adults and children was shown to be responsible for the neurologic disease and was called AIDS Dementia Complex (ADC). This neurologic syndrome, ADC, was the cause of mortality in HIV infected individuals. Although HIV enters the CNS during acute infection, CNS disease manifested mainly during later stages of infection, when individuals were immunosuppressed (16).

Initially, the cause for the late stage development of ADC was not clear, however, later studies of the replication and regulation of HIV in macrophages, the cells infected in brain demonstrated differential regulation of HIV in myeloid cells as compared to lymphocytes (17–19). HIV transcription in macrophages is regulated by the transcription factor c/EBP β and its isoforms (17–22), in contrast to transcriptional regulation of HIV by NF- κ B in CD4⁺ lymphocytes (23, 24). The differential expression of c/EBP β isoforms is modulated in macrophages by IFN β (17–19). Presence of this cytokine in brain causes the translation of a dominant negative form of c/EBP β that down-regulates viral transcription and histone acetylation of the HIV LTR, resulting in transcriptional silencing of HIV *in vitro* and SIV *in vitro* and *in vivo* (20–22, 25, 26). Thus, regulation of HIV transcriptional activation and suppression by IFN β may be one mechanism for establishing HIV latency in macrophages in tissues. Viral latency is a state of reversibly nonproductive infection of individual cells and provides an important mechanism for viral persistence and escape from immune recognition and drug pressure.

In the era of ART, fully suppressed HIV-infected individuals usually control virus replication in blood controlling viral levels below 50 copies of HIV/ml. The occurrence of systemic immunosuppression and HIV-associated dementia has been greatly diminished by treatment. ART does not eliminate the viral provirus from tissues but suppresses virus that becomes latent, the latent reservoir is recognized as a major barrier to curing HIV-1 infection. HIV research is mainly focused on the suppression of virus replication in CD4⁺ lymphocytes (CD4⁺T) and on mechanisms of virus latency and the formation of long-lived CD4⁺T reservoirs (27). The dramatic decrease in CNS dementia suggests that the infected brain macrophages (microglia and perivascular macrophages) are no longer actively infected

during ART. In contrast to the availability of CD4⁺T cells in blood, however, brain macrophages cannot be directly studied in humans because of the difficulty of analysis *in situ*. To circumvent this hurdle, HIV studies performed in cerebrospinal fluid (CSF) and brain (post-mortem) of HIV infected individuals on ART demonstrated that HIV is present in brain despite undetectable virus in the plasma (28, 29). The identification of HIV-neurocognitive disorders (HAND) and HIV RNA in the CSF in HIV-infected individuals on suppressive ART further demonstrates that HIV infection persists in brain in either a latent or persistent form (28, 29). SIV-infected macaques on ART regimens, similar to those used in humans, provide the opportunity to study longitudinal progression of AIDS, CNS infection, disease pathogenesis and viral latency of both CD4⁺T and myeloid cells in blood, CSF, and tissues, including brain.

HIV & SIV Infection in the CNS

Both HIV and SIV infect the CNS as early as the first week after infection, and both viruses are detectable in CSF as well as in the blood of infected individuals during acute, chronic, and late stage disease. Infection of the CNS is caused by entry of infected CD4⁺ T cells and monocytes trafficking across the blood brain barrier (BBB). HIV and SIV infection is then spread to perivascular macrophages that line the BBB and to microglia, the resident brain macrophages. Microglia are embryonically derived cells that self-renew rather than being replenished from circulating monocytes (30, 31). Infected microglial cells have been identified in HIV-infected humans and SIV-infected non-human primates (32–37). However, the role of microglia infection in long-term HIV latency and persistence is controversial despite the detection of HIV DNA in post-mortem brain of ART-suppressed individuals. In SIV infection, microglia isolated from both viremic and ART suppressed macaques contain SIV DNA and RNA (see below for detailed studies).

New Insights on Macrophage Origin and Phenotypes

Depletion of CD4⁺ T cells is the hallmark of HIV-1 infection, and most studies of pathogenesis and latency of HIV and SIV have focused on lymphocytes. Nonetheless, macrophages are a natural host cell for lentiviruses (13, 38–40) and multiple lines of evidence point to the importance of macrophages during HIV infection: 1) The accessory protein Vpx (HIV-2) specifically enhances viral replication in macrophages (41, 42), but not in CD4⁺ T cells. Comparably, Vpr (HIV-1) recruits UNG2 into virions and modulates viral mutation rates in macrophages (43); 2) Many HIV-1 strains replicate efficiently in macrophages, independent of the presence of Vpx (44, 45); 3) AIDS is characterized by dramatic depletion of CD4⁺ T cells, however, despite depletion of these cells high plasma viral load persists, suggesting that viral replication is occurring in cells other than CD4⁺ lymphocytes. In the macaque models for SIV infection, experimental depletion of CD4⁺ T cells results in an increase in viral load and selection *in vivo* of CD4-independent macrophage-tropic SIV phenotypes (46–48); 4) Damage to both lung and brain (interstitial pneumonia and encephalitis) are directly associated with infections of macrophages (49, 50); 5) Finally, activation of monocytes and macrophages during cART suppression in HIV is associated with higher morbidity (51–53).

In the last several years, advances in myeloid cell biology have shown that every tissue harbors distinct populations of macrophages: those arriving during embryogenesis (both yolk-sac and fetal liver-derived), and post-natal bone marrow-derived blood monocytes (31). A similar classification can be extended to humans based on transcriptomic and phenotypic profiling (30). Most resident tissue mononuclear phagocytes - including Kupffer cells in the liver, Langerhans cells in the skin, microglia in brain, and alveolar and peritoneal macrophages –originate from *Myb*-independent progenitor cells that migrated directly or indirectly from the yolk sac to their respective tissues during embryogenesis (30, 54). They are predominantly maintained through self-renewal during steady state, independently of adult hematopoiesis (55). These cells have only recently been thoroughly characterized as distinct from monocyte-derived macrophages, and little is known about their *in vitro* function (54). Resident tissue macrophages, infected with HIV or SIV, have the potential to divide and expand the viral reservoirs in tissues. In addition, HIV and SIV infected macrophages are not efficiently killed by CD8+ T cells unlike infected CD4+ T cells (56, 57). Thus, resident tissue macrophages remain in tissues long-term, are capable of self-renewal, are relatively resistant to the cytopathic effects of HIV infection compared to CD4+ T cells, and may serve as stable viral reservoirs.

Some tissue macrophages are directly derived from blood monocytes, which arise from common monoblasts in bone marrow. Especially during infection or inflammation, circulating monocytes infiltrate tissues via pro-inflammatory mediators, including chemokine gradients, and differentiate into cells with a broad range of functions, depending on the microenvironment (58, 59). Although morphologically similar, macrophages originating from monocytes have distinct transcriptomes, surface markers, and phenotypic profiles from those of embryonic origin (31, 60). Macrophages with the same ontogeny can express different sets of transcripts and may respond differently to pathogens, depending on the tissue location (61).

In addition to distinct ontogenies, *in vitro* studies have demonstrated that monocyte-derived macrophages (MDM) can dramatically change their phenotype, pattern of gene expression, and functionality under different culture conditions (62). Generally, macrophages matured in CSF-1 are the starting point for many murine and human experiments. Any alteration to these culture conditions will present specialized functional properties, referred to as polarization. Historically, post-differentiated MDMs treated with IFN- γ were defined as M1, or classically activated, and MDMs treated with IL-4 termed M2, or alternatively activated. Current perspective acknowledges a spectrum of polarization, with large shifts in gene expression based on stimuli used in culture, and suggests terminology specific to the activator, such as M(IFN- γ + LPS). MDMs activated by IFN- γ alone or in combination with microbial stimuli (LPS) or cytokines (TNF) express copious amounts of inflammatory and effector molecules (IL-6, IL-2, CCL2, CXCL10, iNOS, and ROS), contribute to the induction and maintenance of T_H1 and T_H17 responses. They also have enhanced complement- and antibody-mediated phagocytosis with microbicidal capacity (63). Conversely, MDMs exposed to anti-inflammatory stimuli such as IL-4, IL-13, IL-10, TGF- β , immune complexes, or glucocorticoids are associated with T_H2 responses, high levels of Fc receptors (CD16, CD32, CD64), and the resolution of inflammatory responses (64, 65). These phenotypes are likely to be less distinctive *in vivo* since the microenvironments

that macrophages inhabit are exposed to a broad and changing range of signaling molecules. Also, *in vitro* studies show that macrophages, even after full polarization, can rapidly change phenotypes when exposed to a novel stimulus (66).

In the context of HIV infection, polarization studies demonstrate the antiviral features of M1 MDM (67). This inflammatory phenotype has poor surface expression of CD4 and DC-SIGN, which are important receptors for viral binding. They also inhibit intracellular steps of viral replication due to high levels of *APOBEC3A*, *tetherin*, and *TRIM22* (68, 69). These cells present a transcription profile (65), which, at least in theory, should support HIV RNA expression (70, 71). Thus, while cell activation is directly related to increased viral transcription in CD4⁺ T lymphocytes, this is not the case in macrophages. Polarization has been mainly explored in bone marrow-derived MDMs *in vitro*; while *in vivo* macrophages are incredibly heterogeneous, and likely exist along a continuum of the M1-M2 spectrum.. During inflammatory and infectious processes, there is a major influx of monocytes into tissues (75, 76), making it difficult to demonstrate that shifts in polarization are occurring in resident cells and not in recently arrived monocytes. Recent studies on brain macrophages show that microglia respond to cytokine stimulation similar to MDMs (77–79). While the concepts described by *in vitro* studies have been useful in tissue macrophages (72–74), direct translation of these studies has not been comprehensively explored *in vivo* (80).

SIV Macaque Models

SIV infection in macaques comprehensively reproduces the immunodeficiency symptoms observed in HIV-infected humans, with infection of CD4⁺T cells and monocytes in blood, and of macrophages in tissues such as lymph nodes, bowel, brain, lung, spleen, and heart (81, 82). Antiretroviral drugs have been shown to fully suppress SIV replication in blood (82) and, in limited studies, CSF (83–85) to levels comparable to those in ART-suppressed HIV-infected individuals. SIV-infected macaques carry latently infected CD4⁺T cells that harbor replication competent virus, as shown by quantitative viral outgrowth assays (QVOA) (86) and by the rapid rebound of SIV in plasma when ART is discontinued (87). The role of infection and latency in monocytes and tissue macrophages in ART-suppressed macaques has only been recently addressed. This is important to pursue because, in the era of ART and potential HIV cure approaches, fully characterizing all latently infected cells that may contribute to viral rebound after cessation of ART has become a priority. Initial trials of HIV eradication strategies have focused on viral load (VL) in plasma as an indication of HIV reactivation or change in the latent reservoir, although there is evidence from the “Boston Patients” that virus rebound occurred not only in the blood but also in the brain, based on CNS symptoms prior to virus rebound and presence of HIV in CSF (88). However, the mechanisms that drive latency in macrophages remain unclear and, probably, are distinct from those in CD4⁺ T-cells. Also, new evidence indicates that many latent SIV genomes located in tissues may respond differently to latency reversing agents (LRA) (84).

Several well-characterized SIV macaque models have been used mainly to study the development of AIDS and the pathogenesis of infection using a variety of SIV viral strains and molecular clones. The most commonly used strains are cloned SIVmac239 and SIVmac251 strains, or viruses derived from these strains. Also, there are SIV models

focused on the study of infection and disease progression in the CNS (81). Each model uses distinct mechanisms to achieve SIV encephalitis, which includes infecting macaques with naturally occurring neurotropic and immunosuppressive virus swarms, neurotropic virus adapted by in vivo passage of SIV, and non-neurotropic strains in association with CD8+ lymphocyte depletion (81). A recent review has compared these SIV models concluding that all the models include monocyte/macrophage infection and activation, and increased number of macrophages in the brain of macaques that develop encephalitis (81).

This review focuses on studies using an SIV macaque model in which animals are inoculated with a viral strain swarm (SIVdelta/B670) that contains 22 SIV *env*-defined genotypes and a neurovirulent, molecular clone (SIV/17E) that consistently causes AIDS in 90 days with a high incidence of CNS infection and encephalitis (89–91). This SIV model has been characterized longitudinally, demonstrating that SIV infection in brain occurred in the first week of infection (by 4 d p.i.) and that virus infection in brain was differentially regulated from the periphery (89, 92). Macrophages in brain, including resident microglia and perivascular macrophages are the major target cell in the CNS; SIV and HIV infection of macrophages has been shown to be transcriptionally regulated by C/EBP β isoforms (17–22), which are regulated by innate immune responses as discussed previously in this review. The regulation of SIV transcription in brain macrophages provides a mechanism for silencing of the viral genome in macrophages and is likely to contribute to mechanism of SIV and HIV latency in tissue resident macrophages, particularly in the CNS.

ART regimens in this dual-infection SIV model result in suppression of viral load to undetectable levels in the plasma and CSF (86). There is an extensive literature that addresses the frequency of HIV infection and latency in CD4+T cells in ART suppressed humans, but this same rigorous analysis had not been applied to the ART-suppressed SIV macaque models. Therefore, we developed an SIV rCD4+ QVOA analogous to the HIV quantitative viral outgrowth assay (QVOA) (86) and used it to measure the frequency of rCD4+ cells harboring replication competent SIV latent genomes, not only plasma but also in spleen and multiple lymph nodes and spleen (FIGURE 1) (86). These studies demonstrated that the frequencies of latently infected rCD4+ cells in blood, lymph nodes, and spleen are very similar to those in ART-suppressed HIV-infected individuals. In another study using the same macaque model, it was shown that SIV DNA persists in the brain despite undetectable levels of SIV cellular RNA in the CNS (83).

Evidence for a Functional Viral Reservoir in Brain Macrophages

Our well-characterized and consistent macaque model for AIDS and CNS disease was also used to evaluate the contribution of brain macrophages in SIV latency and reactivation during ART (84). In this study, SIV-infected macaques were fully suppressed with ART for over one year (<30 copies of SIV RNA per ml of plasma). To induce *in vivo* activation of latent reservoirs we tested a combination of two synergistic LRAs: the protein kinase C (PKC) activator ingenol-B and the histone deacetylase (HDAC) inhibitor vorinostat. We had previously shown that the ingenol-B reactivated HIV-1 genomes in two different *in vitro* HIV-1 latency models as well as in CD4+T isolated from HIV-infected individuals (93). Our results show that LRA administration led to an increase in VL in cerebrospinal fluid (CSF)

in one of two SIV-infected macaques. The increase in virus in the CSF was 10-fold higher than virus rebound in the plasma and phylogenetic analyses of viruses demonstrated distinct genotypes in the plasma and CSF, suggesting compartmentalization of virus in the brain. These findings suggest that the CNS harbors latent SIV genomes despite long-term ART suppression and that these reservoirs can be activated with LRAs. Although a small number of animals were assessed, this study is the first *in vivo* demonstration that the brain represents a consequential viral reservoir (84).

Relative Levels of Infection of CD4⁺ T Lymphocytes and Macrophages in SIV infected Macaques

The frequency of HIV or SIV infection of macrophages in tissues has been examined previously in a number of studies by measuring viral DNA in cells isolated from tissues (94). However, this approach overestimates the number of productively infected CD4⁺ T cells due to the presence of a large proportion of defective proviruses *in vivo* (95). Thus, we developed a quantitative viral outgrowth assay similar to the CD4⁺ T cell assay for HIV and for SIV to estimate the size of the potential latent reservoir of monocytes and macrophages (MΦ-QVOA) (94) (Figure 2 **Macrophage QVOA**). To validate this assay, we first examined the number of macrophages and CD4⁺ T cells in blood and tissues of viremic SIV-infected macaques (94). To eliminate the potential contribution of CD4⁺ T cells to the quantitation of infected macrophages, we also assessed the number of CD3⁺ T cells in each assay by measuring *TCRβ* RNA.

The MΦ-QVOA utilized the expression of the integrin CD11b (96) on monocytes and tissue macrophages and separated these myeloid cells from other cell types by sorting with CD11b Miltenyi magnetic beads (94). Like the CD4⁺ T cell QVOA, the MΦ-QVOA involved a serial dilution of selected cells. Antiretroviral drugs were added to the culture to prevent virus spread from any CD4⁺ T cells that might be in the culture during the first couple of days, while macrophage differentiated and matured *in vitro*. Unlike T cells, macrophages do not divide exponentially when activated in culture and strongly adhere to culture plates when grown *in vitro*. Cell supernatants were collected from the MΦ-QVOA wells after 12 days of cultivation (Figure 2). Viral RNA was isolated from replicate wells and quantitated individually by qRT-PCR. The frequency of infectious virus per million (IUPM) was calculated using limiting dilution statistical analyses (97).

Quantitating macrophages with the QVOA from SIV-chronically and late-stage infected macaques demonstrated that the number of productively infected macrophages in a given tissue was surprisingly similar from macaque to macaque, whereas the number of productively infected macrophages varied widely across different tissues from the same SIV-infected macaque. The highest number of infected macrophages (424 IUPM) was measured in spleen demonstrating that splenic macrophages are highly susceptible to SIV infection and harbor high levels of productive genomes (Figure 3 **IUPM CD4⁺ T cells, monocytes, macrophages**). This suggests a role for tissue microenvironments in mediating virus infection of macrophages, since populations of macrophages that reside in each tissue may

be differentially susceptible to SIV/HIV infection based on the cytokine profiles of the organs (98).

The number of infected brain macrophages, including both microglia and perivascular macrophages, were quantitated by MΦ-QVOA in SIV infected macaques during both the chronic and late stage disease. Brain sections of these animals were examined for pathological changes associated with SIV encephalitis and were scored as none, mild, moderate, or severe disease. It was found that the brain of animals with mild to severe CNS disease contained the next highest level of infected cells (median 231 IUPM) compared to spleen. The two macaques with the most productively infected cells brain macrophages (Pm3 and Pm4 with 24,000 IUPM) had severe encephalitis and high levels of viral RNA in brain. The macaques without CNS disease had undetectable numbers of infected microglia/macrophages and little or no detectable viral RNA in the brain. Thus, the number of productively infected cells in the brain correlated with the severity of disease and the level of viral RNA detected in brain by qPCR. This study provided the first estimate of productively infected CD4⁺ T cells and myeloid cells in SIV-infected tissues *in vivo*.

Quantitation of Latently Infected Brain Macrophages in ART Suppressed SIV Macaques

ART has dramatically reduced the severe forms of HAND, but milder forms of neurologic impairment are still observed in HIV-infected individuals virally suppressed on ART. HAND is thought to be a result of chronic central nervous system (CNS) inflammation in the brain (99–102). It is unclear whether inflammation is caused by incomplete penetrance of antiretroviral drugs into the CNS or the persistence of virus in brain macrophages (BrMΦ) in a latent state that reactivate causing sporadic inflammatory responses (103). Indeed, some HIV-infected individuals on ART have no detectable virus in the plasma but have measurable levels of HIV RNA in the CSF (104, 105). Also, HIV was detected after rebound in the CSF of the Boston patients, who had undetectable plasma HIV during ART interruption for several months (88). There is a continuing debate on the sources of virus in the CSF and the cause of the chronic inflammation in brain that leads to HAND.

Using our SIV macaque model with SIV-infected macaques suppressed with four antiretroviral drugs for 100–500 days, we evaluated whether infected cells persist in brain despite ART. SIV-infected pigtailed macaques were virally suppressed with ART, and plasma and CSF VL were analyzed longitudinally to demonstrate viral suppression in the peripheral blood and the CNS. To assess whether virus persisted in brain macrophages (BrMΦ) in these long-term ART suppressed macaques, we used MΦ-QVOA, qPCR, and *in situ* hybridization (ISH) to measure the frequency of infected cells and levels of viral RNA and DNA in brain. Viral RNA in brain tissue of suppressed macaques was undetectable, although viral DNA was observed in all animals. The MΦ-QVOA demonstrated that the majority of suppressed animals contained latently infected BrMΦ. We also showed that virus produced in the MΦ-QVOAs was replication competent, suggesting that latently infected BrMΦ are capable of re-establishing productive infection upon ART interruption. This study provides the first confirmation of replication-competent SIV in BrMΦ of ART-suppressed

macaques and suggests that the highly debated question of viral latency in macrophages, at least in brain, has been addressed in SIV-infected macaques treated with ART.

In this study, we identified latently infected BrMΦ in brain samples containing fewer than 10 copies of SIV DNA per million cells. In animals suppressed for more than 500 days, the number of infected macrophages measured in the MΦ-QVOA ranged from 3.6 to 15 in a hundred million cells, supporting the low level of DNA quantitated by qPCR. Thus, the quantitation of SIV DNA or RNA by PCR in brain tissue does not fully reflect the size of the latent functional reservoir, which is the main target in eradication strategies. (FIGURE 3 **Brain Macrophage IUPM Frequency**)

Most animals in the study harbored latently infected macrophages in regions of the brain that contained no detectable viral RNA. After 1.7 years of viral suppression, three macaques in the study showed no viral RNA in basal ganglia and parietal cortex. Nevertheless, all three macaques had replication competent virus produced in the isolated BrMΦ. Of note, we detected viral RNA by ISH in the brain of one of the macaques in the study treated with a LRA. However, the RNA was detected the occipital cortex, a brain section not used for the BrMΦ QVOA. These results corroborate findings showing that SIV, and potentially HIV, infection in brain is highly focal (84) and can provide variable results depending on the brain region analyzed for each specific assay.

Also, the results from the MΦ-QVOA showing that a small number of replication competent viruses are sporadically released in some latent BrMΦ indicate that parameters we used to define a positive QVOA well supernatants with > 50 SIV RNA copies/mL) underestimate the number of latently infected cells that produce replication competent virus, at least in macrophages. Indeed, viruses collected from most QVOA assay supernatants were able to spread in healthy PBMC.

The demonstration that there is latent replication-competent virus in SIV-infected ART suppressed macaque brain provides a mechanism for the ongoing macrophage activation observed both in the macaques and HIV individuals suppressed on ART. Recent studies have suggested that, while virus does not spread during ART suppression, there is ongoing stochastic activation of virus genomes in latently infected cells (106, 107). Reactivation of virus without spread in the macrophage is likely to induce innate immune responses and cellular activation. Thus, productively infected latent macrophages in brain provide a mechanism for the ongoing inflammation of HIV in a fully suppressed individual. Also, it has been recently demonstrated that defective provirus expressed in rCD4s could be recognized by adaptive immune responses, shaping the proviral landscape (95). It is possible that similar responses might happen with viral proteins generated from defective proviruses in BrMΦ.

Conclusions

The presence of a long-term functional reservoir of SIV in brain macrophages that parallels the biologic and pathologic features of infected individuals with HIV encephalitis suggests that the HIV in brain may be a formidable barrier to strategies to decrease or eliminate latent

reservoirs. Further, the presence of low levels of viral DNA in brain of ART-suppressed macaques can contribute to virus spread in brain and potentially in the periphery during cessation of ART or eradication treatments. While the brain is protected by the blood brain barrier and eradication approaches may not penetrate the brain, immune activation in the periphery could potentially activate virus in the CNS. On the other hand, the lack of CNS penetrance of such eradication therapies would potentially leave the CNS functional reservoir intact and undermine virus eradication. Strategies that include activation of virus in brain may have the effect of increasing inflammation and neuronal toxicity due to increased macrophage activation and production of cytokines, as we observed in a suppressed macaque treated with two cycles of LRAs (84). Our studies demonstrating the presence of a functional latent reservoir in brain macrophages have major implications for SIV eradication studies used to model treatment for HIV individuals. Examining recrudescence of virus in plasma but not CSF may overlook a source of virus that significantly contributes to the virus rebound.

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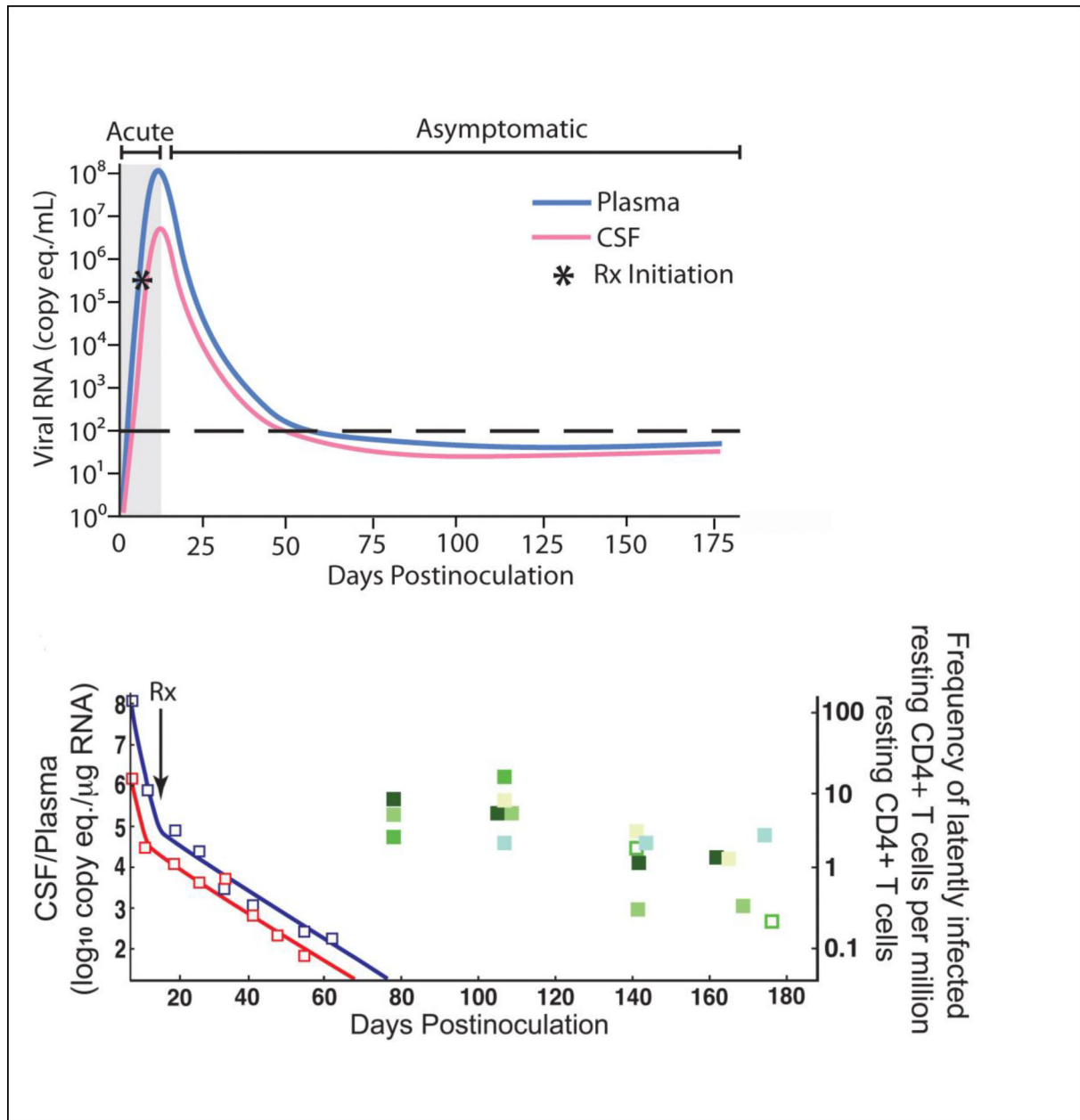


Figure 1.

(a) Viral RNA levels in plasma and CSF increase rapidly during the first 7–10 days prior to ART treatment. Within a few days after initiation of ART, plasma and CSF viral load decline. By approximately 60 days p.i., plasma and CSF viral load have declined to below the level of detection (<50 copy eq./mL) and viral loads remain low during ART. (c) The decline in plasma and CSF viral RNA occurred in two phases: an initial short-term rapid decline followed by a longer term slower decline similar to the two-phase decline seen in the plasma of HIV-infected individuals on HAART. At 80 days p.i., there were 8–10 latently infected resting CD4+ T cells per million resting CD4+ T cells in the blood. These numbers declined gradually to ~ one latently infected resting CD4+ T cells per million by 175 days p.i. Abs., absolute; CSF, cerebrospinal fluid; p.i., post-inoculation; Rx, therapy; vRNA, viral

RNA., plasma vRNA;, CSF vRNA (severe/moderate encephalitis);, CSF vRNA (no/mild encephalitis);, Abs. CD4+ cell counts in blood.

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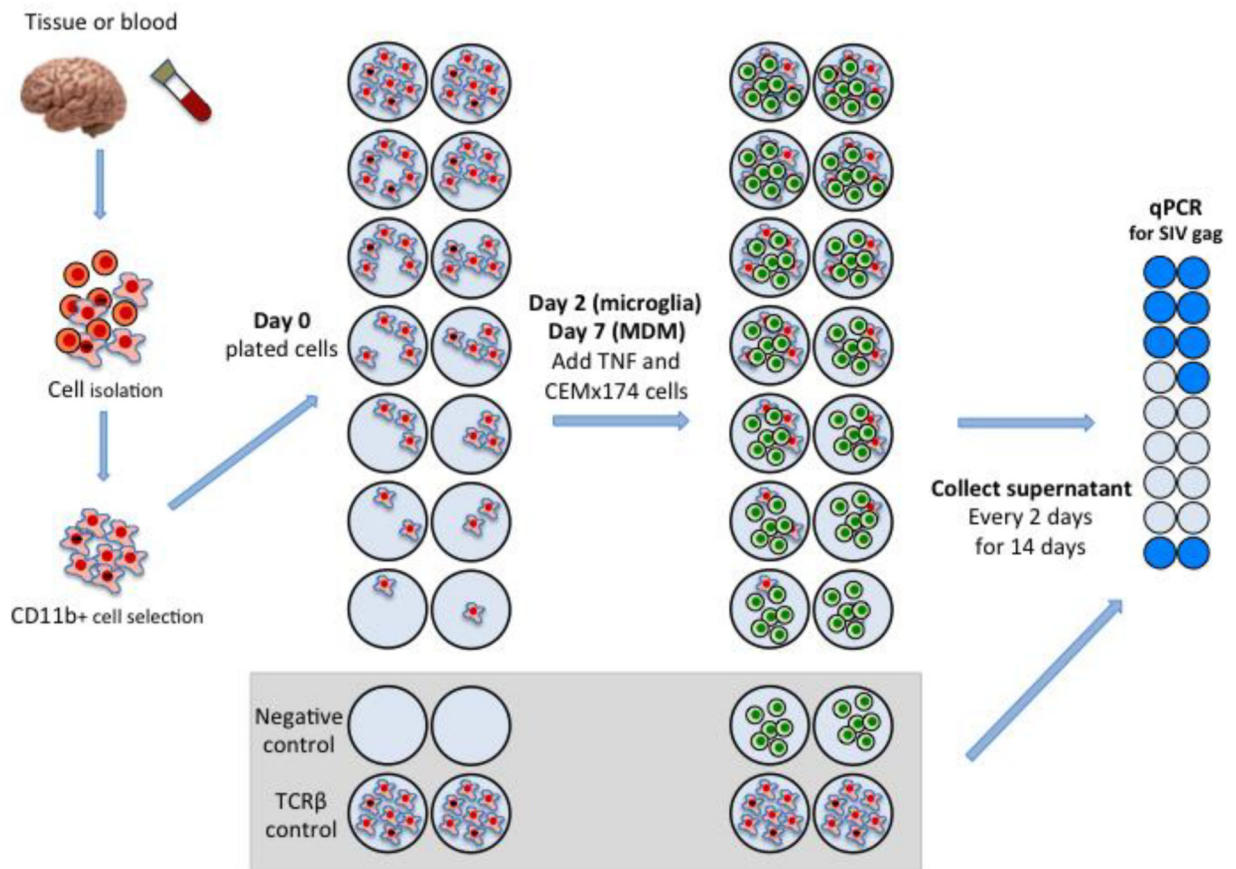


Figure 2.

MΦ-QVOA. Monocytes from blood and macrophages from brain were collected from SIV-infected animals and purified by CD11b-specific bead selection. Macrophages expressing CD11b were plated in serial dilutions in triplicate wells. Cells were cultured with zidovudine (AZT) and darunavir (DRV). Nonadherent cells and the antiretrovirals were removed prior to activation with TNF and co-culture with CEMx174 cells (85, 94).

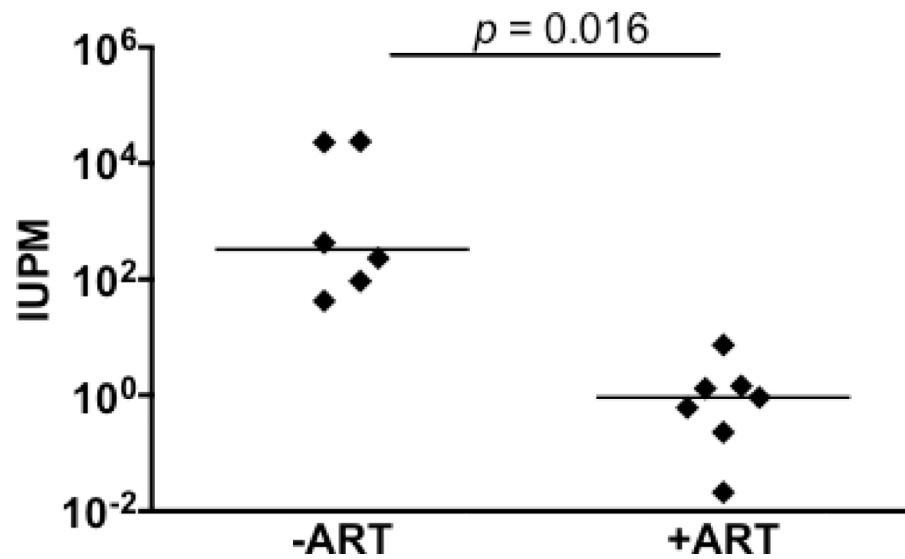


Figure 3.

Quantitation of latently infected brain macrophages in ART-treated macaques by MΦ-QVOA. Quantitation of infected brain macrophages from ART-treated macaques (85). Comparison between the numbers of SIV-infected brain macrophages isolated from animals that were not given ART (-ART) and the numbers isolated from animals that were treated with ART and with viral suppression <10 copies SIV RNA/ml plasma. The horizontal black line represents the median IUPM values. The MΦ QVOA results from SIV-infected animals with and without ART have been reported (85, 94). Significance was determined by Mann-Whitney nonparametric *t* test; a *P* of <0.05 was considered significant.