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SIV Infection Impairs the Central Nervous System in Chinese Rhesus Macaques

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Abstract

The central nervous system (CNS) impairment is a consequence seen in SIV infection of rhesus macaques of Indian-origin, which is more common in infected macaques with rapid disease progression than in those with conventional disease progression. Here, we investigated the CNS damages in SIVmac239-infected Chinese rhesus macaques. We demonstrated that SIV infection of Chinese macaques could cause neuropathological impairments, which was evidenced by appearance of SIV-RNA positive cells, the infiltration of activated macrophages and abundant multinucleated giant cells (MNGCs) in the different regions of the brains. The animals with high viremia and short survival time (average of 16 weeks, rapid progression, RP) had severer neuropathological changes than those with conventional progression (CP). As compared with the RP animals, CP macaques had lower viremia and much longer survival time (average of 154 weeks). These findings indicate that SIVmac239 infection of Chinese rhesus macaque can be used as a suitable animal model and alternative resource for neuroAIDS research.

Keywords

SIVmac239; Chinese rhesus macaque; Neuropathological lesion; Rapid progression; NeuroAIDS

Introduction

Neurological disease is a well-documented complication of HIV-1 infection, especially in the terminal stages of AIDS (Budka 1991). Although the incidence of severe dementia in HIV-infected individuals (HAD) has declined due to highly active antiretroviral therapy (HAART), the cumulative prevalence of HIV-associated neurocognitive disorders (HAND) has continued to increase (Clifford and Ances 2013; Maschke et al. 2000; McArthur et al.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

2003; Sacktor et al. 2002). Mild neurocognitive impairment is currently the most prevalent form of HIV-1 associated central nervous system (CNS) disease (Heaton et al. 2010; Liner et al. 2008; McArthur et al. 2010; Simpson et al. 2006; Smyth et al. 2007). Similar to HIV, Simian immunodeficiency viruses (SIV) and Simian-human immunodeficiency virus (SHIV) (Barber et al. 2006; Chakrabarti et al. 1991; Gonzalez et al. 2000; Horn et al. 1998; Lackner et al. 1991; Sharma et al. 1992) were found to invade the CNS of pig-tailed or rhesus macaques (RMs) of Indian-origin, causing neuropathological lesions that recapitulate the characteristics of HIV neuropathogenesis (Harbison et al. 2014). Moreover, investigators have demonstrated that rapid disease progression is the best correlate of the development of SIVencephalitis in Indian RMs (Westmoreland et al. 1998). Given that AIDS is a disease that affects humans of diverse origins, it is important to examine HIV animal models with different geographical background, including evaluation of the neuropathogenesis of SIV infection in Chinese RMs.

In comparison to Indian macaques, little has been devoted to research on the neuropathogenesis in SIV or SHIV-infected Chinese RMs. We previously reported the severe lesions in the CNS of two SHIV_{KU-1} infected-Chinese RMs with conventional progression (Xiao et al. 2015), demonstrating that the infected animals had the neuropathological damages, which are similar to the features of HIVencephalitis or SIV-induced encephalitis (SIVE) in Indian macaques. In the present study, we further investigate the neurological damages in the brains of SIV-infected Chinese RMs with rapid and conventional progression of disease.

Materials and Methods

Experimental Animals and SIV Challenge

All animals were housed at the Animal BioSafety Level III (ABSL-III) laboratory of the Wuhan University, in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care International. Protocols used in this study were approved by the Institutional Animal Care and Use Committee of Wuhan University School of Medicine. Eleven female RMs (*M. mulatta*) of Chinese origin were enrolled in this study. At the beginning of the study, the animals were screened and found negative for infections of simian retrovirus D, SIV, simian T leukemia virus type 1 (STLV-1) and Herpes virus B. The animals were inoculated with 10^3 – 10^4 TCID₅₀ of SIVmac239 by either intravenous or intravaginal route. Whole blood from the inoculated macaques was collected for monitoring SIV viral load and CD4/CD8 T cell ratio. Animals were euthanized if they lost >20 % of their body weight or developed intractable diarrhea that was unresponsive to supportive or antibiotic treatment, respiratory signs with radiographic evidence of pneumonia, persistent anorexia and lethargy, or neurologic signs. Brain tissues were collected at the time of necropsy.

Quantification of Virus in Plasma

Plasma samples were collected during the course of study. Expression level of SIV RNA in plasma was measured by real-time RT-PCR assay. Total RNA was extracted from cell-free plasma by using TRI Reagent (Molecular Research Center, Inc) as the manufacturer's

instructions. Viral RNA was then reverse transcribed to generate cDNA for real-time quantitative RT-PCR. Real-time PCR was performed using following primers: 5'-GCAGAGGAGGAAATTACCCAGTAC-3' (forward) and 5'-CAATTTTACCCAGGCATTTAATGTT-3' (reverse). A serial of 10-fold dilutions of standard RNA template was used to generate a standard curve for each assay. All amplifications were performed in duplicate. Assay results were normalized to the volume of plasma extracted and expressed as numbers of SIV RNA copy equivalents per milliliter of plasma.

Flow Cytometry

CD4/CD8 T cell ratio was monitored in 50 µl of EDTA anti-coagulated whole blood stained with anti-human CD3-FITC, anti-human CD4-PerCP-Cy5.5 and anti-human CD8-APC. All antibodies were obtained from BD Biosciences (San Diego, CA) and used for cell labeling according to the manufacturer's instructions. The cells were fixed with 0.5 % paraformaldehyde and analyzed on a flow cytometer (BD FACSVerse). Results were analyzed using FlowJo software (TreeStar, v7.6.1).

SIV-Specific In Situ Hybridization

Formalin-fixed, paraffin-embedded brain sections were assayed for SIV viral RNA expression by in situ hybridization (ISH). Briefly, deparaffinized and rehydrated sections were first incubated with pre-hybridization buffer at 50 °C for 30 min to avoid background staining. Tissue sections were hybridized overnight at 50 °C with either sense or antisense digoxigenin-UTP-labeled SIVmac239 probe that spanned the entire genome. The hybridized sections were then blocked with 2 % normal horse serum (Gibco) and 1 % bovine serum albumin (BSA) in 0.1 M Tris/HCl (pH 7.4) for 1 h and incubated with sheep anti-digoxigenin-alkaline phosphatase (Roche Molecular Biochemicals) at 37 °C for 30 min, followed by staining with BCIP/NBT (Sigma) and counter staining with methyl green. ISH-stained sections were visualized and photographed with a camera-assisted microscope (OLYMPUS, BX53).

Immunohistochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded brain tissues obtained from all macaques at necropsy were consecutively cut into 5 µm sections, and mounted on glass slides for immunohistochemistry (IHC) staining. Sections were deparaffinized and rehydrated before heat-mediated epitope retrieval was performed. To avoid non-specific staining in tissue sections, hydrogen peroxide blocking and serum blocking were applied at room temperature. Primary antibodies were diluted in PBS and incubated with the sections at 4 °C overnight. After primary antibody incubation, sections were washed and stained with goat anti-mouse/rabbit IgG secondary antibody (HRP Polymer) for 30 min at room temperature. The color reaction product was developed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with hematoxylin followed by dehydration and mounting. The primary antibodies used were: the pan-macrophage marker CD68 (1/50; Boster), the macrophage scavenger receptor CD163 (1/400; Boster), the microglia activation marker Iba-1 (1/1000; Wako) and SIVmac251 p28 antibody (1/400; MyBioSource). Sections were visualized, and imaged with a camera-assisted microscope (OLYMPUS, BX53). For

immunofluorescence staining, primary antibody labeled brain sections were incubated with either goat-anti mouse IgG-Cy3 secondary antibody (1/100; Boster) or goat-anti rabbit IgG-FITC secondary antibody (1/100; Boster) for 30 min at room temperature. 4', 6-diamidino-2'-phenylindole dihydrochloride (DAPI) staining was used for all fluorescence staining conditions to identify nuclear in cells. Slides were examined and photographed using a camera-assisted fluorescent microscope (OLYMPUS, IX71).

Results

Outcomes of SIVmac239 Infection in Chinese Rhesus Macaques

The animals were inoculated with 10^3 – 10^4 TCID₅₀ of SIVmac239 by either intravenous ($n = 9$) or intravaginal route ($n = 2$) (Table 1). The infected animals were classified as RP and CP macaques based on the viremia and the rate of disease progression (Mellors et al. 1996). As shown in Table 1, three macaques identified as RP macaques were euthanized at 9, 15 and 24 weeks post infection, with the average survival time was only 16 weeks, which is similar to the rapid disease progression reported among Indian RMs (Brown et al. 2007; Williams et al. 2008). In contrast, 7 out of 8 CP macaques survived more than 100 weeks post infection, with an average survival time of 154 weeks. Macaque WSP12 did not have any signs of advanced HIV disease and euthanized at 215 weeks post infection. Statistically, there is a significant difference in survival time between CP and RP macaques (Table 1, $P < 0.05$; log rank test). In addition, disease phenotypes also differed distinctly between RP and CP macaques. Most CP macaques (7/8) commonly succumbed to the gastrointestinal dysfunctions, such as severe diarrhea at the end-stage. In contrast, clinical signs in RP macaques appeared to be quite diverse. The macaque WSL02 gradually showed HAD-like syndrome including ataxia and dyspraxia, while WSL06 exhibited diarrhea and severe wasting and then euthanized at week 15. The RP macaque (WSL01) unexpectedly died at week 9 post infection due to acute heart failure caused by heart thrombus. We classified WSL01 to the RP group because this animal had sustained high level of viremia over the course of infection.

All 11 animals became infected as evidenced by increased levels of plasma SIV RNA (Fig. 1a and c). Peak viremia of 6–8 log₁₀ RNA copies per milliliter plasma was detected in both RP and CP macaques, with no significant difference in the magnitude between the two groups. However, the set point viral loads were significantly higher in the RP macaques than those of the CP macaques (Fig. 1b, $p < 0.001$). As shown in Fig. 1c, all three RP macaques exhibited higher persistent plasma viremia than CP animals over the course of SIV infection, with a slight decline after peak viremia. All the infected animals showed the transient decline of peripheral CD4/CD8 ratio (Fig. 1d). In general, CD4/CD8 ratio in RP macaques is comparable to those in CP macaques over the course of infection, with a median ratio value of 0.5 in both groups at the time of necropsy.

Pathologic Changes in Brains of Infected Chinese Rhesus Macaques

The immunohistological examination was completed in 3 RP macaques (WSL01, WSL02 and WSL06) and 5 CP macaques (WSP08, WSP09, WSP10, WSP11 and WSL03) (Table 2). IHC staining and ISH for SIV-RNA expression in brain tissues obtained at the necropsy of 8

animals revealed distinct neuropathologic characteristics in SIV-induced brain lesions between RP and CP macaques.

Among three RP macaques, two (WSL02 and WSL06) were demonstrated to have the neuropathologic damages in the cerebral cortex and the cerebellum (Table 2, Fig. 2). IHC staining for CD68 and CD163 revealed the robust macrophage activation (Fig. 2b–e), together with a number of MNGCs formation, throughout the brain sections of WSL02. In addition, many of SIV-infected cells were identified as macrophages (Fig. 2a–c, e–f) in these sections. Furthermore, the immunofluorescence demonstrated double staining of SIVp28 antigen (red) and CD68-positive cells (green) in the brain section of macaque WSL02 (Fig. 2g). Compared with WSL02, macaque WSL06 presented milder neuropathologic lesions in the brain, despite the fact that activated CD68⁺/CD163⁺ macrophages and MNGCs as well as SIV-infected cells could be observed in the most regions of the brain (Fig. 2h–k). Moreover, similar to SIV/SHIV-infected Indian macaques and our earlier report (Xiao et al. 2015), the brain lesions in SIVmac239-infected RP macaques could be divided into two main histopathological patterns. The first one represents classical perivascular lesions comprised of CD68⁺/CD163⁺ SIV-infected macrophages and MNGCs (Fig. 2a–g); and the second pattern is characterized by scattered SIV infected cells, with extensive macrophage activation.

As compared with RP macaques, the SIV infection and pathological changes in the brains of CP macaques were significantly less severe (Table 2, Fig. 3). SIV-RNA positive cells detected by ISH scattered over the cerebrum of WSP10 (Fig. 3a), but the majority of SIV-RNA positive cells were rarely identified as macrophages by IHC staining with CD68 and CD163 antibodies (Fig. 3b–d). IHC staining for SIV gag p28 was positive in the brain of WSP08 (Fig. 3e) without CD68-positive macrophages (Fig. 3f), but with activated microglia marked as Iba-1 positive (Fig. 3g). Future research is necessary to determine the target cells responsible for SIV infection in the CNS. Interestingly, scattered but widely distributed CD68⁺ macrophages were seen throughout the cerebellum of WSP09 (Fig. 3h), but SIV-RNA (ISH) was not.

CD4/CD8 Ratio in Peripheral Blood is Associated with SIV Infection of the CNS in CP Macaques

SIV infection in the brain was demonstrated in 4 out of 5 CP macaques with little SIV-induced neuropathological changes. The numbers of SIV-infected cells and activated macrophage in the brains of these CP macaques varied, which was ranked in the following order: WSP10, WSL03 > WSP08, WSP11 > WSP09. The animal WSL03 (Fig. 4a and e) and WSP10 (Fig. 4b and f) had more SIV-RNA positive cells and activated CD68⁺ macrophages than the animals WSP08 (Fig. 4c and g) and WSP11 (Fig. 4d and h). Corresponding to the neuropathologic changes in CP macaques, the early decline of CD4/CD8 ratios was seen in macaque WSP10 and WSL03, with the value of ratio dropping from 0.9 at day 0 to 0.25 at week 25 and week 35, respectively. These animals maintained the low CD4/CD8 ratio (<0.2) until the time of euthanasia (Fig. 4i).

Discussion

Central nervous system disease is a major feature of SIV infection of macaques of Indian origin (Matsuda et al. 2013; Orandle et al. 2001). SIVE was strongly associated with a rapid disease course (Nowlin et al. 2015; O'Neil et al. 2004; Orandle et al. 2001; Westmoreland et al. 1998). However, there is little information about neuropathology in the brains of SIV-infected Chinese RMs. In the present study, we observed that the neuropathological features in RP Chinese RMs were similar to those in RP macaques of Indian origin, including MNGCs formation and SIV expression in macrophages (Baskin et al. 1991; Brown et al. 2007; Zhuang et al. 2014). In addition, we showed that the majority of SIVmac239-infected Chinese RMs at end stage disease had the brain impairments in various degrees. The RP macaques (WSL02 and WSL06) had severe neuropathological damages as evidenced by the positive staining of activated CD68⁺/CD163⁺ macrophages, abundant MNGCs and many SIV-RNA⁺ cells in the brains (Fig. 2). In contrast, the pathological examination of the brains of CP macaques showed few of activated macrophages and only scattered SIV-RNA⁺ cells (Figs. 3 and 4). Although the CP and RP macaques had similar peak viremia, RP macaques maintained higher levels of viral loads than CP macaques during the course of infection.

Although Chinese RM and Indian RM belong to the same species, they demonstrate differences in physiological and immunological responses (Champoux et al. 1997; Clarke and O'Neil 1999; Doxiadis et al. 2003), as well as genetic background (Doxiadis et al. 2003; Otting et al. 2007; Penedo et al. 2005; Viray et al. 2001). Ling et al. showed that compared with Indian RM, SIV pathogenesis in Chinese RM was closer to HIV infection in humans, as the infected Chinese RMs had significantly lower setpoint viral load than Indian RMs (Ling et al. 2002). Such difference in viral load was also observed in SIVmac251-infected Chinese RMs (Reimann et al. 2005; Marthas et al. 2001; Marcondes et al. 2010). Consistent with these reports, we demonstrated that the majority of SIVmac239-infected Chinese RMs had low viremia and long survival time (average 154 weeks). Similar to HIV infection in humans (Forbi and Agwale 2009), inverted CD4/CD8 ratio is associated with the onset of disease in CP macaques. We found that CD4/CD8 ratio in eight CP macaques gradually declined over the course of infection. The CD4/CD8 ratio of macaques (WSP10 and WSL03) dropped significantly (<0.25) at the early stage of infection (at 25 and 35 weeks post infection, respectively), and maintained such lower ratios until the time of euthanasia (week 109 and week 150, respectively). These two animals also had severe SIV infection in the CNS, as demonstrated by SIV-RNA positive cells and infiltrated macrophages in the brains. Interestingly, RP macaques (WSL02 and WSL06) with severer neuropathological damages did not have significant decrease in CD4/CD8 ratio. Thus, it is likely that high and sustained levels of viral loads would be responsible for the SIVmac239-induced brain lesions.

In summary, the present study demonstrated that SIVmac239 infection could induce neurological impairments in the brain of the infected Chinese rhesus macaques. The CNS injury in the infected animals was positively associated with the disease progression. These data indicate that Chinese rhesus macaque is a suitable and alternative non-human primate model for neuroAIDS studies.

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Fig. 1.

Virologic and immunologic parameters in SIVmac239-infected macaques **a.** Comparison of the changes in viral load in RP and CP macaques over the course of infection (100 weeks). **b.** Comparison of peak and set point between RP and CP macaques. Asterisks (***) indicates statistical significance ($p < 0.001$). **c.** Kinetics of virus dissemination within the first 30 weeks of infection in RP and CP macaques. **d.** Comparison of changes in CD4/CD8 ratios in RP and CP macaques over the course of infection (100 weeks). Trend lines in **a** and **d** were created using median viral load or CD4/CD8 ratio of RP ($n = 3$) and CP ($n = 8$) macaques at each time point

Fig. 2.

Neuropathologic lesions of SIVE in brains of SIVmac239-infected Chinese RMs with rapid disease progression **a–c**. Representative regions of SIVE lesions in the brain of WSL02. There was a high expression of SIV-RNA positive signals (**a**, BCIP/NBT; *indigo*) within CD68⁺ (**b**, DAB; *brown*) and CD163⁺ (**c**, DAB; *brown*) perivascular macrophages. **d**. Classic MNGCs with CD68⁺ macrophages surrounded in the brain of WSL02. **e–f**. Massive CD68⁺ macrophages accumulated (**e**) with SIV-RNA positive cells (**f**) in the cerebellum of WSL02. **g**. dual-color immunofluorescence staining of MNGCs formation in the cerebellum of WSL02, with CD68⁺ macrophages (*green*) and SIVp28 (*red*) overlapping. Nuclear staining is DAPI (*blue*). **h–j**. Typical SIV induced brain lesion in WSL06 comprised of SIV-RNA positive cells (**h**) are CD68⁺ (**i**) and CD163⁺ (**j**) macrophages. **k**. MNGCs with CD68⁺ macrophages in the brain of WSL06. **a–c**, **e–f** are from the cerebellum of WSL02; **d** and **g** are from the cerebral cortex of WSL02; **h–k** are from the cerebral cortex of WSL06. Scale bar: 50 μ m

Fig. 3. Neuropathological changes in brains of SIVmac239-infected Chinese RMs with conventional disease progression **a**. In situ hybridization for SIV viral RNA in the cerebellum of WSP10 (magnification, $\times 10$). Cells with SIV-RNA positive signals (BCIP/NBT; *indigo*) are shown with arrows. **b–d**. Enlargements of the area indicated by the rectangle in **a**, that SIV-RNA positive cells (**b**) are CD68[−] (**c**) and CD163[−] cells. (**d**). **e–g**. IHC staining for SIVp28 (DAB; *brown*) was positive in the brain of WSP08 (**e**), with less CD68⁺ macrophages (DAB; *brown*) recruitment (**f**), but obvious activated microglia expression marked by Iba-1 (**g**, DAB; *brown*). **h**. Scattered CD68⁺ macrophages in the cerebellum of WSP09. Scale bar: 50 μ m (except for **a**)

Fig. 4.

Variant neuropathological changes and CD4/CD8 ratio over time in CP macaques **a–d**. ISH for SIV RNA (BCIP/NBT; *indigo*) in brains of CP macaques. More SIV-expressing cells presented in the brain of WSL03 (**a**, temporal cortex) and WSP10 (**b**, occipital cortex) comparing to WSP08 (**c**, occipital cortex) and WSP11 (**d**, cerebellum). **e–h**. Distribution of CD68⁺ macrophages (DAB; *brown*) in brains of CP macaques with SIVmac239 infection. **e**. CD68⁺ macrophage accumulation was seen in the cerebellum of WSL03. **f**. CD68⁺ macrophages scattered in the brain of WSP10. **g–h**. IHC staining of CD68 was not significant in the brain of WSP08 (**g**) and WSP11 (**h**), while ISH for SIV-RNA was positive (**c** and **d**). **i**. CD4/CD8 ratio in 5 CP macaques with a neuropathological diagnosis. Scale bars: 50 μ m

Table 1

Characteristics of SIVmac239-infected Chinese RMs

Disease progression	Animal #	Infection route	Infection dose (TICD ₅₀)	Time to autopsy (week)	Peripheral viremia (copies/ml)	
					Peak	Autopsy
Rapid	WSL01	IV	10,000	9	2.8×10^7	5.8×10^7
	WSL02	IV	10,000	24	9.6×10^7	4.4×10^7
	WSL06	IV	10,000	15	4.4×10^7	1.7×10^9
Conventional	WSL03	IV	10,000	151	3.8×10^7	9.6×10^7
	WSL04	IV	10,000	184	8.9×10^6	2.1×10^6
	WSL05	IV	10,000	181	1.5×10^6	7.3×10^7
	WSP08	IV	1,500	125	1.6×10^7	8.3×10^8
	WSP09	IV	1,500	128	2.9×10^6	4.6×10^7
	WSP10	IV	1,500	96	2.0×10^8	3.5×10^6
	WSP11	I.Vag	1,500	150	5.1×10^7	3.4×10^6
	WSP12	I.Vag	1,500	216	5.3×10^6	1.0×10^7

IV, intravenous; I.Vag, intravaginal

Table 2

Summary of IHC and ISH results

Disease progression	Animal #	Tissue	CD68	CD163	SIV (ISH)
Rapid	WSL01	CC	-	-	-
		CB	+	-	-
	WSL02	CC	+	+	NP
		CB-1	+	+	+
		CB-2	+	+	+
	WSL06	CC	+	+	+
Conventional		CB	+	+	+
	WSL03	TC	-	-	+
		OC	+	-	+
		CB	+	+	NP
	WSP08	TC	-	-	+
		OC	+	-	+
		CB	+	NP	-
	WSP09	TC	+	-	-
		OC	+	+	-
		CB	+	+	-
	WSP10	TC	+	-	+
		OC	+	+	+
		CB	+	+	+
	WSP11	TC	-	-	+
		OC	-	-	+
		CB	+	+	+

+, IHC or ISH positive signals were found in tissues slides; -, no positive signal in tissues slides
CC, cerebral cortex; CB, cerebellum; TC, temporal cortex; OC, occipital cortex; NP, not performed