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Longitudinal cerebral metabolic changes in pig-tailed macaques infected with the neurovirulent virus SIVsmmFGb

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Abstract

Longitudinal cerebral metabolite changes in pig-tailed macaques inoculated with the simian immunodeficiency virus SIVsmmFGb were evaluated with *in vivo* proton MRS at 3T. Blood sample collection, and MRS were carried out before and 2, 4, 8, 12, 16, 20 and 24 weeks after SIV inoculation. Significant reduction of N-acetylaspartate (NAA)/creatinine (Cr) and Choline (Cho)/Cr ratios in prefrontal grey matter (PGM) and glutamate/glutamine (Glx)/Cr ratios in striatum, and increase of myo-inositol (mI)/Cr in striatum were observed during acute SIV infection. The metabolite alterations during the SIVsmmFGb infection are largely in agreement with previous findings in other non-human primate models and HIV patients. Also, NAA/Cr in PGM and striatum and Glx/Cr in striatum are negatively correlated with the percentage of CD8+ T cells after the SIV infection, suggesting the interaction between brain metabolite and immune dysfunction. The present study complements previous studies by describing the time course of alterations of brain metabolites during SIVsmmFGb infection. The findings further demonstrate the efficacy of the SIVsmmFGb-infected macaque as a model to characterize central nervous system infection using novel neuroimaging approaches and also as a tool for exploration of novel and advanced neuroimaging techniques in HIV/AIDS studies.

Keywords

neuro-AIDS; SIV; *in vivo* proton MRS; macaque monkey; immune dysfunction

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Introduction

Non-human primates (NHPs) infected with Simian Immunodeficiency Virus (SIV) exhibit neuropathological symptoms similar to those seen in HIV⁺ patients, and have been employed extensively to study the mechanisms of the disease or therapeutical treatment (Apetrei *et al*, 2012; Haigwood, 2004; Hu, 2005; Staprans and Feinberg, 2004; Van Rompay, 2012; Williams *et al*, 2008). SIVsmmFGb is a novel strain of SIV, which induces neuropathology (as measured by significant levels of SIV in the brain parenchyma, multi-nucleated giant cells (MNGCs), and SIV-encephalitis (SIVE)) in 90–100% of infected pig-tailed macaques while most SIV strains in 25–40% of infected macaques (Novembre *et al*, 1998; O'Neil *et al*, 2004; Reeve *et al*, 2009), suggesting SIVsmmFGb infected pig-tailed macaques are an excellent model system for studies on neuropathogenesis and therapeutic development.

Neurochemical alterations associated with the inflammatory process and neuronal loss have been commonly observed in previous magnetic resonance spectroscopy (MRS) studies of HIV patients (Chang, 1995; Chang *et al*, 1999; Ernst *et al*, 2003; Ernst *et al*, 2010; Lentz *et al*, 2011; Salvan *et al*, 1997; Tracey *et al*, 1996; Vion-Dury *et al*, 1998). Also, rhesus macaques inoculated with a SIV251 swarm (Kodama *et al*, 1993; Strickland *et al*, 2011), have been employed to investigate the metabolite alterations of brain injury during SIV infection (Fuller *et al*, 2004; Gonzalez *et al*, 2000; Greco *et al*, 2004; Lentz *et al*, 2005; Williams *et al*, 2005; Wu *et al*, 2013a; Wu *et al*, 2013b), demonstrating in vivo MRS is a promising approach to evaluate progressive neurochemical changes in the brain injury of SIV NeuroAIDS. Our previous studies have demonstrated that SIVsmmFGb is highly neuropathogenic in pig-tailed macaques, but the temporal evolution of brain infection remains not fully understood. In the present study, in vivo MRS was used to investigate temporal cerebral metabolite alterations in pig-tailed macaques infected with SIVsmmFGb.

Materials and Methods

Pig-tailed macaques (*Macacanemestrina*) (male, 4 years old, n=5), were infected by intravenous inoculation with 100 TCID₅₀ of the SIVsmmFGb isolate (Novembre *et al*, 1998; O'Neil *et al*, 2004).

In vivo proton MRS examination

The single voxel spectra were acquired with the point-resolved spectroscopy (PRESS) sequence with Chemical Shift Selective (CHESS) water suppression and TR/TE =1500ms/30ms by using a circular surface coil (ID=7cm) on a Siemens 3T Trio scanner (Siemens Medical Solutions, PA, USA). A volume of 6mm × 6mm × 6 mm (or 0.22 ml) within prefrontal grey matter (PGM) or striatum was chosen as regions of interest (ROI) (Fig 1A and 1B) and scanned before SIV inoculation (baseline) and at 2, 4, 8, 12, 16, 20 and 24 wpi. For each scan, animals were anesthetized and maintained with 1–1.5% isoflurane mixed with 100% oxygen and the animal head was immobilized with a custom-built head holder. Vital physiological parameters including heart rate, breathing rate, rectal temperature, blood pressure, oxygen saturation, etc, were monitored continuously during scanning. Baseline

MRS data were acquired in three separate scanning sessions for each animal, with at least one week between sessions. Animals were euthanized after their final scanning session.

CD4+ and CD8+ T-cell monitoring

Blood samples were collected from anesthetized animals for quantitation of circulating CD4+ and CD8+ T-cell subsets, one day before each MRI scan and a complete blood (CBC) count with differential and lymphocyte phenotype analysis was performed. Absolute numbers and percentages of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T lymphocytes were measured, in whole blood, using four-color flow cytometry and gating on the lymphocyte population. All monoclonal antibodies (CD3, CD20, CD4 and CD8) used here were purchased from BD company as previously described (Novembre *et al*, 1998; O'Neil *et al*, 2004).

Data analysis

In vivo MRS data were processed using the LC-Model software (Provencher, 1993). Total creatine (Cr, plus phosphocreatine) levels were used as an internal reference (Fuller *et al*, 2004; Greco *et al*, 2002; Haga *et al*, 2009; Herndon *et al*, 1998; Kaiser *et al*, 2005; Ratai *et al*, 2009; Wiebenga *et al*, 2014). Metabolite ratios including NAA/Cr, Cho/Cr, mI/Cr and glutamate/glutamine (Glx)/Cr were calculated. Representative spectra of the post-inoculation proton MRS in PGM and striatum were shown (Fig 1c). Also, the pre-inoculation data of each animal were processed separately and the metabolite ratios with smallest standard deviation percentage in the LC-model fitting were used as the reference baseline for the following data analysis of that animal.

Statistical Analysis

Analysis of variance (ANOVA) with post hoc testing was employed for the statistical analysis of repeated measurements across different time points by using SPSS 20.0. Student's t-test was applied to compare the results between the baseline and that in the post-inoculation. Relationships between the percentages of T cells (or CD4/CD8 ratio) and metabolite changes were examined using linear mixed regression analysis (Blackwell *et al*, 2006). P-values less than 0.05 were considered statistically significant. All the p-values were adjusted for multiple comparisons by means of the false discovery rate (FDR) procedure (Benjamini, 1995).

Results

CD4+ and CD8+ T cell percentage changes post SIV infection

Longitudinal changes in circulating CD4+ and CD8+ T-cell percentages and numbers are illustrated in Fig. 2. CD4+ T cell percentage declined progressively as expected, differing significantly from baseline at 12, 16, 20 and 24 wpi. In the acute phase (<1 month), the CD8+ T cell percentages increased significantly and then became steady and declined slightly in the chronic phase after SIV infection (Fig 2).

Cerebral Metabolite ratio changes post SIV inoculation

Compared to the baselines, mean NAA/Cr and Glx/Cr ratio (averaged cross time post-inoculation) were reduced $13.6\% \pm 6.5\%$ (SD) and $13.1\% \pm 9.3\%$ (SD), respectively in PGM and $13.2\% \pm 8.0\%$ (SD) and $9.6\% \pm 1.5\%$ (SD) in striatum, although these declines were not statistically significant (Fig 3). Also, in comparison with the baselines, the mean Cho/Cr and mI/Cr ratios remained almost unchanged in PGM and striatum (Fig 3).

In the longitudinal examination, ANOVA analysis indicated there were no significant changes observed in all interested metabolites in PGM and striatum at most time points after the SIV infection except in the acute stage. Specifically, significant changes of the NAA/Cr ratio in PGM and Glx/Cr and mI/Cr ratios in striatum were observed at 2 wpi (Fig 4a and 4b). Significant reduction of Cho/Cr and Glx/Cr ratios in PGM occurred at 4 and 12 wpi (Fig 4a). The Glx/Cr ratio in PGM continued to decrease over the time, while the Glx/Cr ratio in striatum had a transient drop at 2 wpi. The NAA/Cr ratio in PMG and striatum correlated with the Glx/Cr ratio in striatum ($r = 0.36$, $p = 0.03$).

Relationships of the metabolite alterations with immunologic dysfunction

As seen in Fig 4 and Table 1, the progressive changes of the NAA/Cr ratios in PGM and striatum and the Glx/Cr ratio in striatum were correlated with the percentage of CD8+ T cells during SIV infection, as indicated by linear mixed effects regression analyses (Table 1). Standardized beta values (slopes) were significantly greater than 0 for the relationship between the NAA/Cr ratio and CD8 percentage in PGM ($F_{(1,13.87)} = 14.55$, $P = .002$), and for the relationship between the NAA/Cr ratio and CD8 percentage in striatum ($F_{(1,15.19)} = 9.57$, $p = 0.007$), and for the relationship between the Glx/Cr ratio and CD8 percentage in striatum ($F_{(1,18.85)} = 16.85$, $p = 0.001$). No significant correlation between any metabolite ratios and the CD4+ T cell depletion (or CD4/CD8 ratio) was observed.

Discussion

The present in vivo MRS study reveals that the cerebral metabolites in prefrontal grey matter and striatum of pig-tailed macaques are altered progressively during the SIVsmmFGb infection and largely in agreement with those seen in SIVmac251-infected rhesus macaques as well as in HIV-infected patients. Notably, the temporal metabolite changes are associated with the progressive immune dysfunction. In addition, the Glx/Cr ratio changed with different temporal profiles in striatum vs. prefrontal grey matter, indicating that the glutamatergic system might be affected differently in these two regions following SIV infection. As SIVsmmFGb enters the brain in about 90% of the infected animals, we believe that SIVsmmFGb has advantages over SIVmac251 for neuroimaging studies designed to characterize CNS injury following SIV infection of macaque monkeys.

Brain metabolite changes in PGM and striatum

N-acetyl-aspartate (NAA)—NAA is a neuronal marker widely used to assess brain injury. Previous studies have demonstrated that no significant changes in NAA/Cr were seen in basal ganglia, frontal cortex and centrum semiovale at any time point after 2 weeks in rhesus monkeys ($n=8$) with SIVmac251 infection (Fuller *et al*, 2004). In contrast, the present study

revealed significant reduction in NAA/Cr of PGM at 2 wpi (Fig 4a), consistent with the NAA reduction in frontal cortical grey matter seen in the prior studies of HIV⁺ patients in the early stages of infection (Lentz *et al*, 2009) and the NAA/Cr decrease in frontal cortex at 13 days post inoculation in SIVmac251-infected macaques (n=10) (Greco *et al*, 2004) and those seen at 12 days (n=3) and 14 days (n=3) post inoculation in an ex vivo NMR study (Lentz *et al*, 2005). Such transient reduction of NAA/Cr correlated with the changes in synaptophysin and might reflect the reversible neuronal injury during the primary SIV infection (Lentz *et al*, 2005). Also, the NAA/Cr alteration is in agreement with previous finding of whole brain fractional anisotropy (FA) in SIVsmmFGB-infected macaque brains examined with diffusion tensor imaging (DTI) (Li *et al*, 2011).

It is of use to compare the present macaque experiment done at 3T with previous ones at 1.5T. Even though current 3T offers higher (nearly two-fold) sensitivity than 1.5T in in vivo MRS and a dedicated surface coil was used, the voxel size used in the present study was 6mm × 6mm × 6 mm (0.22ml), much smaller (15-fold) than that (15mm × 15mm × 15mm, or 3.4ml) used in previous rhesus macaque studies. In consideration of the spectrum SNR and the sample size difference, the NAA/Cr reduction at 2 weeks post inoculation was seen at the present study with n=5 and previous study with n=10 (Greco *et al*, 2004) but not with n=8 (Fuller *et al*, 2004), the present MRS results suggest that SIVsmmFGB in pig-tailed macaques is highly neuropathogenic in comparison with SIVmac251 in rhesus macaques.

Glutamate and glutamine (Glx)—Neurons and glial cells are believed to be the primary sources of glutamate and glutamine in which glutamate is considered as the principal excitatory neurotransmitter in brain. Disturbance of these substances could reflect dysfunction in one or both of these cell types (Lentz *et al*, 2009). Significant Glx reduction has been observed in the frontal cortical gray matter of HIV patients at early and chronic stages (Lentz *et al*, 2009) and in basal ganglia at chronic stage (Lentz *et al*, 2011). Also, no changes in Glx or Glx/Cr were reported in the frontal cortex and centrum semiovale but the reduction was nearly significant in basal ganglia in previous macaque study (Ratai *et al*, 2009). In contrast, Glx/Cr in PGM demonstrated continuous decline, and significant reduction at 12 wpi, consistent with the changes seen in HIV patients with early infection (Lentz *et al*, 2009). Also, significant Glx/Cr reduction in striatum was seen in 2 wpi in the present study while such change was also observed in basal ganglia in HIV patients at chronic stage (Lentz *et al*, 2011), consistent with that seen in the SIVmac251 infected macaques with CD8 depletion (Ratai *et al*, 2009). In addition, the difference of Glx/Cr evolutions in PGM and striatum of the SIVsmmFGB infected macaques suggests that the glutamatergic system may be affected differently in these regions probably due to the higher viral load in PGM than in striatum in the unique SIV model of neuroAIDS (O'Neil *et al*, 2004).

Choline (Cho)—As a marker for inflammation and membrane disruption, increased Cho levels, seen in patients with chronic HIV infection, are typically thought to represent increased lipid membrane turnover due to glial activation in response to the HIV infection (Lentz *et al*, 2011). This interpretation is supported by the observation of pathologic changes during early HIV and SIV infection (Gray *et al*, 1996; Kim *et al*, 2005).

Elevation in Cho has been observed in the frontal white matter of chronic HIV infection (Chang *et al*, 1999; Meyerhoff *et al*, 1999) and in Cho/Cr of the frontal cortex and white matter of HIV patients in early and chronic stage (Lentz *et al*, 2011). Ratai and colleagues reported Cho and Cho/Cr increased at 2 wpi and decrease at 4 wpi in the frontal cortex, basal ganglia during SIV infection (Ratai *et al*, 2009). In contrast, reduction of Cho/Cr in PGM was only seen at 4 wpi in the present study.

myo-inositol (mI)—Because mI exists primarily in glia, its elevation is considered to reflect increased glial cell activity and a marker of inflammation (Brand *et al*, 1993). Higher mI has been commonly observed in the white matter of asymptomatic HIV⁺ patients (Chang *et al*, 2004). Also, such mI increase was seen in frontal cortex in SIVmac251-infected macaques (n=10) during acute infection (Ratai *et al*, 2009). In contrast, no changes in mI/Cr were reported in the frontal cortex and basal ganglia of SIVmac251-infected macaques in (n=8) in early and chronic stage (Fuller *et al*, 2004). In the present study, mI/Cr was stable in PGM in acute stage but significantly elevated in striatum at 2 wpi and then reduced at 4 wpi, different from that seen in the SIVmac251 infected macaques (Ratai *et al*, 2009).

Correlation between temporal changes of brain metabolite ratios in PGM and striatum

Region-specific metabolic responses have been reported previously in SIV-infected macaques (Ratai *et al*, 2009). The temporal changes in NAA/Cr and Glx/Cr ratios of striatum, and NAA/Cr in PGM were correlated with each other, suggesting that the temporal association of neuronal injury in the frontal cortex and basal ganglia during SIVsmmFGB infection. In contrast, significant correlation was observed in Glx but not NAA in frontal cortex and basal ganglia in Ratai *et al*'s study.

T cell phenotypes and their association with brain metabolite alterations

Immune system responses to HIV or SIV infection have been reported previously in both SIV and HIV immunology literature (Marcondes *et al*, 2001; von Geldern *et al*, 2007). Depletion of CD4⁺ T cell and accumulation of CD8⁺ T lymphocytes are the most significant predictor of the clinical disease severity (Chang *et al*, 2000; Grassi *et al*, 1999; Langford *et al*, 2007). Down-regulation of viral replication in the brain coincides with increased CD8⁺ T cell infiltration and reduced glial and macrophage activation have been observed in SIV infected macaques (Clements *et al*, 2002). The results of the temporal correlations between NAA/Cr in PGM and striatum and Glx/Cr in striatum and the percentage of CD8⁺ T cells during the SIV infection is consistent with the findings in patients with early HIV⁺ infection (Lentz *et al*, 2009), suggesting that recruited CD8⁺ T cells may damage bystander neurons during down-regulated virus expression, as indicated by reduced NAA/Cr and Glx/Cr probably due to neuronal loss or dysfunction (Crews *et al*, 2008; De Stefano *et al*, 1995; Guimaraes *et al*, 1995; Urenjak *et al*, 1993).

Previous MRS studies of HIV patients showed such relationship was driven only by the early infection cohort, and no correlation between any CD8⁺ T-cell population and the NAA or Glx levels in chronic infection cohort were reported (Lentz *et al*, 2009). However, in the present study of SIV-infected monkeys, it is seen that NAA/Cr and Glx/Cr correlated with the percentage of CD8⁺ T cells, but not with the percentage of CD4⁺ T cells. The difference

in MRS findings between the SIV monkeys and HIV patients may be attributed to the unknown or variable factors including the mode and time course of infection and the medication in HIV patients.

Macaque models are widely used in HIV/AIDS studies and therapeutic development due to the difficulty of studying the disease in humans. The neurochemical alteration during SIV infection has previously been investigated extensively in macaques infected with SIVmac251. Here we report similar findings in SIVsmmFGB infected macaques. However, as demonstrated in our previous studies (Reeve *et al.*, 2009; Reeve *et al.*, 2010), SIVsmmFGB induces neuropathology in over 90% of the infected pig-tailed macaques, a much higher infection rate than seen in macaques infected with SIVmac251 and other strains (Westmoreland *et al.*, 1998). Although the sample size (n=5) in the present study was smaller than those used in previous MRS studies of SIVmac251-infected macaques (n= 8–18) (Fuller *et al.*, 2004; Greco *et al.*, 2004; Ratai *et al.*, 2009), we were still able to detect significant metabolite changes, as described above. Further, these changes were detected in a shorter time course than seen in other models (6-month vs. 24-month endpoint) (Fuller *et al.*, 2004). Therefore, our results suggest that SIVsmmFGB-infected macaques are a more sensitive and responsive model than others for characterizing neuronal injury during SIV infection.

HIV-infection leads to immune deficiency and a range of neurological disorders due to the infection of central nervous system and associated dementia. MRI holds great potential to evaluate the physiological, microstructural, neurochemical, and functional changes in HIV patients (Chang, 1995; Chang *et al.*, 2004; Offiah and Turnbull, 2006; Shyam babu *et al.*, 2013; Tucker *et al.*, 2004; Zhang and Li, 2013). However, to date, MRI is still not a sensitive approach in screening HIV patients without neurological symptoms (Nishijima *et al.*, 2014). Our previous study has demonstrated the sensitivity of DTI and perfusion techniques in SIVsmmFGB-infected brains using high-field MRI (Li *et al.*, 2011). Currently, high-field (3T) and ultra-high field (7T and above) MRI techniques exhibit improved image quality and expanded range of applications in preclinical and clinical studies (Duyn, 2012; Oz *et al.*, 2013). In particular, some novel diffusion, perfusion, and multi-parameter MRI methods may be used to facilitate better detection of brain injury in SIV-infected macaques (Li *et al.*, 2014; Meng and Zhang, 2014; Zhang *et al.*, 2014). Taken together with the present MRS results at 3T, our findings suggest that SIVsmmFGB infected macaques could serve as an excellent platform for exploring advanced imaging techniques in HIV/AIDS studies.

Conclusions

The temporal cerebral metabolite changes during the SIVsmmFGB infection are evaluated with in vivo MRS at 3T. The MRS results demonstrate the metabolite alterations are largely consistent with previous findings of neuronal injury in SIVmac251-infected rhesus monkeys and HIV patients, and, in particular, associated with progressive immune dysfunction. The findings suggest that the SIVsmmFGB infected pig-tailed macaques are an excellent model for characterizing CNS infection using novel neuroimaging approaches and exploring advanced neuroimaging techniques as well.

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Fig. 1.

Voxels of interest illustrated in A): prefrontal grey matter (PGM) and B): Striatum (caudate and putamen) on the T₂-weighted structural images of a pig-tailed adult macaque monkey. C) Representative proton MR spectrum in PGM with fitted by the LCModel software (post SIV inoculation) with the residual shown on the top.

Abbreviations: Cr, total creatine; Glx, glutamate/glutamine; mI, myo-inositol; NAA, N-acetylaspartate; Cho, choline.

Fig. 2. CD4+ and CD8+ T-cell percentages (a) and absolute numbers (b) of the lymphocyte changes post SIV inoculation. Error bars represent Standard Error of the Mean (SEM). *: $P < 0.05$, compared with the baseline (pre SIV inoculation).

Fig. 3.

Cerebral metabolite ratio changes (averaged across time) in prefrontal grey matter (PGM) (a) and striatum (b) after SIV inoculation. Error bars represent Standard Error of the Mean (SEM). *: $P < 0.05$, compared with baseline. Metabolites include: N-acetylaspartate (NAA/Cr), total creatine (Cr), total choline (Cho), myo-inositol (mI), Glutamate/Glutamin (Glx). Total creatine (Cr) is used as internal reference.

Fig. 4. Longitudinal cerebral metabolite ratio changes in prefrontal grey matter (PGM) (a) and striatum (b) of pig-tailed macaques after SIV inoculation. Error bars represent Standard Error of the Mean (SEM). *: $P < 0.05$, compared with the baseline; “----”: the temporal changes of metabolites/Cr significantly negatively correlated with CD8+ T-cell percentages change.

Table 1

Standardized β value between CD4+, CD8+ T-cell percentages and CD4/CD8 with cerebral metabolites in prefrontal grey matter (PGM) and striatum, *;
 $P < 0.05$ after FDR correction.

Location	%	ml/Cr	NAA/Cr	Cho/Cr	Glx/Cr
PGM	CD4	-0.37	-0.02	0.33	0.18
	CD8	0.24	*-0.51	0.16	-0.20
	CD4/CD8	-0.26	0.29	0.11	0.29
Striatum	CD4	-0.04	-0.27	0.12	-0.09
	CD8	-0.06	*-0.43	0.13	*-0.52
	CD4/CD8	0.05	0.18	0.09	0.37

F(1,13.87) NAA:CD8(PGM)=14.55, $p=0.002$
F(1,15.19) NAA:CD8(striatum)=9.57, $p=0.007$
F(1,18.85) GLX:CD8(striatum)=16.85, $p=0.001$