Persistent Hijacking of Brain Proteasomes in HIV-Associated Dementia

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Immunoproteasome induction sustains class 1 antigen presentation and immunological vigilance against HIV-1 in the brain. Investigation of HIV-1-associated alterations in brain protein turnover by the ubiquitin-proteasome system was performed by (1) determining proteasome subunit changes associated with persistent brain inflammation due to HIV-1; (2) determining whether these changes are related to HIV-1 neurocognitive disturbances, encephalitis, and viral loads; and (3) localizing proteasome subunits in brain cells and synapses. On the basis of neurocognitive performance, virological, and immunological measurements obtained within 6 months before death, 153 autopsy cases were selected. Semiquantitative immunoblot analysis performed in the dorsolateral prefrontal cortex revealed up to threefold induction of immunoproteasome subunits LMP7 and PA28 α in HIV-1-infected subjects and was strongly related to diagnoses of neuropsychological impairment and HIV encephalitis. Low performance on neurocognitive tests specific for dorsolateral prefrontal cortex functioning domains was selectively correlated with immunoproteasome induction. Immunohistochemistry and laser confocal microscopy were then used to localize immunoproteasome subunits to glial and neuronal elements including perikarya, dystrophic axons, and synapses. In addition, HIV loads in brain tissue, cerebrospinal fluid, and blood plasma were robustly correlated to immunoproteasome levels. This persistent “hijacking” of the proteasome by HIV-1-mediated inflammatory response and immunoproteasome induction in the brain is hypothesized to impede turnover of folded proteins in brain cells. This would disrupt neuronal and synaptic protein dynamics, contributing to HIV-1 neurocognitive disturbances. (*Am J Pathol* 2010; 176:893–902; DOI: 10.2353/ajpath.2010.090390)

including HIV-associated dementia (HAD) and mild cognitive and motor disturbance (MCMD). Highly active antiretroviral therapy suppresses HIV-1 replication, prevents dementia, and prolongs survival, but does not eradicate HIV-1 infection.¹ Inflammation is the putative driving force behind MCMD and HAD.^{2,3} HIV-1 enters the central nervous system (CNS) via infected macrophages and triggers inflammatory changes including the release of cytokines, neurotoxins, and toxic viral proteins. HIV-1 produces inflammatory changes neuropathologically that are known as HIV encephalitis (HIVE).⁴ HIVE and HAD are correlated with each other, which supports a proinflammatory mechanism for the pathophysiology of dementia in many, but not all cases.⁵

Inflammation has an influence on protein turnover through the ubiquitin proteasome system (UPS).^{6–8} The proteasome is a multicatalytic proteinase that is the main route of cellular protein degradation and turnover.⁹ Inflammatory mediators including interferon- γ (IFN- γ) and tumor necrosis factor α modify expression of proteasome subunits to promote the synthesis of the immunoproteasome complex (IPS).^{6–8,10–15} This causes switching from the synthesis of “standard” constitutive proteasome complexes (CPS), which process folded proteins through the UPS, to IPS complexes, which are specialized for processing unfolded polypeptides for class 1 antigen presentation in viral defense.^{10,15} The “borrowing” of the UPS by IPS induction is not pathological to cells because it subsides quickly after an infected host eradicates the pathogen.⁷ Eradication of HIV-1 in the CNS, however, is not achieved and a vigilant immune defense must be maintained.^{15–17} This persistent inflammatory drive in HIV/AIDS could exert a potentially harmful slowing of protein turnover through the UPS. That in turn could have a profound influence in the CNS because impairment of protein turnover interferes with synaptic function and impairs learning and memory formation.^{18,19} A persistent slowing of protein turnover via the UPS probably leads to

Supported by grants R01 MH69200 and U01-MH083507 from the National Institutes of Health.

Accepted for publication October 20, 2009.

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People infected with HIV-1 are vulnerable to syndromes of neurocognitive impairment at a relatively young age,

Table 1. Immunoblotting Primary Antibodies

Antibody (clone)	Source	ID	Type	Dilution
20S α -type				
$\alpha 4$ (MCP79)	Biomol	PW9140	Mouse mAb	1:1000
$\alpha 6$ (MCP20)	Biomol	PW8100	Mouse mAb	1:1000
$\alpha 7$ (MCP72)	Biomol	PW8110	Mouse mAb	1:1000
20S β -type				
X/ $\beta 5$	Affinity Bioreagents	PA1-977	Rabbit pAb	1:1000
Y/ $\beta 1$	Affinity Bioreagents	PA1-978	Rabbit pAb	1:1000
Z/ $\beta 2$	Affinity Bioreagents	PA1-979	Rabbit pAb	1:1000
LMP7/ $\beta 5i$ (LMP7-1)	Biomol	PW8845	Mouse mAb	1:1000
LMP2/ $\beta 1i$ (LMP2-13)	Biomol	PW8840	Mouse mAb	1:1000
MECL-1/ $\beta 2i$	Biomol	PW8150	Rabbit pAb	1:1000
19S regulator ATPase				
Rpt1	Biomol	PW8315	Rabbit pAb	1:1000
Rpt5	Affinity Bioreagents	PA1-967	Rabbit pAb	1:500
19S regulator non-ATPase				
Rpn2	Affinity Bioreagents	PA1-973	Rabbit pAb	1:1000
Rpn8	Affinity Bioreagents	PA1-1963	Rabbit pAb	1:1000
11S regulator				
PA28 α	Biomol	PW8185	Rabbit pAb	1:1000

mAb, monoclonal antibody; pAb, polyclonal antibody.

accumulation of misfolded ubiquitinated proteins in pathological aging, which is a hallmark neuropathological change in neurodegenerative diseases.^{20–27} An increase in ubiquitin-protein conjugates was reported in HIV/AIDS brains that was associated with inflammation and altered synaptic protein content.²⁸ Here we report that HIV-1 infection exerts a strong influence on brain UPS that is associated with neurocognitive impairment and neuropathological changes.

Materials and Methods

Study Subjects

Eighty-eight HIV-positive (HIV⁺) subjects were selected from the National NeuroAIDS Tissue Consortium²⁹ and/or the Texas NeuroAIDS Research Center. Forty-seven HIV⁺ subjects had neuropsychological impairment (NPI), including 23 subjects with HAD and 24 subjects with MCMD. Eleven HIV⁺ subjects did not have syndromic impairment. Twenty HIV⁺ subjects had NPI combined with other conditions (NPI-O), which precluded a diagnosis of HAD or MCMD. Ten HIV⁺ decedents were included that did not have neurocognitive diagnoses. Twenty subjects had HIV[−]. All HIV⁺ patients were treated with antiretroviral therapy. Sixty-five HIV-negative (HIV[−]) subjects of comparable age, gender, and race with no significant neuropathological findings were included. The protection of human subjects was approved by the institutional review board of the University of Texas Medical Branch at Galveston under protocol 98-402.

Brain Tissue Preparation and Western Blots

Samples from the dorsolateral prefrontal cortex (DLPFC) and frontal white matter (WM) from fresh-frozen brain slices stored at -80°C were homogenized by silica bead beating and sonication in 10 mmol/L Tris-HCl, 0.5 mmol/L

Dithiothreitol, 0.03% Triton X-100, 5 mmol/L MgCl_2 , and pH 7.8. Homogenates (10 to 30 μg total protein) were added to 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) with 5% β -mercaptoethanol, boiled, and loaded into Criterion Precast Tris-HCL gels (Bio-Rad Laboratories) for SDS-polyacrylamide gel electrophoresis. Protein was transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% nonfat dry milk. Primary antibodies from Biomol International, Inc. (Plymouth Meeting, PA) and Affinity Bioreagents (Golden, CO) (Table 1), anti-rabbit or anti-mouse secondary antibodies and Enhanced Chemiluminescence Detection Reagent (Amersham Biosciences, Piscataway, NJ), were applied. Exposed X-ray film band densities were quantified with One-Scan (BD Biosciences Bioimaging, Rockville, MD).

Neurocognitive Testing

A battery of tests was designed by the National NeuroAIDS Tissue Consortium to evaluate domains of cognitive functioning in MCMD and HAD.^{29,30} The Wisconsin Card Sorting Test-64 (WCST-64) assesses abstract and executive functioning driven primarily by frontal lobe circuitry. The Wechsler Adult Intelligence Scale III subtests, Digit Symbol and Symbol Search, provide an index of information processing speed. Also included were the Hopkins Verbal Learning Test Revised, the Brief Visuospatial Memory Test Revised, the Paced Auditory Serial Addition Test, the F-A-S Test, and the Wide Range Assessment Test. A neuropsychologist rendered the neuropsychological diagnosis.

Viral Load

Laboratory values of plasma CD4⁺ lymphocyte count and viral loads (VL) in plasma and cerebrospinal fluid (CSF) were obtained within 6 months of death. Plasma

Table 2. Immunofluorescence Primary Antibodies

Antibody (Clone)	Source	ID	Type	Dilution
Immunoproteasome				
LMP2/ β 1i	Abcam Cambridge, MA	ab3328	Rabbit pAb	1:1000
PA28 α	Biomol	PW8185	Rabbit pAb	1:1000
Neurons				
NeuN (A60)	Millipore Billerica, MA	MAB377	Mouse mAb	1:100
Neurofilament (SPM204)	Abcam	ab17832	Mouse mAb	1:50
Synaptophysin (SVP-38)	Sigma Aldrich	S5768	Mouse mAb	1:200
Astrocytes				
GFAP (GA5)	Millipore	MAB360	Mouse mAb	1:400
Oligodendrocytes				
OMG (4A9)	Lifespan Seattle, WA	LS-C27282	Mouse mAb	1:300
Macrophage/microglia				
CD68 (KP1)	Dako	M0814	Mouse mAb	1:100

mAb, monoclonal antibody; pAb, polyclonal antibody.

and CSF were analyzed by using the Roche AMPLICOR HIV-1 Monitor Test (Branchburg, NJ). Brain tissue RNA was extracted by using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) for HIV-1 RNA single copy detection as described by Palmer et al.³¹ One microgram of brain RNA and 1 μ mol/L of antisense primer 84R were used in 20 μ l reaction (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA). Four microliters of cDNA was used for 25 μ l real-time PCR by using JumpStart Taq ReadyMix for Quantitative PCR (Sigma Aldrich, St. Louis, MO) and SmartCycler (Cepheid, Sunnyvale, CA). Results were standardized against a known brain secondary standard.

Microscopy

Immunoperoxidase histochemistry was performed on formalin-fixed paraffin-embedded tissue sections that were quenched with 3% hydrogen peroxide in methanol, irradiated with microwaves in sodium citrate buffer (10 mmol/L sodium citrate, 0.05% Tween-20, pH 6.0), and blocked with 0.1% nonfat dry milk and 1% normal goat serum. Rabbit polyclonal anti-LMP7 (1:1000) or anti-PA28 α (1:1000) (BIOMOL), secondary antibody, and Vectastain avidin-biotin complex and Peroxidase substrate diaminobenzidine kits (Vector Laboratories, Burlingame, CA) were applied. Immunohistochemistry for CD8⁺ lymphocytes was similarly performed. Antigen retrieval was performed in 1 mmol/L EDTA, pH 8.0. Mouse anti-CD8 (clone 4B11, 1:40) (Novocastra Labs, Newcastle on Tyne, UK) was applied, followed by the labeled streptavidin-biotin method and diaminobenzidine staining (Dako, Glostrup, Denmark). Slides were then counterstained with Harris Hematoxylin (Fisher Scientific, Pittsburgh, PA). For immunofluorescence, sections were steamed in sodium citrate buffer and blocked with Image-iT FX signal enhancer (Invitrogen Molecular Probes, Eugene, OR), 5% bovine serum albumin, and 5% normal goat serum. Primary antibodies (Table 2) and Alexa-Fluor fluorochrome-conjugated secondary antibodies (Invitrogen Molecular Probes) were applied. Autofluorescence was quenched with 1% Sudan Black B (Sigma Aldrich) in 70% ethanol. Coverslips were mounted with Slow Fade Gold with 4',6-diamidino-2-phenylindole (Invitrogen Molecular Probes). Confocal images of single

optical sections of 500-nm thickness were acquired with a Zeiss LSM 510 UV META laser scanning confocal microscope consisting of an Axiovert 200M Inverted Microscope equipped with Argon, dual HeNe, and UV lasers, fluorescence filters set for DAPI, fluorescein isothiocyanate, and tetramethylrhodamine isothiocyanate, a scanning module with visible and UV acousto optical tunable filters, two independent photomultiplier tubes (PMT) array (Carl Zeiss MicroImaging, Inc, Thornwood, NY).

Statistics

SAS/STAT 9.1.3 PROC REG (SAS Institute, Inc, Cary, NC), Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA), and GraphPad InStat 3.06 (GraphPad Software, Inc, La Jolla, CA) were used. Analysis of variance was used for comparisons between more than two groups. Data with normal distribution were analyzed by using one-way analysis of variance followed by the Tukey-Kramer Multiple Comparisons Test, or Student's *t* test. Otherwise, the Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test was used. Error bars represent SEM. Regression analysis was used to determine effects of age and HIV status.

Results

Proteasome and Immunoproteasome Subunits

Initial immunoblot screening compared eight HIV⁻ subjects and eight HIV⁺ subjects with HIVE and/or neurocognitive impairment to identify proteasome subunits with altered protein levels for further analysis. Screening results showed that levels of IPS 20S β subunits LMP2, LMP7, and MECL-1 were increased substantially in DLPFC and WM of HIV⁺ subjects compared with the basal IPS levels in HIV⁻ subjects (Figure 1B). PA28 α , an inducible subunit of the 11S regulatory complex, was also increased in HIV⁺ subjects (Figure 1D). CPS 20S β (Figure 1A) and α (Figure 1E) subunits were not changed. The majority of CPS 19S subunits screened were unchanged (Figure 1C). However, one delta 19S non-ATPase subunit known as Rpn2, a likely chaperonin, was decreased in DLPFC of HIV⁺ subjects.

Figure 1. Western blots illustrate altered proteasome subunit concentrations in DLPFC and WM from eight HIV⁺ subjects with HIV and/or neurocognitive impairment compared with eight HIV[−] subjects. **A:** Constitutive 20S proteasome subunits X(β 5), Y(β 1), and Z(β 2) were not changed in HIV⁺ subjects in DLPFC or WM. **B:** Three inducible 20S immunoproteasome β subunits were increased in DLPFC and WM of HIV⁺ subjects. **C:** The majority of constitutively expressed 19S proteasome regulatory complex subunits remained unchanged, except for Rpn2, which showed a decrease in the DLPFC of HIV⁺ subjects. **D:** The immunoproteasome 11S regulatory subunit PA28 α was increased in DLPFC and WM of HIV⁺ subjects. **E:** 20S α subunits were not changed.

CD8⁺ Lymphocytes

In our screening panel, we explored whether CD8⁺ lymphocytes, which can produce IFN- γ , might be more prevalent with IPS induction. Histologically stained CD8⁺ lymphocytes were unexpectedly less prevalent in subjects with IPS induction compared with those without IPS induction (13.73 ± 12.7 cells/cm² versus 35.9 ± 20.8 cells/cm², $df = 15$, $P = 0.025$, not illustrated).

Association with Neuropathology and Neuropsychological Diagnoses

LMP7 (Figure 2, A and B) and PA28 α (Figure 2, C and D) were measured in DLPFC and WM samples from all subjects by using serial Western blotting with loading controls and densitometrical analysis. The average levels of these inducible subunits in HIV⁺ subjects were significantly increased approximately twofold to threefold compared with HIV[−] subjects.

LMP7 (Figure 3, A and B) and PA28 α (Figure 3, C and D) were significantly increased in those subjects with HIV in DLPFC and WM. The average increase in HIV was three- to fivefold compared with HIV[−] subjects. In HIV⁺ subjects without HIV, a more modest yet significant increase was present that was approximately two-fold higher than HIV[−] subjects.

Subjects with NPI had significantly increased LMP7 (Figure 4, A and B) and PA28 α (Figure 4, C and D) in DLPFC and WM. Average increases were approximately two- to fourfold, with the most pronounced affect seen with WM PA28 α measurements. HIV⁺ subjects without NPI had little or no increase in either inducible subunit. HIV⁺ NPI-O subjects also had significantly increased IPS subunits, but the increase was less pronounced than those with NPI.

Figure 2. Immunoproteasome subunit proteins in 88 HIV⁺ and 65 HIV[−] subjects were quantified by using densitometry of calibrated Western blots with loading controls. Averaged LMP7 was increased 99% in DLPFC (**A**) and 184% in WM (**B**). PA28 α was increased by 204% in DLPFC (**C**) and 233% in WM (**D**). Statistics: Student's t test; * $P < 10^{-7}$; ** $P < 10^{-10}$. OD = optical density.

The decrease in DLPFC constitutive 19S regulatory complex subunit Rpn2 seen in proteasome subunit screening was investigated. A slight decrease in HIV⁺ subjects was not significant (not shown). Further analysis

Figure 3. Significantly increased immunoproteasome subunit LMP7 and PA28 α concentration in HIV⁺ subjects with and without HIV. **A:** DLPFC LMP7 levels in HIV⁺ subjects with and without HIV were increased 173% and 81%, respectively, compared with HIV[−] subjects. **B:** WM LMP7 in HIV was increased 414% and 114% compared with HIV[−] subjects and HIV⁺ subjects without HIV, respectively. LMP7 levels of HIV⁺ subjects without HIV were 140% greater than HIV[−] subjects. **C:** DLPFC PA28 α levels of HIV⁺ subjects with and without HIV were 349% and 166% higher than HIV[−] subjects, respectively. **D:** In WM, PA28 α was increased 443% and 185% in HIV⁺ subjects with and without HIV, respectively, compared with HIV[−] subjects. Statistics: one-way analysis of variance with Tukey-Kramer Multiple Comparisons Test; * $P < 0.05$; *** $P < 0.001$. OD = optical density.

Figure 4. Increased immunoproteasome subunit LMP7 and PA28 α concentration significantly related to NPI. Subunit level increases in HIV⁺ subjects with NPI-O were less pronounced. No substantial change was observed with HIV⁺ subjects without NPI. **A:** DLPFC LMP7 levels in HIV⁺ NPI subjects were increased 133% and 110% compared with HIV⁻ subjects and HIV⁺ subjects without NPI, respectively. **B:** WM LMP7 increased 243% in those with NPI and 154% in those with NPI-O compared with HIV⁻ subjects. **C:** DLPFC PA28 α in HIV⁺ NPI subjects was increased 259% compared with HIV⁻ subjects and 216% compared with HIV⁺ subjects without NPI. HIV⁺ NPI-O subjects had a 126% increase compared with HIV⁻ subjects. **D:** WM PA28 α was increased 85% in HIV⁺ subjects without NPI, 305% in HIV⁺ NPI subjects, and 159% in HIV⁺ NPI-O subjects compared with HIV⁻ subjects. Statistics: one-way analysis of variance with Tukey-Kramer Multiple Comparisons Test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. OD = optical density.

revealed mild decreases in Rpn2 associated with NPI and HIV (not shown) that may account for the decrease seen in screening, but with the accompanying data of comparable 19S subunits, provided only minimal evidence for alteration in the constitutively expressed 19S regulatory complex.

Effects of Age

Regression analysis was used to determine the effect of age on immunoproteasome subunit concentrations in HIV⁺ and HIV⁻ subjects (Table 3). DLPFC PA28 α and LMP7 regressions with age were equivalent between

Figure 5. Immunoproteasome induction in DLPFC, but not WM, was correlated with worse performance in the abstract and executive functioning and speed of information processing neurocognitive domains. Decreased performance on the WCST-64 reflects abstract and executive function, and it was correlated with PA28 α (**A–C**) and LMP7 (not illustrated). Scores for (**A**) categories completed, (**B**) total errors made, and (**C**) perseverative responses are shown. PA28 α , but not LMP7, also was correlated with decreased performance on the Wechsler Adult Intelligence Scale III (WAIS-III) scores for (**D**) Digit-Symbol and (**E**) Symbol Search subtests, which reflects a decrease in the speed of information processing. Other neurocognitive domains that were tested were not correlated significantly. Statistics: Pearson's correlation.

HIV⁻ and HIV⁺ subjects and not significantly different from zero, except for LMP7 in HIV⁻ subjects. White matter regressions were unequal between HIV⁺ and HIV⁻ subjects for PA28 and LMP7, with significant negative regression with age in HIV⁺ subjects and no significant regressions for HIV⁻ subjects.

Correlation with Neurocognitive Test Performance

DLPFC PA28 α (Figure 5, A–C) and LMP7 (not illustrated) were negatively correlated with WCST-64 scores, which are mediated in the DLPFC^{32,33} and reflect impairment of abstract and executive functioning. This relationship was specific to the DLPFC and was not evident for WM (not illustrated). DLPFC PA28 α was also negatively correlated with Wechsler Adult Intelligence Scale III subtests includ-

Table 3. Aging and Proteasome Subunit Levels Regression Analysis Using Log-transformed Values

Subunit	Status	Slope	Interaction with age		HIV ⁻ vs. HIV ⁺ P
			SE	P	
Dorsolateral prefrontal cortex	LMP7	HIV ⁺	−0.0036	0.0064	NS
		HIV ⁻	0.0108	0.0041	<0.01
	PA28 α	HIV ⁺	−0.0081	0.0092	NS
		HIV ⁻	0.0056	0.0059	NS
White matter	LMP7	HIV ⁺	−0.0250	0.0070	<0.001
		HIV ⁻	−0.0016	0.0046	NS
	PA28 α	HIV ⁺	−0.0306	0.0073	<0.0001
		HIV ⁻	0.0000	0.0047	NS

Figure 6. Immunoproteasome induction in DLPFC (A–D) and WM (E–H) was correlated significantly with virological and immunological status of HIV-infected subjects. HIV-1 RNA concentrations in brain (A and E), CSF (B and F), and blood plasma (C and G) were positively correlated with PA28α levels. CD4⁺ lymphocyte counts in blood plasma were negatively correlated with PA28α levels in DLPFC (D), but not WM (H). Equivalent results for LMP7 were obtained (not illustrated). Statistics: Pearson's correlation.

ing Digit Symbol (Figure 5D) and Symbol Search (Figure 5E), which reflect a decrease in the speed of information processing. The same relationship was not present in WM (not illustrated). No correlations between IPS levels and other functional domains evaluated by the National NeuroAIDS Tissue Consortium battery were observed (not illustrated).

Correlation with Clinical Virology and Immunology

Correlations between IPS subunit levels and VL in brain tissue, CSF, and plasma, and plasma CD4⁺ T-cell count were assessed. DLPFC PA28α levels were robustly and positively correlated with VL in all tissue compartments measured, and were negatively correlated with CD4⁺ T-cell counts (Figure 6, A–D). WM PA28α levels also correlated with VL (Figure 6, E–G) but were not correlated with CD4⁺ T-cell count (Figure 6H). Analysis with LMP7 levels produced results equivalent to PA28α (not illustrated). Assessment of IFN-γ levels in brain tissue by enzyme-linked immunosorbent assay showed an increasing trend with HIV⁺ subjects, but marked variability precluded further analysis (not shown).

Localization in Neurons

Glial and neuronal elements were positively stained for IPS subunits in HIV. In general, immunoreactivity in white matter was more intense than in the cortex. Pathological structures characteristically present in HIV were heavily stained. Microglia, macrophages, and oligodendrocyte nuclei were stained in microglial nodules and diffusely (Figure 7A). Neuronal cell bodies in neocortical laminae III and IV had positive staining for IPS subunits (Figure 7, B and D). WM axons often contained IPS

Figure 7. Immunoperoxidase histochemistry illustrates immunoproteasome subunit staining for PA28α in glial and neuronal elements that are pathological in HIV encephalitis. Microglia, macrophages, and oligodendrocyte nuclei are stained in a microglial nodule (A). Neuronal cell bodies in neocortical neurons were stained in focal pathology (B) and were evident in laminae III and IV generally, though admittedly lighter than focal pathology (D). White matter axons often contained immunoproteasome subunits (C). Low immunoreactivity was observed in the (E) cortex and (F) white matter of HIV⁻ subjects.

subunits (Figure 7C). HIV⁻ sections of DLPFC (Figure 7E) and WM (Figure 7F) lacked focal immunostaining. Colocalization with neuronal markers (NeuN and neurofilament) was present in HIV (Figure 8, A and B) but not HIV⁻ sections (Figure 8 inserts). LMP2 was present primarily in perikaryal cytoplasm, whereas PA28α was more evident in neuronal nuclei. LMP2 and PA28α localized with neurofilament protein in WM axons. Dystrophic swollen axons, which are a neuropathological anomaly in HIV,^{34–37} contained IPS (Figure 8B). Synaptic morphology is disturbed in HIV,³⁸ and these structures also contained IPS. Presynaptic boutons stained for synaptophysin showed the typical punctate staining pattern of synapses. Numerous synaptic boutons contained IPS (Figure 9A). Complete overlapping of IPS and synaptophysin suggested localization in presynaptic boutons (Figure 9B). Incomplete overlapping (Figure 9, C and D) suggested that IPS might be present in postsynaptic densities or synaptic astrocyte foot processes. Markers for glial cells including glial fibrillary acidic protein for hypertrophic astrocytes, CD68 for microglia/macrophages, and oligodendrocyte and myelin glycoprotein confirmed that IPS subunits were present in those cells as suggested in Figure 7 (not illustrated).

Discussion

Immunoproteasomes are induced widely in the brains of HIV⁺ people compared with baseline levels in HIV⁻ sub-

Figure 8. Immunoproteasome subunits expressed in neurons in HIV encephalitis. Dual indirect immunofluorescence staining for LMP2 (**A**) or PA28α (**B**) shows colocalization with the neuronal markers NeuN and neurofilament in single optical sections from confocal microscopy. LMP2 is present in granular deposits in the parikaryon (**A**). PA28α is more prominent in the nucleus (**B**). A pathologically swollen axon in white matter contains PA28α (arrows in **B**), as do several other neurofilament-containing processes. Immunofluorescence stainings of HIV⁻ sections (insets) reveal an absence of LMP2 and PA28α. Scale bar = 10 μm.

jects, and this abnormality is clinically and pathologically relevant. Induction of IPS with switching of 20S catalytic subunits and 11S regulatory subunits of the CPS was present in about half of the HIV⁺ decedents and pro-

Figure 9. Immunofluorescence and laser confocal microscopy reveal that immunoproteasome subunit LMP2 is localized to some neocortical synapses in HIV encephalitis. **A:** LMP2 was colocalized within punctate deposits of synaptophysin, which is an established cell marker of presynaptic boutons (arrows). About 12% of labeled synapses in the figure contain immunoproteasome antigenicity. Complete overlapping of these two antigens was evident in some synapses and suggests that immunoproteasomes often are present in presynaptic boutons (**B**). Some synapses had incomplete overlapping (**C** and **D**), which suggests that immunoproteasomes may be present in adjacent structures, including the postsynaptic density or in synaptic astrocyte foot processes. Scale bar = 10 μm.

duced a three- to fourfold increase in IPS subunit concentration. IPS synthesis was relevant neuropathologically because it was significantly more prevalent in people with a postmortem diagnosis of HIVE, although HIVE was not required. The high prevalence of IPS induction in people with HIVE indicates that the neuropathologically evident inflammatory response to replicating HIV-1 in the brain is important, although it was not the sole risk factor. HIV-1 VL in brain tissue and CSF were correlated, as was HIV-1 VL in the plasma compartment. The latter observation agrees with studies that show linkage between HAD and abnormalities in the vascular compartment, including anemia and altered monocyte populations.^{1,39,40} A relationship between IPS induction and weakened systemic immunity was implied by the lower plasma CD4⁺ lymphocyte counts in affected subjects, and suggests that having advanced HIV/AIDS is a risk factor.¹ The broad clinical-pathological impression that emerges from these results is that inflammatory defense against virus,¹⁵ systemically and in the brain, are involved in IPS induction. Nevertheless, HIVE was not required for IPS induction in the brain; other risk factors are involved.

IPS synthesis was significantly more prevalent in people with MCMD or HAD. IPS synthesis was present in the DLPFC and WM, although significant correlation with neurocognitive impairment was most evident in DLPFC. Indeed, IPS induction measured in the DLPFC was correlated specifically with performance on WCST-64 and Wechsler Adult Intelligence Scale III processing speed subtests, which are driven by circuits involved with executive function and speed of information processing located in Brodmann areas 9 and 46 of the DLPFC.^{32,33} IPS changes in WM were not correlated with these functional domains even though they were significantly different in infected people. The correlation between increased IPS in DLPFC and specific functional impairments driven by the DLPFC circuitry illustrates that region- and circuit-specific changes drive specific facets of the overall syndrome of HIV-associated neurocognitive decline (HAND). “Task-specific” changes that correlate selectively with a change in gray matter versus white matter have been reported in HIV-1 infected people, and suggest that neocortical and subcortical pathologies both contribute to HAND.⁴¹

Authors of several reports have observed changes in constitutive proteasomes and immunoproteasomes associated with advanced age.^{21,42,43} Potential influence of age on immunoproteasome induction associated with HIV infection was investigated by performing regression analysis with HIV status and age regressed on the quantified immunoproteasome subunits. The results indicated that DLPFC immunoproteasome subunit concentrations in HIV⁺ subjects were not significantly influenced by age. WM subunit concentrations were associated with a slight decrease with increasing age of approximately 3% per year. Thus, advanced age does not cause or contribute to the immunoproteasome induction, the three- to fourfold increase in immunoproteasome subunits, or the correlation between DLPFC immunoproteasome subunit concentrations and neurocognitive performance in HIV⁺ subjects.

IFN- γ mediated IPS induction in inflammatory cells and glia is consistent with a role in antigen presentation and host defense against virus infection.^{10,15} Our carefully documented observation that IPS synthesis occurs in neuronal perikarya, dystrophic axons, and synapses is emphasized because it is quite novel. The ability of neurons to undergo IPS induction is not widely appreciated^{10,11,44} and could suggest that neurons may participate in self antigen presentation or are passively involved in a potentially harmful metabolic shift. Neuronal IPS induction has been observed in only two other neurodegenerative diseases, Huntington's disease and Alzheimer's disease.^{43,44} Neurons express IFN- γ receptors and can synthesize Class I histocompatibility complexes in response to IFN- γ ,^{43,44} which probably requires IPS induction. As well, exposure of neurons to IFN- γ produces distinct neurophysiological changes in synaptic transmission pertaining to learning and memory.⁴⁵ It is notable that correlation was present between HAND and IPS induction in brain cortex (predominantly neuronal), but not white matter (mostly glial). Thus, IPS synthesis in neurons could be particularly important for neocortical dysfunction due to HIV-1 infection. For example, synapses and dendrites are abnormal morphologically³⁸ and biochemically^{28,46} in HIVE and are potentially important targets for therapeutic intervention.⁴⁷ Synapses and dendrites contain proteasomes and undergo localized protein turnover.^{18,19,48,49} Polyribosomes, mRNA, and the UPS all are present locally in synaptic and dendritic compartments, and they play a pivotal role in regulating synaptic function.^{19,49,50} Experimental clogging of protein turnover in these compartments can be especially devastating because proteins involved in memory formation in synapses and dendrites turn over with astonishing speed (minutes to hours).¹⁹ Local protein turnover in the synaptic compartment is necessary because synaptic transmission and memory formation require rapid local adjustments that require changes in protein synthesis and degradation.¹⁸ Blocking protein turnover at the level of synthesis in dendritic polyribosomes, or degradation via the UPS, alters the electrophysiological changes that drive learning and memory and impairs performance on tests of learning and memory.^{26,51}

The accumulation of ubiquitinated pathological protein is a virtual "signature" of pathological brain aging, reflecting unsuccessful removal of abnormal protein via the UPS.^{23–25,27} The slowing down of the UPS in normal and pathological brain aging is potentially damaging because effete and oxidatively damaged cellular proteins depend on UPS for efficient removal.^{9,21} Inherited defects of the UPS lead to Parkinson's disease and Angelman's syndrome.^{52,53} UPS protein processing is decreased sharply in Alzheimer's disease and during brain aging.^{20,21,54} As well, experimental disruption produces synaptic dysfunction and deficits in learning and memory.^{18–27,48,49} Likewise, the finding of increased accumulation of ubiquitin-stained deposits and high molecular weight ubiquitin conjugates in HIV/AIDS brains suggest UPS dysfunction.²⁸ Neuronal autophagy, which aids in ubiquitin-protein aggregate degradation, is also reduced in HIV infection and may further exacerbate the accumu-

lation of ubiquitinated protein conjugates due to UPS dysfunction.^{55,56}

HAND represents a novel example of an acquired defect in the UPS linked to synaptic dysfunction.^{38,46} Though the proteasome subunit analysis does not examine the structure or quantity of fully assembled proteasome complexes, the HIV-associated increases in IPS and 11S subunits implicate increasing integration of these subunits into newly formed IPS complexes at the expense of constitutively expressed counterparts, which are assembled four times slower than IPS.^{6,57,58} The replacement of constitutively expressed 19S regulator complexes on the proteasome core by the increased presence of 11S activator complexes, which are integral to IPS formation and antigen processing,^{59,60} is important because 19S processes many thousands of polyubiquitinated protein species during routine turnover and cellular maintenance. After a folded protein has been tagged for destruction with polyubiquitin, it binds to a subunit of 19S. The 19S complex contains ATPases, chaperonins, unfoldases, and isopeptidases and caps the 20S core complex to form the 26S proteasome complex. The substrate is energized by ATPases, polyubiquitin is removed and recycled, and the polypeptide is unfolded and chaperoned "single file" into the 20S core for hydrolysis. Virtually all folded proteins turn over by the 26S proteasome in that manner. The 11S activator, however, does not contain ATPases or isopeptidases. Thus, it can neither process folded polyubiquitinated protein nor recycle ubiquitin monomers, which are absolutely essential for normal operation of the UPS.^{61–63} IPS induc-

Figure 10. Persistent "hijacking" of brain proteasomes in HIV-1-infected people may lead to neuronal dysfunction. Normally, proteasome complexes rapidly turn over ubiquitinated proteins. Persistent infection with HIV-1 produces inflammatory cytokines such as IFN- γ that induce a temporary shift to immunoproteasome synthesis. In turn, the protein substrate repertoire is temporarily shifted toward the processing of unfolded peptides for class I antigen presentation. "Borrowing" the proteasome apparatus for heightened antigen presentation persists until the pathogen is eradicated, after which normal brain protein turnover should resume. Since HIV-1 infection is not eradicated in the brain, there is a persistent "hijacking" of the proteasome. Normal turnover of folded proteins is disrupted chronically. This leads eventually to the accumulation of pathologically misfolded proteins, neuronal dysfunction, and dementia.

tion also coincides with destabilization and decrease in constitutive 26S proteasome complexes.^{12,64} The persistent replacement of 19S complexes on 20S complexes by increasing 11S subunits due to HIV-1 infection is potentially devastating because thousands of cellular components can be perturbed by the changes in substrate repertoire.^{9,12–14}

Pervasive anomalies of the UPS in HAND suggest a novel concept regarding neuroinflammatory modulation of brain function (Figure 10). As 11S increasingly displaces 19S regulatory complexes, the neuronal proteasome population shifts to accommodate newly synthesized 11S/IPS complexes at the expense of the 26S proteasomes. The IPS complexes cannot perform “routine” turnover of polyubiquitinated protein, which is the primary route for the turning over of most folded proteins.⁹ Instead, the IPS is specifically synthesized to process unfolded protein and small, unconjugated polypeptides with high efficiency. The best known function of the IPS is processing unfolded peptides for antigen presentation in Class I histocompatibility complexes.^{10,11} IFN- γ -induced increases of IPS synthesis reflect heightened immune surveillance in response to the presence of foreign antigen.¹⁵ A temporary “borrowing” of the UPS by IPS is tolerable physiologically because pathogens are normally eradicated in due time, and the IPS disappears quickly due to its inherent structural instability.⁷ The shift to IPS synthesis, however, is not transient in the HIV-1 infected brain because the CNS is a reservoir of persistently replicating HIV-1.^{16,17} HIV-1 provokes a long-lasting sustained cytokine response that includes tumor necrosis factor α and IFN- γ expression,^{65,66} synthesis of inflammatory response protein^{28,67} and heightened expression of several IFN- γ inducible genes.⁶⁸ Instead of the typical “borrowing” of the UPS for immune defense,⁷ a persistent “hijacking” of the UPS characterizes aptly the nature of the changes in HIV-1-infected brain tissue.

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