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Plasma gelsolin accumulates in macrophage nodules in brains of simian immunodeficiency virus infected rhesus macaques

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Abstract Plasma gelsolin (pGSN), an isoform 1, is secreted by various types of cells in the central nervous system (CNS) and periphery, but not by the liver. pGSN circulates in blood and cerebrospinal fluid (CSF); however, its concentration in CSF is approximately twenty times lower than in plasma. It has been shown that several types of cells such as oligodendrocytes, neurons, and/or astrocytes contribute to the overall pool of pGSN in the CNS. Further, it has been postulated that pGSN plays multiple roles during microbial infection and modulates inflammatory responses; however, the exact mechanism of regulation is not known. We previously showed that levels of pGSN in CSF of individuals with advanced neurocognitive impairment due to HIV infection of the brain are decreased. Here, we show that macrophages express significant amounts of pGSN in response to HIV infection in vitro. Using immunohistochemistry of simian immunodeficiency virus infected rhesus monkey brains, we show that increased levels of pGSN are present in macrophage nodules creating locally a high level of this protein within the brain. This may not be reflected by the overall decreased level in the distinct CSF compartment.

Keywords Gelsolin · Macrophage · SIV · HIV · Brain · Neuroinflammation

Introduction

HIV-1 invades the brain early after exposure to the virus (Gartner 2000) and remains there for the rest of the host's life. Low levels or restricted infection of naïve CD4+ T cells and mononuclear phagocytes (MP; monocytes, tissue macrophages, and dendritic cells) creates cellular reservoirs for HIV. Therefore, current treatment can control infection to some extent but cannot eradicate it (Letendre et al. 2010). HIV-infected MP become activated creating low level of persistent proinflammatory environment which, along with production of neurotoxic viral proteins, is postulated to be a major contributor of HIV-associated neurocognitive disorders (HAND) (Kraft-Terry et al. 2009; Valcour et al. 2011). In fact, over the course of HIV infection, approximately 50% of individuals develop various levels of HAND (Ances and Ellis 2007). Because HIV-infected individuals now live longer benefiting from multidrug therapy (combined antiretroviral therapy, cART), HAND has become an increasing problem of disease management (Wright 2011). Paradoxically, introduction of cART therapy reduced the number of the most severe form of HAND which is HIV-1-associated dementia; but due to increase longevity, it appears to increase the prevalence of milder forms of neurocognitive deficits. Despite many years of extensive research on various aspects of HIV infection of the brain, the pathogenesis of HIV-associated neuroinflammation is not fully understood thereby limiting our ability to develop new treatments (Burdo et al. 2008; Chao and Ghorpade 2009; Gannon et al. 2011; Grovit-Ferbas and Harris-White 2010; Kraft-Terry et al. 2009; Reynolds et al. 2007; Williams et al. 2008).

Gelsolin (GSN) has been a subject of increasing research due to its role in capping and severing actin filaments that regulate cell movement (Yin and Stossel 1979). There are two main forms of this protein, plasma gelsolin (pGSN) and cytoplasmic gelsolin (cGSN), pGSN being structurally similar to cGSN but longer by 52 a.a at the N-terminal end (Yin et al. 1984). GSN is coded by a single gene, and isoforms 1 and 2 (pGSN and cGSN, respectively) result from alternative initiation sites (Kwiatkowski et al. 1986). It has been

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postulated that a third isoform is made by oligodendrocytes (Vouyiouklis and Brophy 1997), but the protein product has not been characterized yet. Subsequent studies showed that although both known isoforms are involved in severing actin filaments, they are much more functionally versatile molecules, and many regulatory functions have been described (Bucki et al. 2008a, b; Kulakowska et al. 2008). These functions are just beginning to be investigated in more focused and mechanistic studies in the context of various diseases. pGSN is distributed by body fluids, and its half-life time is comparable to that of albumin. pGELS is not synthesized in the liver, and very little is known about regulation of its expression. It has been proposed that neurons and oligodendrocytes are substantial sources of gelsolin in the central nervous system (CNS) (Paunio et al. 1998; Vouyiouklis and Brophy 1997; Yuan and Desiderio 2003). We also present here that HIV-infected macrophages secrete pGSN. The level of GSN in plasma ranges from approximately 100 to 300 μg/ml (Kulakowska et al. 2011), while in cerebrospinal fluid (CSF), it is approximately 20 times lower and varies from 1.2 to 15 μg/ml (Kulakowska et al. 2008). Based on these reports as well as the fact that pGSN is secreted by cells of the CNS, we speculate that the exchange of pGSN through the blood–brain barrier from blood to CSF is minimal if any.

It has been postulated that pGSN interacts with other proteins such as fibronectin (Lind and Janmey 1984) or vitamin D binding protein (Harper et al. 1987) or proteins expressed on the surface of cells such as integrins (Bohgaki et al. 2011). However, detailed interactions and resulting function(s) have not been determined yet, nor do we know if these affect plasma or CSF levels of pGSN.

Among many diseases of the CNS, the role of pGSN in stroke and Alzheimer's disease (AD) has been most extensively studied. In stroke, pGSN's postulated role is to remove actin filaments to prevent formation of secondary occlusions in microcapillaries (Guo et al. 2011). In AD, the role of pGSN may be related to its function as a modulator of inflammation (Guntert et al. 2010). Spinardi and Witke postulate that pGSN plays an important role in acute as well as chronic inflammatory responses although both mechanisms are different (Spinardi and Witke 2007). If this broad hypothesis is correct, one may expect that modulation of pGSN in chronic inflammation of an HIV-infected brain might be an important modulator of neuroinflammation and as such could be a potential target for new pharmacological interventions to treat HAND. The majority of studies published to date investigated average levels of pGSN in the CSF, and very few focused on a detailed pattern of expression (Tanaka and Sobue 1994). This study is the first attempt to use immunohistochemistry to investigate patterns of pGSN expression in the cortex of the brains of SIV-infected rhesus monkeys.

Materials and methods

Monkeys and SIV infection

Animal experiments followed the National Institutes of Health guidelines and were performed with approval from the Scripps Research Institute's and University of Nebraska Medical Center's Institutional Animal Care and Use Committees. Specific pathogen-free (SIV, simian retrovirus type D and Cercopithecine herpesvirus 1 negative) rhesus macaques of Chinese origin were purchased from Valley Biosystems (West Sacramento, CA, USA) or Alpha Genesis (Yemassee, SC, USA). SIV-infected animals were inoculated intravenously with 0.25 ml cell-free SIV derived from SIVmac251 stock as described previously (Burdo et al. 2005). Necropsy was performed after terminal anesthesia. During necropsy, animals were intracardially perfused with sterile PBS containing 1 U/ml heparin to clear blood-borne cells. Tissue samples were then taken and fixed in 10% neutral buffered formalin.

Antibodies

In this study, the following primary antibodies were used for both histopathological and immunofluorescence: rabbit gelsolin precursor monoclonal antibody (mAb) (gelsolin marker; 1:50; LifeSpan Biosciences, Seattle, WA, USA), mouse CD163mAb (macrophage marker; 1:50; Vector Laboratories, Burlingame, CA, USA), chicken GFAP polyclonal antibody (pAb) (astrocytes marker; 1:1,000; Abcam, Cambridge, MA, USA), chicken MAP2 pAb (neuron marker; 1:1,000; Abcam), and mouse monoclonal SIV p27 gag antibody (FA2) (SIV marker; 1:500, hybridoma supernatant, cells obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases). The following secondary antibodies were used for this study: anti-rabbit, chicken, and mouse antibodies conjugated to fluorescent probes—Alexa 488 and 594 (Invitrogen, Eugene, OR, USA) and DAKO EnVision system, horseradish peroxidase (HRP)-labeled polymer anti-rabbit and mouse (DAKO, Carpinteria, CA, USA).

Reagents

ProLong Gold anti-fade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen), Trilogy™ solution (Cell Marque, Rocklin, CA, USA), normal goat serum (NGS) (Vector Laboratories, Burlingame, CA, USA), DAKO liquid 3,3′-diaminobenzidine (DAB)+substrate chromogen system, Mayer's hemotoxylin (DAKO), Cytoseal™ mounting media (Thermo Scientific, Kalamazoo, MI, USA), and Tris-buffered saline (Thermo Scientific, Fair Lawn, NJ, USA) were used in this study.

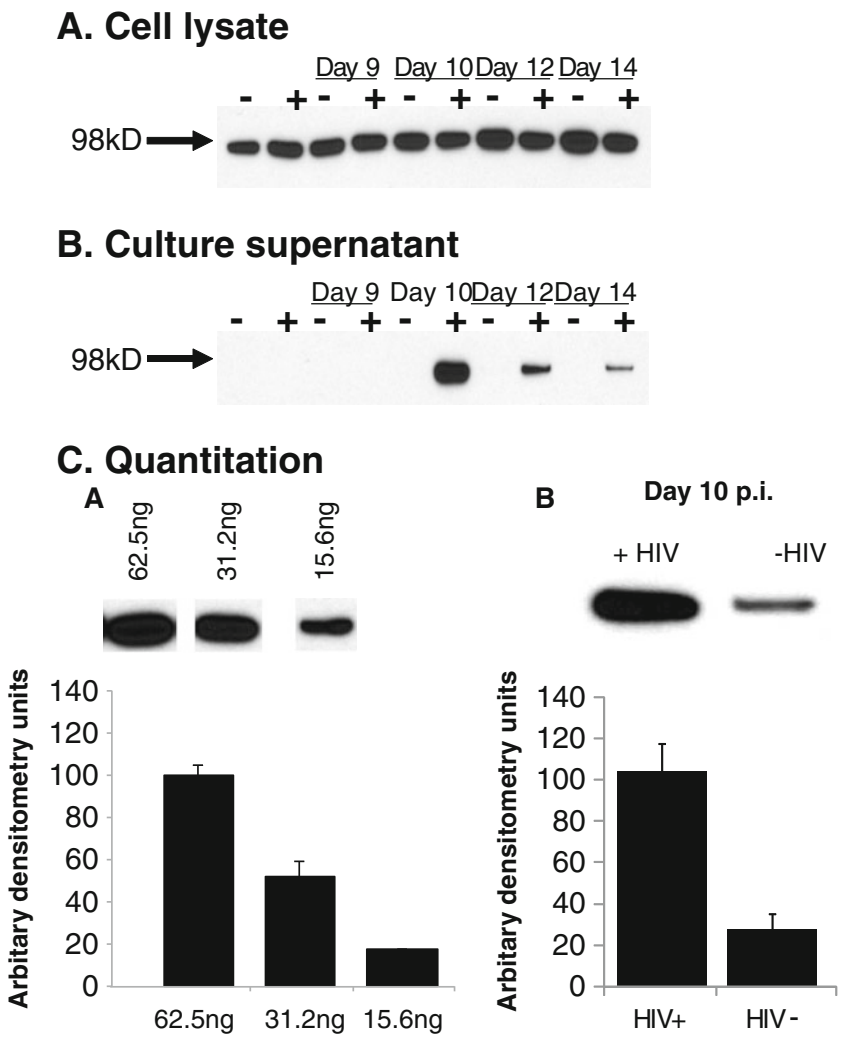

Fig. 1 Expression of GSN in HIV-1_{ADA}-infected monocyte-derived macrophages (MDM). Quantitative changes in the expression of pGSN, cGSN, and vimentin in cell lysates **a** and culture supernatants **b** of HIV-infected MDM. cGSN is marginally downregulated in infected MDM. pGSN is secreted by infected cells starting 3 days after infection (day 10 of culture). Rate of secretion subsides during the following 5 days. **c** Quantitative Western blot and bar graphs showing the amount of pGSN secreted by MPs in the culture supernatant from day 10 after in vitro HIV infection of MDM. Amount of protein loaded (20 μg/lane) represents amount of protein produced by 3×10^5 cells during 24 h of serum-free culture showing that they produced approximately 60 ng of pGSN. Faint band in noninfected culture may be around or less than 1 ng

Histopathological evaluations

Formalin-fixed tissue was paraffin-embedded, and 5-μm sections were stained with hematoxylin and eosine (H&E) and examined microscopically for histopathology. Encephalitis was defined by the presence of macrophage infiltrates and multinucleated giant cells in the brain. Paraffin-embedded formalin-fixed sections of the rhesus macaques brains were incubated with primary antibodies overnight at 4°C. The polymer-based HRP-conjugated anti-mouse and anti-rabbit

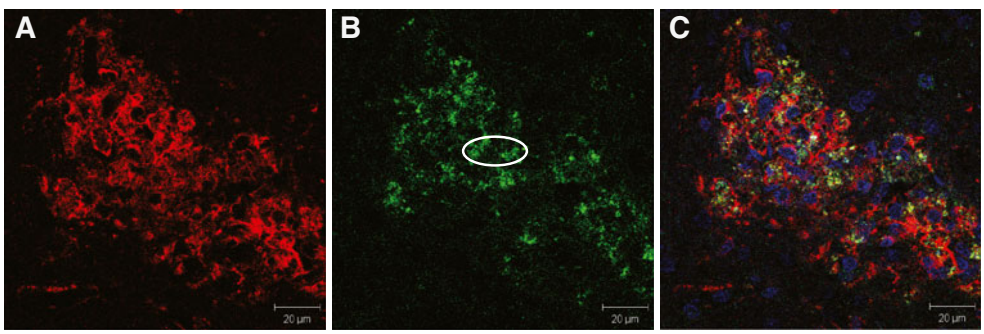

Fig. 2 Immunohistochemical detection of pGSN in macrophage nodule in the cortex of SIV infected monkey's brain. **a** Region stained for anti-CD163 macrophage marker. **b** pGSN staining is noted extracellularly indicating release and accumulation in the macrophage nodules. **c** An overlay with nuclei stain with DAPI. One region (as *marked*) was used for co-localization analysis and shows overlap coefficient $R=1.0$

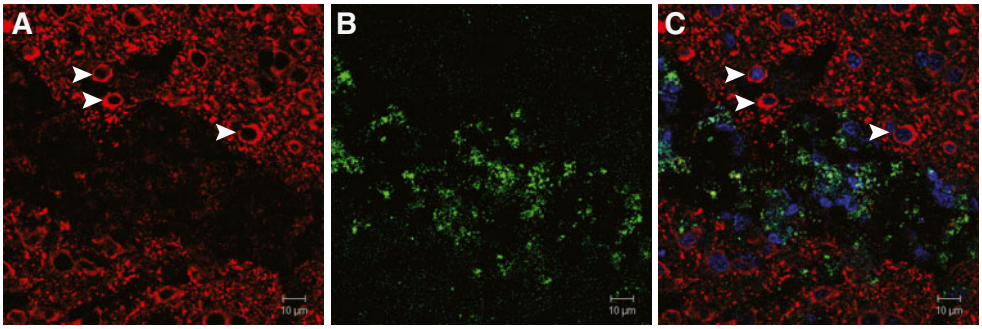

Fig. 3 Immunohistochemical detection of pGSN and neurons. Section from the cortex. **a** Neurons (*red*) residing around macrophage nodule (*arrowhead*). **b** pGSN (*green*) accumulation is seen in the area of macrophage nodules. **c** An overlay shows no accumulation of pGSN around neurons. Nuclei are visualized with DAPI (*blue*)

DAKO EnVision systems were used as secondary detection reagents and were developed with DAB. All sections were counterstained with Mayer's hematoxylin, dehydrated in graded alcohols, and mounted with cytoseal. Images were obtained with Nikon DS-Fi1 camera fixed to a Nikon Eclipse (Nikon instrumental using NIS-Elements F3.0 software).

Immunofluorescence

Sections were deparaffinized and rehydrated in Trilogy™, loaded onto a pressure cooker, and heated to 125°C. After 10-min cooling, sections were blocked with Tris buffer saline with 0.2% Tween-20 (TTBS) containing 10% NGS for 30 min at room temperature (RT) and then double immunofluorescence-stained by using paired combinations of primary antibodies gelsolin/CD163, gelsolin/GFAP, gelsolin/MAP2, and gelsolin/SIV o/n at 4°C. The sections were rinsed three times with TTBS.

The primary antibodies were bound to the respective secondary antibodies conjugated to fluorescent probes Alexa fluor 488 (green) and 594 (red). The sections were rinsed twice with TTBS and once with Milli-Q water. Sections were mounted with ProLong Gold anti-fade reagent containing DAPI to stain nuclei, allowed to dry for 24 h at RT, and then stored at −20°C for future use.

Laser-scanning images were obtained using a Zeiss Meta 510 confocal microscope with a ×63 oil immersion lens. Image analysis was performed using Carl Zeiss AIM Software Release (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA) following the manufacturer recommendations. Briefly, each analyzed section of an image intensity of red (channel 2) and green (channel 1) has been measured, and an overlap coefficient (R) as proposed by Manders et al. has been calculated (Manders et al. 1993). R indicates an actual overlap of the signals and is considered to represent the true degree of co-localization. R ranges from 0 to 1.0, and e.g., 0.4 implies that 40% of both selected channels co-localize (Zinchuk and Zinchuk 2008).

Results and discussion

In response to activating stimuli or infection, mononuclear phagocytes upregulate and secrete a number of proteins including those that are considered as components of the cytoskeleton such as gelsolin, filamin, profilin-1, cofilin-1, vimentin (Bitko et al. 2003; Ciborowski et al. 2007; Jimenez-Baranda et al. 2007; Matarrese and Malorni 2005; Mor-Vaknin et al. 2003; Naghavi et al. 2007; Rozek et al. 2007). Moreover, it has been postulated that these proteins may have regulatory capabilities. The presence of these proteins and their up/downregulation in the environment outside of the cell may profoundly affect the surrounding environment and the functions of neighboring MP and/or other cells of the CNS. In this context, pGSN has been studied as an immunomodulator, an actin scavenger, and a component of amyloid formation, as well as in other roles. The levels of pGSN in plasma and CSF have been found to

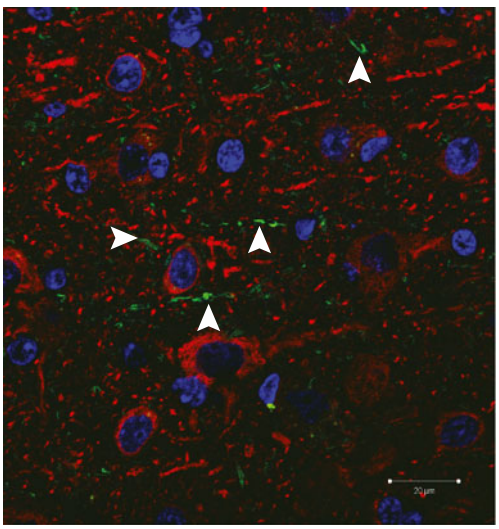

Fig. 4 Immunohistochemical detection of pGSN and neurons in encephalitic brain. An overlay of immunostaining for neurons (*red*), pGSN (*green*), and nuclei (*blue*) of section of the cortex from encephalitic brain. *Arrowheads* show spots of high concentration of pGSN. Expression pGSN is not associated with neurons

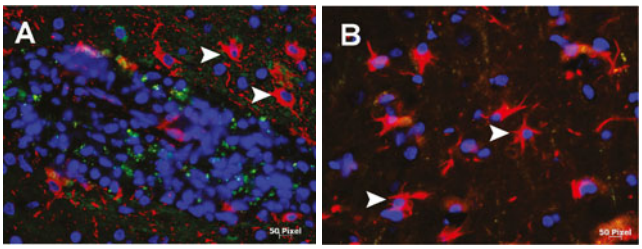

Fig. 5 Immunohistochemical detection of pGSN and astrocytes. Section from the cortex. **a** pGSN (*green*) accumulation is seen in the area of macrophage nodules. Astrocytes (*red*) residing around neurons do not show accumulation of pGSN. **b** Another area of the cortex from the same animal as in (**a**) showing staining for astrocytes, but not for pGSN. Nuclei are visualized with DAPI (*blue*)

be altered (up or down) in various diseases leading to its utility as a biomarker (Kulakowska et al. 2008). These studies were focused on the relative level of pGSN in comparison to controls and did not take into account that this protein can reach locally high concentrations.

Our first observation, which is presented in Fig. 1b, shows secretion of pGSN after in vitro infection of monocyte-derived macrophages (MDMs) with laboratory-adapted HIV-1_{ADA} strain. MDMs expressed most of pGSN 3 days after infection, and then, expression subsided. This pattern is reproducible; however, in some experiments, expression of pGSN can be detected as early as 1 day after infection, and lowering secretion of pGSN is not significant during 14 days in a culture after infection. These differences can be attributed to the dynamic of infection that depends on the efficiency of initial and secondary infections. In addition, we speculate that the rate of downregulation of pGSN might be also associated with the extent of formation of multinucleated giant cells; however, this aspect has not yet been experimentally tested. As pGSN production increases, we observe a slight decrease of cGSN expression as shown in Fig. 1a, and this was previously reported by our laboratory (Kraft-Terry et al. 2011). This is an expected effect, since the same gene expresses both isoforms and more of the GSN transcript is unprocessed to produce pGSN; at the same time, less is available to be processed for expression of cGSN. Because one-allele knockout cells do not show any functional impairment, we conclude that such small downregulation of cGSN in this case will not have functional consequences for intracellular mechanisms (Kangas et al. 1996). In Fig. 1c, we show quantitative Western blot measurements of pGSN in a culture supernatant of HIV-infected MDM. During 24 h of culture in a serum-free medium, 3×10^5 cells produce approximately 60 ng of pGSN, which is 0.30% of the total secreted protein. Noninfected MDM shows either none or 100 times lower release of GSN. In this case, it could be due to the release of cGSN from either dying cells or few cells that have been activated and secreting pGSN. In another study, we showed that pGSN is the isoform circulating in body fluids, and the level of cGSN was under detection limit (Pottiez et al. 2010).

The next questions that we asked were whether expression of pGSN by MP in in vitro experiments also occurs in vivo and, if so, whether such expression is only a contribution to the overall level of pGSN or an impact on the local CNS environment by itself. To address these two questions, we used sections of the cerebral cortex of the SIV-infected monkey brains that showed SIV encephalitis.

Double staining of a macrophage nodule with anti-CD163 (red) and anti-GSN (green) antibody (Fig. 2a, b,

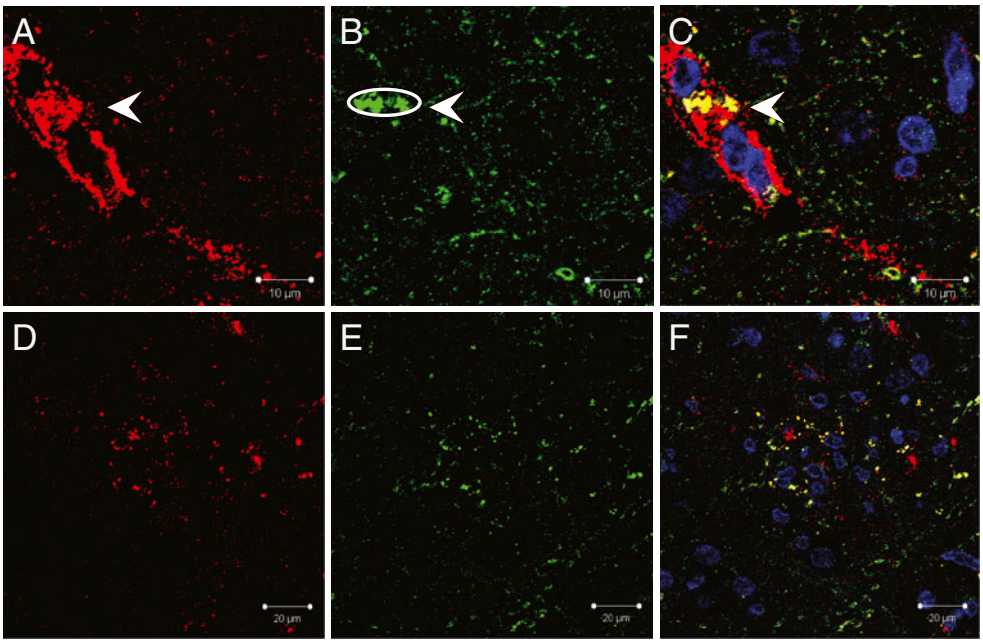

Fig. 6 Immunohistochemical detection of pGSN and gag27 (SIV). Section from the cortex. **a** Two adjacent SIV-infected macrophages show expression of gag27 protein (*red*). **b** pGSN (*green*) shows accumulation of pGSN. **c** An overlay showing co-localization of p27 and pGSN (*yellow*). All indicated by *white arrows*. Another area of the cortex viewed at lower magnification from the same animal shows staining for gag27 (**d**), pGSN (**e**), and an overlay (**f**) and *blue* for nuclei showing co-localization of viral protein and pGSN. **a**, **b** magnification ×63; **d**, **e** magnification ×40 One region (as *marked*) was used for co-localization analysis and shows overlap coefficient $R=1.0$

respectively) shows accumulation of pGSN around MP in an overlay (Fig. 2c). This pattern of pGSN expression was observed in all studied macrophage nodules.

Although it has been postulated that neurons are the major source of pGSN in the CNS, we did not observe local accumulation of this protein around neurons as we observe in macrophage nodules. Figure 3 shows double staining of the brain section for a MAP2 neuronal marker (Fig. 3a), pGSN (Fig. 3b), and an overlay with DAPI (blue staining) for nuclei (Fig. 3c). pGSN is localized in the center where the macrophage nodule is found, and cells are not stained except nuclei. In the subsequent double staining of the cortex of encephalitic brain (Fig. 4), we do not observe accumulation of pGSN (green) around neurons (red, anti-MAP2); however, spotty accumulation of pGSN can be detected in the space between neurons. This suggests that neurons do not secrete high amounts of pGSN but can be exposed to high concentrations of this protein. Our unpublished data show that in in vitro experiments, pGSN protects neurons from toxic effects of the HIV protein Tat; however, it is not clear whether pGSN acts directly by Tat or indirectly by competing to putative receptors on the surface of neurons. Because it has been postulated that pGSN interacts with $\alpha 5\beta 1$ integrin (Bohgaki et al. 2011) and shown that Tat interacts and binds to $\alpha v\beta 5$ integrin (Barillari et al. 1993; Vogel et al. 1993), it is possible that pGSN protection from Tat neurotoxicity can be in part mediated by blocking Tat–integrin interactions. At this point, such hypothesis is speculative and will require further experiments.

In the next step, we performed double staining for pGSN and with anti-GFAP for detection of astrocytes. Figure 5 shows staining for pGSN and astrocytes from two sites. One site (Fig. 5a) is around the macrophage nodule showing again accumulation of pGSN (green) around macrophages visualized with DAPI (blue). The other side (Fig. 5b) is from another region of the same section and shows a typical picture of astrocytes, but no presence of pGSN.

Finally, we performed double fluorescent staining of macrophage nodules for SIV and pGSN. Results of this experiment are presented in Fig. 6. Figure 6a shows two macrophages stained positively for SIV, Fig. 6b shows staining for pGSN, and Fig. 6c shows an overlay indicating accumulation of pGSN around the infected macrophage. Fig. 6d, e is the lower magnification from another tissue section showing spotty expression of SIV protein and overlaying pGSN.

Conclusions

This is the first study to show accumulation of pGSN in macrophage nodules formed in response to SIV infection of monkey brains. It has to be noted that the brains obtained from monkeys represent a snapshot at the time of necropsy. We further speculate that local elevation of pGSN in any spot of the brain may fluctuate over time, which is suggested by various degree of pGSN staining. Taking this further, we conclude that if indeed elevated levels of pGSN promote neuroinflammation, it would be in the form of "pulses" rather than a continuous process. This scenario would fit better to the general scheme of low level but prolonged inflammation ultimately leading to neurocognitive impairment. Based on the monkey model used in this study reflecting HIV infection of the brain in humans, we extrapolate our finding to postulate that although the level of pGSN is decreased in the CSF of patients with advanced HAND, local cells of the CNS can be exposed to high levels of this protein.

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Conflict of interests The authors declare that they have no conflict of interest.

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