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HIV Tat causes synapse loss in a mouse model of HIV-associated neurocognitive disorder that is independent of the classical complement cascade component C1q.

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Abstract

Microglial activation, increased pro-inflammatory cytokine production, and a reduction in synaptic density are key pathological features associated with HIV-associated neurocognitive disorders (HAND). Even with combination antiretroviral therapy (cART), more than 50% of HIV-positive individuals experience some type of cognitive impairment. Although viral replication is inhibited by cART, HIV proteins such as Tat are still produced within the nervous system that are neurotoxic, involved in synapse elimination, and provoke enduring neuroinflammation. As complement deposition on synapses followed by microglial engulfment has been shown during normal development and disease to be a mechanism for pruning synapses, we have tested whether complement is required for the loss of synapses that occurs after a cortical Tat injection mouse model of HAND. In Tat-injected animals evaluated 7 or 28 days after injection, levels of early complement pathway components, C1q and C3 are significantly elevated and associated with microgliosis and a loss of synapses. However, C1qa knockout mice have the same level of Tat-induced synapse loss as wild-type (WT) mice, showing that the C1q-initiated classical complement cascade is not driving synapse removal during HIV1 Tat-induced neuroinflammation.

Graphical Abstract

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Conflicts of interest

The authors declare no competing financial interests.

Keywords

complement; HIV; Tat; C1q; synapse

Introduction:

Although the field of neuroAIDS has been transformed by antiretroviral therapies, cognitive impairments associated with HIV infection remain a major concern. 50% of HIV+ patients on cART have neurological symptoms such as impaired learning and memory and psychomotor slowing (Spudich, 2013). The degree of cognitive impairments in HAND correlates with increased populations of activated microglia/macrophages and with synaptic and dendritic injury rather than neuronal loss (An et al., 1996; Everall et al., 1999; Sa et al., 2004). Thus, adjunctive treatments are needed to protect the central nervous system (CNS) from inflammatory damage with associated cognitive decline and the complement pathway offers an attractive set of targets for therapeutic intervention.

HIV enters the CNS via infected monocytes and lymphocytes that traverse the blood brain barrier (Jones & Power, 2006). Although cART suppresses viral replication, some viral proteins such as Tat, are still made by infected macrophages, microglia, and astrocytes. Viral proteins released from infected glia can harm adjacent neurons directly or indirectly by activating the immune system and triggering the production of cytokines, complement proteins, and reactive oxygen species (Bansal et al., 2000; Buscemi, Ramonet, & Geiger, 2007; N. C. Chen, Partridge, Sell, Torres, & Martin-Garcia, 2017; Jadhav, Krause, & Singh, 2014; Marker et al., 2013; Nitkiewicz et al., 2017; Williams et al., 2010; Youn, Ju, Choi, & Park, 2015). Simply exposing the brain to Tat, causes neuropathologic changes that are

relevant to HAND. Specifically, transgenic mice that express Tat in astrocytes have impaired learning and memory, dendritic spine reductions, changes in synaptic protein expression, microglia and astrocyte activation, increased oxidative stress, reduced excitability or numbers of specific neuron subpopulations, and reductions in grey matter volumes (Bruce-Keller et al., 2008; Carey et al., 2013; Chivero et al., 2017; Dickens et al., 2017; Fatima et al., 2017; Fitting et al., 2013; Leibrand et al., 2017; Marks et al., 2016; Paris, Zou, Hahn, Knapp, & Hauser, 2016; Schier et al., 2017).

Synapse loss and an enduring neuroinflammatory response also occur following a single injection of Tat (Aksenov et al., 2003; Lu et al., 2011; Pu et al., 2003). The synapse loss begins as early as 24 hours after Tat exposure and is still prominent after 4 weeks. Microglia in Tat injected brains have more phagocytic inclusions than in controls and some inclusions clearly contain synaptic vesicles (Lu et al., 2011; Marker et al., 2013; Raybuck, Hargus, & Thayer, 2017; Tremblay et al., 2013). This led us to investigate whether complement opsonization drives this microglia-mediated synapse phagocytosis as complement has been implicated in synaptic pruning or synaptic debris clearance in development, Alzheimer's, schizophrenia, frontotemporal dementia, aging, West Nile virus neuroinvasive disease, and following optic nerve injury (Hong et al., 2016; Lui et al., 2016; Norris et al., 2018; Sekar et al., 2016; Shi et al., 2015; Vasek et al., 2016). Microglial-mediated synaptic pruning requires the early complement cascade components C1q, C3, C4, and the microglia-specific complement receptor 3 (Schafer & Stevens, 2010; Stevens et al., 2007).

Previous work has provided correlative support for complement species being dysregulated in the CNS after HIV-1 infection. Elevated levels of C1q were found in the cerebrospinal fluid (CSF) of young, cognitively impaired, HIV+ patients when compared to seropositive patients that were cognitively normal (McGuire, Gill, Douglas, Kolson, & Group, 2016). In contrast, the reports on C3 levels in the CSF of HIV+ patients have been mixed with some studies showing increased levels (Jongen et al., 2000) and others decreased levels (Reboul et al., 1989; Rozek et al., 2007) that likely reflect the complex interplay between increased expression and enhanced activation/degradation of C3. Within brain parenchyma, multiple complement transcripts are up-regulated in patients with HIV-1 encephalitis (Gelman et al., 2012; Mahajan et al., 2017), a neuropathologic entity that occurs infrequently since the advent of cART. Whether aberrant complement deposition represents a penultimate step in synapse removal associated with HIV infection remains an essential unanswered question because synaptic architecture and function are the key pathologic substrates for HAND.

In the present study, we demonstrate that the neuroinflammatory response after Tat injection into the cortex of mice is associated with increased C1q and C3 expression. However, genetic deletion of C1q does not prevent Tat induced synapse loss. In turn, our data provide a counterpoint to the idea that complement deposition is a “final common pathway” event to synapse loss in the context of neuroinflammation.

Methods:

Animals

Animal care and use were carried out in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals and the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Protocols were approved by the University Committee on Animal Resources at the University of Rochester. C57BL/6J and CD45.1 were obtained from Jackson Laboratories. C1qa knockout (KO) mice were obtained from Dr. Andrea Tenner (University of California, Irvine; (Botto et al., 1998)).

Experimental Design and Statistical Analysis

Mice were 8–14 weeks at the start of all experiments. Male mice were used for chimera immunohistochemistry (IHC) experiments in Figure 1. A mix of male and female mice was used for the remaining C1qa knockout vs WT experiments. *N* values (number of animals) for each experiment are reported in the figure legends. GraphPad Prism software was used to perform all statistics. We defined significance as $p < 0.05$. Student's *t*-test was used when comparing only two groups. One-way ANOVA followed by Tukey's multiple comparisons test was done for western blot data. Two-way ANOVAs were done when comparing IHC data from WT and C1qa KO mice injected with Tat or Saline. The two-way ANOVAs were followed by Holms-Sidak's multiple comparisons test with 4 comparisons done per family (WT-Saline vs WT-Tat; C1qa KO-Saline vs C1qa KO-Tat; WT-Saline vs C1qa KO – Saline; WT-Tat vs C1qa KO Tat). All data are expressed as the mean $\pm$ standard error of the mean (SEM).

HIV-1 Tat_{1–101} purification

His-tagged Tat_{1–101} protein was made by first sub-cloning Tat cDNA into the p#T28a vector using *Nhe*I and *Xho*I restriction sites and the PCR primers: 5'–CCCTAGCTAGCATGGAACCGGTGGA TCCGAACC–3' and 5'–GGCCGCTCGAGTTATTAATGCACCGGATCGGTTTC GG–3'. His-tagged Tat_{1–101} protein was purified from BL21-Gold (DE3) bacteria transformed with the p#T28a-Tat plasmid by lysing cells with a French press into a solution of 5mM imidazole, 200 μ M PMSF, 200 μ M DTT, in dPBS, pH = 8 and running supernatant-containing His-tagged Tat_{1–101} on a Bio-Rad Profinia with IMAC column (BioRad, 732–4610) and eluting with 300mM imidazole in dPBS, pH = 8. Endotoxin was removed by adding CaCl₂ and sodium citrate to a final concentration of 1mM and passing sample through an EndoTrap HD column (Hyglos, # 800053). Excess salt and imidazole were removed by passing samples through PD-10 columns (GE Healthcare, #17-0851-0), and eluting with 100mM ammonium bicarbonate in endotoxin-free H₂O (Hyclone, SH30529.02). Purified protein was aliquoted using siliconized tips, lyophilized overnight, and stored at –80°C.

Bone marrow chimeras

CD45.1 mice were engrafted with heterozygous CX₃CR1/GFP^{+/–} bone marrow as described previously (Lu et al., 2011; Marker et al., 2013). Briefly, 8-week-old CD45.1 host mice were

lethally irradiated with 9 Gy of gamma radiation from a cesium source. Mice were head shielded to prevent any radiation-induced neuroinflammation or damage (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; Mildner et al., 2007). 8- to 12-week-old CX₃CR1/GFP^{+/−} donor mice were euthanized by CO₂ asphyxiation and the bone marrow from the femurs and tibias were collected in Dulbecco's PBS (dPBS; Cellgro; 21–030-CV). Red blood cells were lysed with ACK lysing buffer (Lonza; 10–548E), and the remaining bone marrow cells were washed twice in sterile dPBS and resuspended at a concentration of 5×10^7 cells/ml. 200µl of this cell suspension was then injected into the tail veins of the irradiated host mice. The mice recovered for 4 weeks prior to Tat injection.

Stereotactic injection of Tat or Saline

3µl of HIV-1 Tat or Saline (0.9% NaCl) was stereotactically injected into the cortex at the coordinates 1.0 mm posterior to bregma, 1.0 mm lateral left of bregma, and 0.7 mm ventral to the pial surface as described previously ((Lu et al., 2011; Marker, Tremblay, Lu, Majewska, & Gelbard, 2010; Marker et al., 2013)). Tat or saline was injected at a flow rate of 80 nl/min controlled by a micro-syringe pump (World Precision Instruments; Micro4). The 35 gauge needles and 10µl NanoFil syringes (World Precision Instruments) were pre-coated with Sigmacote (MilliporeSigma; SL-2) to prevent the Tat from sticking to the inside of the syringe. For CX₃CR1-GFP chimera experiments, a 3µg/µl solution of HIV-1 Tat_{1–72} (gift of Dr. Philip Ray (University of Kentucky), (Hollman et al., 2005)), dissolved in saline was used. For all other experiments a 1.5µg/µl solution of HIV-1 Tat_{1–101} was used. Mice were allowed to recover for 28 days prior to euthanization and IHC.

For 7 day recovery post injection experiments; mice were injected 1.0 mm posterior to bregma and bilaterally 1.5mm left and right of bregma with 2µl for each cortical hemisphere of 1.5µg/µl of HIV1-Tat_{1–101} dissolved in saline with 0.1% Evans blue. Seven days later, mice were perfused briefly with PBS and brains were quickly removed. A 3mm biopsy punch was centered over each injection site (visible due to the Evans blue dye) to collect a 3mm round piece of cortex. The two cortical biopsies from each mouse were pooled and immediately frozen on dry ice and stored at −80° C.

Western Blots

Cortical biopsies were homogenized with a pestle on ice in RIPA lysis buffer containing a protease inhibitor cocktail (MilliporeSigma: Calbiochem, SetV). The cell lysate was kept on ice with periodic vortexing for 30 minutes then centrifuged at 13,000 rpm for 10 minutes. The supernatant was centrifuged again at 13,000 rpm for 10 minutes. The protein concentration of the final supernatant lysate was assayed with a detergent-compatible Bradford assay (Pierce). Equivalent amounts of protein between samples were then mixed with loading dye and run on an 8 or 12% SDS-PAGE gel and transferred to PVDF. Membranes were blocked with 5% milk in TBSt for 30 minutes and probed with primary antibodies (either PSD95 (NeuroMAB, 75–028), vGlut1 (Cell Signaling Technology, 12331S), vGAT (Synaptic Systems, 131004), Synapsin (Cell Signaling Technology, 5297S), Synaptophysin (MilliporeSigma, MAB5258-I), C1q (gift of Andrea Tenner, 1151, (Huang et al., 1999)), C3 (MP Biomedicals, 55730), C3d (R&D systems, AF2655) actin (Santa Cruz, SC-47778), IBA1 (Wako Biochemicals, 019–19741), IL-10 (AbD Serotec MCA1302G),

Arg1 (Santa Cruz, SC-18354), IL-1 β (Genzyme, 1886–01) in 3% BSA in TBSt at 4 degrees overnight. Membranes were washed 3 times in TBSt and then incubated with HRP secondary antibodies for 1 hour in 5% milk in TBSt. After washing we applied ECL substrate (Pierce) and developed membranes on film or using a digital imager (Azure Biosystems). Membranes were stripped using buffer consisting of 200mM glycine; 0.1% SDS, 1% Tween 20, pH 2.2 and re-probed up to 5 times.

IHC

Animals were transcardially perfused with PBS then 4% PFA in PBS. Brains were post-fixed for 18–24 hours in 4% PFA then stored in PBS at 4°C. Brains were cut into 40 μ m-thick sections using a vibratome (Leica V1000) and stored in a cryoprotectant mixture of 30% PEG300, 30% glycerol, 20% 0.1 M phosphate buffer, and 20% ddH₂O at –20°C. We performed free-floating-section IHC. The sections were washed three times for 30 min in PBS to remove the cryoprotectant. Then sections were incubated in 100mM glycine in PBS for 30 minutes followed by citrate antigen unmasking at 37°C for 30 minutes (Vector, H3300 with 0.05% tween 20). Primary antibodies were diluted in blocking buffer consisting of 1.5% BSA (MilliporeSigma; A3294), 3% normal goat serum (Vector Laboratories; NGS; S1000) or 3% normal donkey serum (MilliporeSigma, 566460), 0.5% Triton X-100 (Promega, H5142), and 1.8% NaCl in 1 $\times$ PBS. We used the following antibodies in these experiments: rabbit anti-Iba1 at 1:1000 (Wako Biochemicals, 019–19741) rabbit anti-C1q (Abcam Ab182451 clone 4.8, KO verified in (Stephan et al., 2013)), goat anti-C3 (MP Biomedicals 55730, KO verified in (Schafer et al., 2012)), and guinea pig anti-piccolo (Synaptic Systems, 142104). Sections were incubated in the primary antibody mixture for 2–3 days at room temperature with agitation. Sections were then washed three times for 30 min in 1 $\times$ PBS with 1.8% NaCl and then incubated overnight at room temperature in Alexa Fluor-conjugated secondary antibodies (1:500; Invitrogen or 1:250 Jackson Immuno Research) in blocking buffer. Finally, sections were washed 3 times with 1 $\times$ PBS with 1.8% NaCl, mounted on slides with Prolong Gold or Diamond mounting agents with Dapi (Life Technologies; P36935 and P36961). For sections that received co-staining against C1q and GFP, we first performed primary and secondary staining against C1q (and C3) as described above. Sections were then incubated overnight with rabbit anti-GFP Alexa Fluor 488-conjugated antibodies (1:1000; Life Technologies, A11122) in blocking buffer consisting of 1.5% BSA, 3% normal donkey serum, 3% normal rabbit serum (Vector Laboratories, S5000), 0.5% Triton X-100, and 1.8% NaCl in 1 $\times$ PBS. Sections were washed and mounted on slides.

Imaging

IHC sections were imaged with a Hamamatsu ORCA-ER camera on an Olympus BX-51 upright microscope with Quioptic Optigrid optical sectioning hardware. The acquisition was controlled with Volocity 3DM software (PerkinElmer Life and Analytical Sciences). Objectives: 4 $\times$, 0.13 NA; 10 $\times$, NA 0.4; 20 $\times$, NA 0.7; 60 $\times$ NA 1.4. Image analysis was done using Volocity. Differences in z-axis registration of different fluors were corrected by calibration with multicolor fluorescent beads. When using 4 $\times$ -20 $\times$ objectives, one image per mouse was taken at each location relative to the injection site using identical light intensity and exposure settings for all animals within each image set. For 60 $\times$ images, 3 image stacks

(10 μ m thick; z-step = 0.3 μ m) were collected at each location (at the injection site, 150 μ m lateral, and 300 μ m lateral). All image stacks are displayed as maximum intensity projections. IBA1+ cell morphology measurements (surface area and volume) were obtained using an intensity-based automated segmentation algorithm in Volocity (“find objects”) followed by a fine filter. Piccolo puncta were identified using the “find spots” algorithm in Volocity software, which identifies puncta based on local intensity maxima that also exceed an intensity threshold value. These two functions were combined to identify the piccolo puncta that co-localize with IBA1+ cells.

Results:

C1q and C3 deposit in Tat but not Saline injected brains

We and others have previously shown that mice receiving a single cortical injection of HIV-1 Tat protein display a long lasting (at least 28 day) neuroinflammation involving influx of peripheral monocytes and neutrophils, microglial activation, elevated cytokine and chemokine production, synapse loss, and increased numbers of microglial phagocytic inclusions (Lu et al., 2011; Marker et al., 2013; Puccini et al., 2015; Tremblay et al., 2013). To determine if Tat exposure also results in increased complement production and deposition that could make synapses vulnerable to phagocytosis by microglia or other immune cells, we performed IHC using anti-C1q and anti-C3 antibodies (Figure 1). We used CD45.1 mice with allografted CX₃CR1-GFP^{+/-} bone marrow as these chimeric CX₃CR1-GFP mice specifically highlight the peripheral monocyte cell population that infiltrates the brain in response to intraparenchymal injections of Tat. Tat-injected animals analyzed 28 days after injection, had a 2.4 fold increase in C1q immunoreactivity at the injection site compared to saline-injected controls (student's t test: $p < 0.001$; Figure 1 A–D). We also observed that C1q was elevated broadly across the Tat injected brains even at locations remote from the injection site where other signs of neuroinflammation were absent (Figure 1 A). Specifically, C1q immunoreactivity in the cortex 500 μ m–1mm away from the Tat-injection site was elevated 2.0 fold above controls ($p = 0.015$; Figure 1 A, D). This 500 μ m–1mm region had minimal to no peripheral monocyte infiltration unlike the Tat injection site where significant infiltration occurred. C3 immunoreactivity was also concentrated at the Tat injection site measuring 2.5 fold above controls ($p = 0.045$), with a 1.4 fold increase above controls found at 500 μ m away from the injection site. As C1q and C3 are secreted proteins, most of the signal from anti-C1q or anti-C3 antibodies was diffusely localized throughout the neuropil and not concentrated within specific cells (Figure 1C). Infiltrating peripheral monocytes sometimes co-localized with regions of higher C1q expression, suggesting that these cells either contribute to the production of C1q at the injection site and/or have phagocytosed significant amounts of C1q tagged debris. The few cells expressing higher C3 levels than the already elevated diffuse expression in the neuropil do not co-localize with infiltrating cells and may be damaged neurons or reactive astrocytes. In addition to the injection site, the corpus callosum was also an important site of C3 deposition and peripheral monocyte infiltration due to Tat exposure suggesting that white matter tracts may be especially vulnerable to Tat.

Tat exposure results in synapse loss and an increase in IBA1+ cells that are not prevented by knockout of C1q.

Due to the significant deposition of C1q and C3 in the cortex of Tat-injected mice, we investigated whether blockade of the classical complement cascade by knockout of C1qa would protect synapses or alter immune cell activation or infiltration during Tat-induced neuroinflammation. WT and C1qa knockout (KO) mice were injected with saline or HIV1 Tat and processed for IHC after 28 days using antibodies against IBA1 to label both microglia and infiltrating monocyte-derived macrophages and antibodies against the presynaptic protein, piccolo, to label synapses (Figure 2). Anti-C1q antibodies were also used to verify that C1qa KO animals expressed no C1q (Figure 2G).

Tat injection induced a 5–6-fold increase in the IBA1+ cell volume at the injection site in both WT and C1qa KO mice compare to saline injected controls (Figure 2A,B; IBA1 volume in WT-saline = 3199 $\pm$ 837; WT-Tat = 20,383 $\pm$ 3942; C1qa KO-Saline = 4049 $\pm$ 1028; C1qa KO-Tat = 21,396 $\pm$ 9327 μ m³). Two-way ANOVA tests showed there was no significant interaction between genotype and injection treatment on IBA1+ cell volume ($F < 0.001$; $p = 0.988$); but the Tat vs saline injection main effects on IBA1+ cell volume were highly significant ($F_{(1,22)} = 10.12$ $p = 0.004$). Significant Tat induced increases in IBA1+ cell volumes were also seen at locations 150 μ m and 300 μ m lateral to the injection site although to a lesser degree with each increase in distance. The Tat-induced increase in IBA1 volume reflects both an increase in IBA1+ cell number as well as a more reactive cell morphology in which IBA1 cells are hypertrophied or even amoeboid compared to the highly ramified microglia found in saline-injected controls. This reactive morphology was quantified using the surface area to volume ratio of IBA1+ cells. Tat-injected brains have a lower IBA1 surface area/volume ratio compared to saline injected brains indicative of a reactive cell morphology (Figure 2C). There was no significant difference in the IBA1+ cell surface area/volume ratio between WT and C1qa KO animals injected with Tat, although Tat-treated C1qa KO animals did trend marginally higher than WT. Thus, absence of C1q does not notably modify the inflammatory milieu of the brain in a manner that affects the phenotypic correlates of microglial activation and/or monocyte infiltration in response to Tat.

Using the presynaptic protein piccolo as a marker of synapses, we observed that Tat, but not saline injection, results in a loss of piccolo immunoreactivity specifically in regions with high IBA1+ cell volumes (Figure 2A,D). C1qa KO mice exhibit a similar loss of piccolo immunoreactivity in response to Tat as WT mice. Quantification and two-way ANOVA analysis (Figure 2D, E) of the number of piccolo puncta per field of view (FOV) at 60 $\times$ magnification shows that Tat causes a 30% reduction of synapses at the injection site in both WT (mean synapses/FOV = 11900 $\pm$ 1841) and C1qa KO mice (mean synapses/FOV = 12055 $\pm$ 1874) compared to saline injected controls (mean synapses/FOV for WT-Saline: 17501 $\pm$ 695; C1qa KO-Saline 16831 $\pm$ 1022). The Tat vs Saline injection main effects were: $F_{(1,22)} = 12.45$; $p = 0.002$. At 150 μ m from the injection site, Tat caused a 14% reduction of synapses in both WT and C1qa KO mice (Tat vs Saline injection main effect: $F_{(1,22)} = 6.058$; $p = 0.022$). No significant synapse loss occurred at 300 μ m from the injection site or more distant locations (Tat vs Saline injection main effect $F_{(1,22)} = 1.358$ $p = 0.256$). There was no

significant change in piccolo puncta numbers due to genotype nor any significant interaction between genotype and injection type at any location measured (Injection site: Genotype $F_{(1,22)}=0.031$, $p=0.863$; Interaction $F_{(1,22)}=0.079$, $p=0.781$. 150 μ m lateral: Genotype $F_{(1,22)}=0.046$, $p=0.832$ Interaction $F_{(1,22)}=0.003$, $p=0.955$. 300 μ m lateral: Genotype $F_{(1,22)}=0.262$, $p=0.614$; Interaction $F_{(1,22)}=0.072$, $p=0.791$). In summary, genetic deletion of C1qa was unable to protect synapses after Tat exposure. Despite the correlation between high levels of C1q and C3 and synapse loss at the injection site, the C1q initiated complement pathway is not a primary driver of synapse removal in this model system.

Along with the Tat-induced decrease in piccolo puncta in WT and C1qa KO mice, we observed that Tat exposure resulted in a 3-fold increase in large, bright piccolo aggregates with fluorescence values seven standard deviations above the mean (Figure 2 D, F; average piccolo aggregate volume/FOV for WT-saline = 47.3 $\pm$ 12.4; C1qa KO-Saline = 50.0 $\pm$ 10.2; WT-Tat = 153.0 $\pm$ 31.2; C1qa KO-Tat = 125 $\pm$ 23.2. Tat vs Saline two-way ANOVA main effects $F_{(1,22)}=19.11$, $p<0.001$). These aggregates could represent synaptic debris or simply the remaining synapses that acquire more piccolo as neighboring synapses from the same axon are lost. WT and C1qa KO mice had similarly increased volumes of these piccolo aggregates after Tat exposure at the injection site (ANOVA results indicate no variation attributed to genotype or interactions between genotype and injection treatment).

C1qa KO does not prevent loss of multiple synaptic markers 7 days after Tat injection

Next we used western blots of cortical lysates from control and Tat-injected WT and C1qa KO mice to determine if there were changes in synaptic protein levels or markers of immune cell activation (Figure 3). We collected 3mm cortical biopsies surrounding the injection site, 7 days after Tat injection from WT or C1qa KO mice. We observed that Tat-injected WT mice have an 10-fold increase in C1q expression compared to WT controls ($p=0.029$; Figure 3 A,B). As expected, C1qa KO mice do not express C1q protein in response to Tat. WT and C1qa KO mice injected with Tat, had a 20–30% reduction in synaptic protein levels compared to WT controls (Figure 3 C,D) of the excitatory, postsynaptic protein: PSD95 (WT-Control = 1 $\pm$ 0.01; WT-Tat = 0.75 $\pm$ 0.04, $p=0.002$; C1qa KO-Tat 0.74 $\pm$ 0.06, $p=0.003$), the presynaptic proteins vGlut1 (WT-Control 1 $\pm$ 0.06; WT-Tat 0.72 $\pm$ 0.02 $p=0.003$; C1qa KO-Tat 0.63 $\pm$ 0.03 $p=0.001$), synapsin (WT-Control 1 $\pm$ 0.05; WT-Tat 0.77 $\pm$ 0.07 $p=0.039$; C1qa KO-Tat 0.70 $\pm$ 0.05 $p=0.011$), and synaptophysin (WT-Control 1 $\pm$ 0.05; WT-Tat 0.90 $\pm$ 0.03 $p=0.215$; C1qa KO-Tat 0.80 $\pm$ 0.03 $p=0.013$). We also observed a similar 15% reduction that was shy of statistical significance in the levels of the presynaptic, inhibitory protein, vGAT. There was no significant difference in the levels of actin showing equivalent levels of protein were loaded for each sample. Importantly, there were no significant differences in the levels of synaptic proteins between Tat-injected WT and Tat-injected C1qa KO groups, again demonstrating that C1q is not necessary for synapse loss in response to Tat-induced neuroinflammation.

For markers of microglial activation, we measured relative expression of IBA1 and IL-1 β as well as at putative markers/stimulators of phagocytic, alternatively activated microglia, namely Arg1 and IL-10 respectively (Figure 3 E, F). In general, protein levels were elevated for IBA1 (2–3 fold), IL-1 β (9 fold), Arg1 (40–60 fold), and IL-10 (1.5 fold) in both WT and

C1qa KO Tat-injected animals compared to saline injected controls, although there was substantial animal-to-animal variability. Genetic deletion of C1qa did not protect against Tat-induced increases in IL-1 β or Arg1, but C1q KO mice had less IBA1 expression than WT (WT-Tat = 3.33 fold vs C1qaKO-Tat = 2.27 fold increase over control; $p=0.0576$ (Tukey's)).

Piccolo puncta and aggregates overlapping IBA1+ cells

Previous studies have suggested that C1q helps microglia engulf synaptic elements. Thus, we investigated whether there was a difference in the number of piccolo puncta co-localized with microglia in WT and C1qa KO mice injected with saline or Tat (Figure 4). We found the percent of piccolo puncta co-localizing with IBA1+ cells increased from 1.68 $\pm$ 0.4% in WT saline-injected controls to 13.3 $\pm$ 2.6% in WT Tat-injected animals (Figure 4C). C1qa KO mice showed the same trend with 2.4 $\pm$ 0.7% in saline injected controls and 12.3 $\pm$ 5.5% in Tat-injected animals. Two-way ANOVA shows there is no significant main effects of genotype on the co-localization of piccolo+ synapses with IBA1+ cells ($F_{(1,22)}=0.001$, $p=0.974$) and no significant interaction of genotype with injection treatment ($F_{(1,22)}=0.066$, $p=0.799$). Only the Tat vs saline main effect reported significant differences on co-localization ($F_{(1,22)}=11.06$ $p=0.003$). The percent of bright piccolo aggregates co-localizing with microglia/monocyte-derived macrophages increased from 3.9% in WT-saline controls to 28.2% in WT-Tat injected animals (Figure 4E). Again, C1qa KO animals showed the same trends (C1qa KO Saline = 6.7% C1qa KO-Tat = 27.1%) and there was no significant difference between WT and C1qa KO animals in the percent of bright piccolo aggregates co-localizing with IBA+ cells in response to Tat. It is important to note that when the number of piccolo puncta overlapping IBA1+ cells was normalized to the IBA1+ cell volume, the IBA1 co-localized piccolo puncta population was lower in Tat injected animals than Saline injected animals and was representative of the whole field of view (compare Figure 4D to Figure 2E). This suggests that individual IBA+ cells in Tat-injected animals do not possess more phagocytic activity or contact more piccolo+ synaptic elements than in control animals at this 28day time point, but rather the large increase in IBA+ cell volume we observed likely accounts for the increase in piccolo puncta overlapping microglia/monocyte-derived macrophages.

Both C1qa KO and WT animals have elevated C3 expression in response to TAT exposure, but C1qa KOs have significantly less C3 activation.

The complement cascade can be initiated through three separate pathways (the classical pathway that requires C1q, the alternative pathway, and the lectin pathway) that all converge with activation of a C3 convertase that cleaves C3 to create the chemokine, C3a, and the opsonin that aids phagocytosis, C3b. C3b is inactivated by further cleavage that creates the fragments iC3b, C3c, and C3d (see diagram in Figure 5C). We next investigated whether C1qa KO altered C3 expression or activation in response to Tat (Figure 5). Using IHC at 28 days after injection, Tat-treated WT mice had significantly more C3 at the injection site than saline injected animals (C3 Fluorescence Intensity: WT-Tat: 1519 $\pm$ 400; WT-Saline: 402 $\pm$ 77; $p<0.0178$). Tat-injected C1qa KO mice also had more C3 than saline-injected C1qa KO controls (C1qa KO-Tat 917 $\pm$ 275; C1qa KO-Saline 399 $\pm$ 43.6; $p=0.248$); but they also had less C3 than Tat-injected WT mice; (C1qa KO-Tat vs WT-Tat $p=0.248$). These latter

comparisons suggest that loss of C1q partially protects against C3 deposition; however, the data only show trends as they do not reach statistical significance. With IHC we cannot tell whether the detected C3 is full length C3 or whether it is a cleaved form of C3 downstream of activation. Therefore, we further analyzed C3 levels by western blot using samples collected at 7 days post injection. Tat injection results in elevated levels of full length C3 (α chain) in both WT and C1qa KO mice compared to WT saline-injected controls (Figure 5 D, E (WT-Tat=1.9 fold above control; $p=0.001$; C1qa KO-Tat= 2.4 fold above control; $p<0.001$). Using an antibody to the C3d region (which also detects full length C3 and the α' 1 fragment of iC3b), we found that WT mice injected with Tat rather than saline have a significant increase in C3 cleavage products representing chronic complement pathway activation (α' 1 = 13.2 fold increase $p=0.018$; C3d = 11.1 fold increase $p=0.01$ (Tukey's)). While C1qKO mice injected with Tat also had some elevated levels of the α' 1 and C3d cleavage products, the increase was no longer significant from saline-injected controls (α' 1=5.83 fold increase $p=0.40$ C3d=5.8 fold increase $p=0.224$) and trended less than Tat-injected WT mice (α' 1 $p=0.14$; C3d $p=0.17$). The decreased C3 activation products in the Tat-injected C1qaKO mice compared to WT mice indicates that the C1q dependent classical pathway is partially responsible for activating C3 downstream of Tat. As there is still some C3 cleavage occurring in C1qa KO mice, the alternative and/or lectin pathways remain active. Taken together, we conclude that Tat injection results in increased C3 expression, but that knockout of C1qa partially prevents C3 activation. This lower level of activation at 1 week post injection in C1qa KO animals could contribute to the decreased C3 fluorescence we observed at 28 days by IHC.

Discussion:

Our previous research studies designed to investigate mechanisms responsible for HAND pathology using the simplified Tat-injection mouse model, allowed us to determine that microglia/infiltrating monocyte-derived macrophages were responsible for clearing synapses or synaptic debris via phagocytosis (Marker et al., 2013; Tremblay et al., 2013). As complement has been widely implicated in synaptic pruning in development and multiple neuroinflammatory diseases, we investigated whether complement activation was also involved in HIV1 Tat-induced synapse loss.

We report here the complement proteins C1q and C3 were both highly elevated at the Tat injection site where synapse loss and infiltrating peripheral monocytes were maximal. Elevated C1q protein levels were also found at locations more distant from the injection site where synapse numbers were unaffected. Complement proteins are known to be up-regulated by many inflammatory signals. Expression of the C1q genes is controlled by the transcription factor MafB, as well as, by IRF8, PU.1, and multiple nuclear receptors, such as PPAR δ , RXR α , RAR, and LXR α (G. Chen, Tan, Teh, & Lu, 2011; Mukundan et al., 2009; Tran et al., 2017). C3 has been shown to be induced in astrocytes in vitro by HIV Tat downstream of NF- κ B and IL-6 (Nitkiewicz et al., 2017). We found at the Tat injection site, C1q and C3 were both diffusely located throughout the neuropil.

By comparing synapse loss in response to Tat exposure in WT and C1qa KO animals we found that Tat-induced synapse loss occurred independent of C1q and was largely restricted

to the central injection site and adjacent regions up to 150 μ m away. Specifically, we found that the levels of multiple synaptic proteins were decreased after Tat exposure in both WT and C1qa KO mice, and in particular, the number of piccolo-positive synaptic puncta was significantly decreased after Tat injection independent of genotype (WT vs C1qa KO). The percent of piccolo puncta or aggregates co-localizing with microglia or monocyte-derived macrophages, representing both engulfed and contacted synapses, were increased by Tat exposure, but again independent of genotype (WT vs C1qa KO). Thus, synapse loss can occur during Tat-induced inflammation in the absence of C1q, and elevated C1q expression is not directly linked to synapse elimination. These results caution against concluding that when elevated C1q and decreased synaptic density are correlated in human pathology that the relationship is always causative.

Tat injection results in persistent neuroinflammation that involves not only activation of microglia, but also infiltration of peripheral immune cells (Lu et al., 2011; Marker et al., 2013). Complement can have multiple effects on inflammatory pathways. In addition to promoting phagocytosis, complement activation results in the production of anaphylatoxins, C3a and C5a, that cause chemotaxis of immune cells and glia, promote pro-inflammatory polarization and cytokine production, and stimulate oxidative bursts (reviewed (Schraufstatter, Khaldoyanidi, & DiScipio, 2015; Thielens, Tedesco, Bohlson, Gaboriaud, & Tenner, 2017). C1q also has immunoregulatory properties that suppress pro-inflammatory cytokine production and limit inflammation when clearing apoptotic cells and debris, but how this anti-inflammatory effect is received by immune cells may depend on their differentiation state and the surrounding inflammatory milieu (Colonna, Parry, Panicker, & Elkon, 2016; Fraser, Laust, Nelson, & Tenner, 2009).

Our results suggest that C1q does not significantly contribute to the Tat-induced inflammatory milieu in either a positive or negative manner; although, C1q does contribute to complement activation downstream of Tat. C1qa KO did not affect the recruitment of IBA1+ peripheral monocytes/microglia to the Tat injection site, nor did genetic deletion of C1qa significantly alter the morphology of IBA1+ cells as measured by their surface area/volume ratio. Western blot results showed that expression levels of inflammatory markers and cytokines were highly variable in response to Tat, but generally increased in both WT and C1qa KO animals. We did however see that Tat-injected C1qa KO animals had less IBA1 protein expression than Tat-injected WT animals. Although C3 levels were still elevated in C1qa KO animals in response to TAT, we observed less C3 activation. Tat-injected C1qa KO mice had less deposition of C3 cleavage products (α' 1 and C3d) than Tat-injected WT mice. Thus, C1q instigates activation of the classical complement cascade downstream of Tat. Yet this complement activity is not necessary for Tat-induced synapse loss nor microglia/monocyte recruitment and activation.

The 28 day post Tat injection time point was chosen for IHC experiments as it evaluates a state of chronic neuroinflammation rather than an acute response. We acknowledge that many signaling events have occurred with intra- and extracellular consequences in the four weeks following Tat injection. Specifically, we know inflammation begins as early as 12 hours post-Tat injection, synapse loss can be seen at 24 hours, and a significant increase in microglial phagocytic inclusions has been observed at 1 day and 7 days post Tat injection

(Lu et al., 2011; Marker et al., 2013; Tremblay et al., 2013). Thus, it is possible that C1q is involved in synapse removal or clearance of cellular debris at the injection site early on, but that other “eat me” signals also exist that can fully compensate for the loss of C1q. The 7 day time point used for western blots was aimed at assessing synapse loss at an earlier time point, but it also leaves time for compensatory mechanisms to compensate for the loss of C1q. Nevertheless, by analyzing the later time points of 7 days and 28 days, we can conclude that loss of C1q is not sufficient to rescue synapses and that targeting C1q as a synaptic protection strategy would not work in this case.

As C1q, as well as other members of the early complement cascade namely C3, C4, C3aR, and CR3 have been repeatedly shown in development and different disease models of mediating synapse loss by phagocytic microglia (Hong et al., 2016; Lui et al., 2016; Norris et al., 2018; Shi et al., 2015; Vasek et al., 2016), it is curious that C1q is not required for even a portion of the significant synapse elimination that occurs in the Tat injection model of HAND. Yet genetic loss of C1q has also failed to protect against disease progression and reduced synaptic density in the mutant SOD1 mouse model of ALS (Lobsiger et al., 2013). At this point, we cannot completely rule out a role for C3, the central hub of the complement cascade, or other complement proteins in Tat-induced synapse loss. Indeed, we did see that C3 levels were elevated by Tat at the injection site. However, as C1qa KO did partially limit C3 activation (measured as a decrease in C3d and $\alpha'1$), but did not protect synapses, it seems unlikely that C3 is a primary driver for Tat-induced synapse loss via the lectin or alternative complement pathways. Other factors that may explain why C1q plays no role in this neuroinflammatory model, as opposed to other diseases with a neuroinflammatory component, include the significant infiltration of peripheral immune cells, differences in synaptic vulnerability in affected brain regions, possible redundant eat-me signals, and the direct neurotoxicity of Tat, which has been linked to NMDAR activation, Ca^{2+} influx, mitochondrial dysfunction, and activation of the ubiquitin–proteasome pathway (reviewed in (Ru & Tang, 2017)).

Going forward, it is important that we better understand the mechanisms by which HIV1 viral proteins such as Tat, that are present in the CNS of HIV+ patients even with virally suppressive cART, contribute to inflammation, dysregulated neural circuits, and synapse loss so that we can successfully design adjunctive therapies to protect the brain. Ruling out mechanisms such as C1q-dependent, complement mediated elimination of synapses is an important part of this process that goes hand in hand with identifying the true molecular drivers of disease.

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References:

- Ajami B, Bennett JL, Krieger C, Tetzlaff W, & Rossi FM (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci*, 10(12), 1538–1543. doi: 10.1038/nn2014 [PubMed: 18026097]
- Aksenov MY, Hasselrot U, Wu G, Nath A, Anderson C, Mactutus CF, & Booze RM (2003). Temporal relationships between HIV-1 Tat-induced neuronal degeneration, OX-42 immunoreactivity, reactive astrogliosis, and protein oxidation in the rat striatum. *Brain Res*, 987(1), 1–9. [PubMed: 14499939]
- An SF, Giometto B, Scaravilli T, Tavalato B, Gray F, & Scaravilli F (1996). Programmed cell death in brains of HIV-1-positive AIDS and pre-AIDS patients. *Acta Neuropathol*, 91(2), 169–173. [PubMed: 8787150]
- Bansal AK, Mactutus CF, Nath A, Maragos W, Hauser KF, & Booze RM (2000). Neurotoxicity of HIV-1 proteins gp120 and Tat in the rat striatum. *Brain Res*, 879(1–2), 42–49. [PubMed: 11011004]
- Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, ... Walport MJ (1998). Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat Genet*, 19(1), 56–59. doi: 10.1038/ng0598-56 [PubMed: 9590289]
- Bruce-Keller AJ, Turchan-Cholewo J, Smart EJ, Geurin T, Chauhan A, Reid R, ... Hauser KF (2008). Morphine causes rapid increases in glial activation and neuronal injury in the striatum of inducible HIV-1 Tat transgenic mice. *Glia*, 56(13), 1414–1427. doi: 10.1002/glia.20708 [PubMed: 18551626]
- Buscemi L, Ramonet D, & Geiger JD (2007). Human immunodeficiency virus type-1 protein Tat induces tumor necrosis factor- α -mediated neurotoxicity. *Neurobiol Dis*, 26(3), 661–670. doi: 10.1016/j.nbd.2007.03.004 [PubMed: 17451964]
- Carey AN, Liu X, Mintzopoulos D, Paris JJ, Muschamp JW, McLaughlin JP, & Kaufman MJ (2013). Conditional Tat protein expression in the GT-tg bigenic mouse brain induces gray matter density reductions. *Prog Neuropsychopharmacol Biol Psychiatry*, 43, 49–54. doi: 10.1016/j.pnpbp.2012.12.018 [PubMed: 23269344]
- Chen G, Tan CS, Teh BK, & Lu J (2011). Molecular mechanisms for synchronized transcription of three complement C1q subunit genes in dendritic cells and macrophages. *J Biol Chem*, 286(40), 34941–34950. doi: 10.1074/jbc.M111.286427 [PubMed: 21862594]
- Chen NC, Partridge AT, Sell C, Torres C, & Martin-Garcia J (2017). Fate of microglia during HIV-1 infection: From activation to senescence? *Glia*, 65(3), 431–446. doi: 10.1002/glia.23081 [PubMed: 27888531]
- Chivero ET, Guo ML, Periyasamy P, Liao K, Callen SE, & Buch S (2017). HIV-1 Tat Primes and Activates Microglial NLRP3 Inflammasome-Mediated Neuroinflammation. *J Neurosci*, 37(13), 3599–3609. doi: 10.1523/JNEUROSCI.3045-16.2017 [PubMed: 28270571]
- Colonna L, Parry GC, Panicker S, & Elkon KB (2016). Uncoupling complement C1s activation from C1q binding in apoptotic cell phagocytosis and immunosuppressive capacity. *Clin Immunol*, 163, 84–90. doi: 10.1016/j.clim.2015.12.017 [PubMed: 26769276]
- Dickens AM, Yoo SW, Chin AC, Xu J, Johnson TP, Trout AL, ... Haughey NJ (2017). Chronic low-level expression of HIV-1 Tat promotes a neurodegenerative phenotype with aging. *Sci Rep*, 7(1), 7748. doi: 10.1038/s41598-017-07570-5 [PubMed: 28798382]
- Everall IP, Heaton RK, Marcotte TD, Ellis RJ, McCutchan JA, Atkinson JH, ... Masliah E (1999). Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV Neurobehavioral Research Center. *Brain Pathol*, 9(2), 209–217. [PubMed: 10219738]
- Fatima M, Prajapati B, Saleem K, Kumari R, Mohindar Singh Singal C, & Seth P (2017). Novel insights into role of miR-320a-VDAC1 axis in astrocyte-mediated neuronal damage in neuroAIDS. *Glia*, 65(2), 250–263. doi: 10.1002/glia.23089 [PubMed: 27761954]
- Fitting S, Ignatowska-Jankowska BM, Bull C, Skoff RP, Lichtman AH, Wise LE, ... Hauser KF (2013). Synaptic dysfunction in the hippocampus accompanies learning and memory deficits in human immunodeficiency virus type-1 Tat transgenic mice. *Biol Psychiatry*, 73(5), 443–453. doi: 10.1016/j.biopsych.2012.09.026 [PubMed: 23218253]
- Fraser DA, Laust AK, Nelson EL, & Tenner AJ (2009). C1q differentially modulates phagocytosis and cytokine responses during ingestion of apoptotic cells by human monocytes, macrophages, and

- dendritic cells. *J Immunol*, 183(10), 6175–6185. doi: 10.4049/jimmunol.0902232 [PubMed: 19864605]
- Gelman BB, Chen T, Lisinicchia JG, Soukup VM, Carmical JR, Starkey JM, ... National Neuro, A. T. C. (2012). The National NeuroAIDS Tissue Consortium brain gene array: two types of HIV-associated neurocognitive impairment. *PLoS One*, 7(9), e46178. doi: 10.1371/journal.pone.0046178 [PubMed: 23049970]
- Hollman AM, Christian DA, Ray PD, Galey D, Turchan J, Nath A, & Bhattacharyya D (2005). Selective isolation and purification of tat protein via affinity membrane separation. *Biotechnol Prog*, 21(2), 451–459. doi: 10.1021/bp049804z [PubMed: 15801785]
- Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, ... Stevens B (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science*, 352(6286), 712–716. doi: 10.1126/science.aad8373 [PubMed: 27033548]
- Huang J, Kim LJ, Mealey R, Marsh HC, Jr., Zhang Y, Tenner AJ, ... Pinsky DJ (1999). Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein. *Science*, 285(5427), 595–599. [PubMed: 10417391]
- Jadhav VS, Krause KH, & Singh SK (2014). HIV-1 Tat C modulates NOX2 and NOX4 expressions through miR-17 in a human microglial cell line. *J Neurochem*, 131(6), 803–815. doi: 10.1111/jnc.12933 [PubMed: 25146963]
- Jones G, & Power C (2006). Regulation of neural cell survival by HIV-1 infection. *Neurobiol Dis*, 21(1), 1–17. doi: 10.1016/j.nbd.2005.07.018 [PubMed: 16298136]
- Jongen PJ, Doesburg WH, Ibrahim-Stappers JL, Lemmens WA, Hommes OR, & Lamers KJ (2000). Cerebrospinal fluid C3 and C4 indexes in immunological disorders of the central nervous system. *Acta Neurol Scand*, 101(2), 116–121. [PubMed: 10685859]
- Leibrand CR, Paris JJ, Ghandour MS, Knapp PE, Kim WK, Hauser KF, & McRae M (2017). HIV-1 Tat disrupts blood-brain barrier integrity and increases phagocytic perivascular macrophages and microglia in the dorsal striatum of transgenic mice. *Neurosci Lett*, 640, 136–143. doi: 10.1016/j.neulet.2016.12.073 [PubMed: 28057474]
- Lobsiger CS, Boillee S, Pozniak C, Khan AM, McAlonis-Downes M, Lewcock JW, & Cleveland DW (2013). C1q induction and global complement pathway activation do not contribute to ALS toxicity in mutant SOD1 mice. *Proc Natl Acad Sci U S A*, 110(46), E4385–4392. doi: 10.1073/pnas.1318309110 [PubMed: 24170856]
- Lu SM, Tremblay ME, King IL, Qi J, Reynolds HM, Marker DF, ... Gelbard HA (2011). HIV-1 Tat-induced microgliosis and synaptic damage via interactions between peripheral and central myeloid cells. *PLoS One*, 6(9), e23915. doi: 10.1371/journal.pone.0023915 [PubMed: 21912650]
- Lui H, Zhang J, Makinson SR, Cahill MK, Kelley KW, Huang HY, ... Huang EJ (2016). Progranulin Deficiency Promotes Circuit-Specific Synaptic Pruning by Microglia via Complement Activation. *Cell*, 165(4), 921–935. doi: 10.1016/j.cell.2016.04.001 [PubMed: 27114033]
- Mahajan SD, Aalinkeel R, Parikh NU, Jacob A, Cwiklinski K, Sandhu P, ... Alexander JJ (2017). Immunomodulatory Role of Complement Proteins in the Neuropathology Associated with Opiate Abuse and HIV-1 Co-Morbidity. *Immunol Invest*, 46(8), 816–832. doi: 10.1080/08820139.2017.1371891 [PubMed: 29058550]
- Marker DF, Tremblay ME, Lu SM, Majewska AK, & Gelbard HA (2010). A thin-skull window technique for chronic two-photon in vivo imaging of murine microglia in models of neuroinflammation. *J Vis Exp* (43). doi: 10.3791/2059
- Marker DF, Tremblay ME, Puccini JM, Barbieri J, Gantz Marker MA, Loweth CJ, ... Gelbard HA (2013). The new small-molecule mixed-lineage kinase 3 inhibitor URM-099 is neuroprotective and anti-inflammatory in models of human immunodeficiency virus-associated neurocognitive disorders. *J Neurosci*, 33(24), 9998–10010. doi: 10.1523/JNEUROSCI.0598-13.2013 [PubMed: 23761895]
- Marks WD, Paris JJ, Schier CJ, Denton MD, Fitting S, McQuiston AR, ... Hauser KF (2016). HIV-1 Tat causes cognitive deficits and selective loss of parvalbumin, somatostatin, and neuronal nitric oxide synthase expressing hippocampal CA1 interneuron subpopulations. *J Neurovirol*, 22(6), 747–762. doi: 10.1007/s13365-016-0447-2 [PubMed: 27178324]

- McGuire JL, Gill AJ, Douglas SD, Kolson DL, & Group, C. H. A. T. E. R. (2016). The complement system, neuronal injury, and cognitive function in horizontally-acquired HIV-infected youth. *J Neurovirol*. doi: 10.1007/s13365-016-0460-5
- Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, ... Prinz M (2007). Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nat Neurosci*, 10(12), 1544–1553. doi: 10.1038/nn2015 [PubMed: 18026096]
- Mukundan L, Odegaard JI, Morel CR, Heredia JE, Mwangi JW, Ricardo-Gonzalez RR, ... Chawla A (2009). PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance. *Nat Med*, 15(11), 1266–1272. doi: 10.1038/nm.2048 [PubMed: 19838202]
- Nitkiewicz J, Borjabad A, Morgello S, Murray J, Chao W, Emdad L, ... Volsky DJ (2017). HIV induces expression of complement component C3 in astrocytes by NF-kappaB-dependent activation of interleukin-6 synthesis. *J Neuroinflammation*, 14(1), 23. doi: 10.1186/s12974-017-0794-9 [PubMed: 28122624]
- Norris GT, Smirnov I, Filiano AJ, Shadowen HM, Cody KR, Thompson JA, ... Kipnis J (2018). Neuronal integrity and complement control synaptic material clearance by microglia after CNS injury. *J Exp Med*, 215(7), 1789–1801. doi: 10.1084/jem.20172244 [PubMed: 29941548]
- Paris JJ, Zou S, Hahn YK, Knapp PE, & Hauser KF (2016). 5alpha-reduced progestogens ameliorate mood-related behavioral pathology, neurotoxicity, and microgliosis associated with exposure to HIV-1 Tat. *Brain Behav Immun*, 55, 202–214. doi: 10.1016/j.bbi.2016.01.007 [PubMed: 26774528]
- Pu H, Tian J, Flora G, Lee YW, Nath A, Hennig B, & Toborek M (2003). HIV-1 Tat protein upregulates inflammatory mediators and induces monocyte invasion into the brain. *Mol Cell Neurosci*, 24(1), 224–237. [PubMed: 14550782]
- Puccini JM, Marker DF, Fitzgerald T, Barbieri J, Kim CS, Miller-Rhodes P, ... Gelbard HA (2015). Leucine-rich repeat kinase 2 modulates neuroinflammation and neurotoxicity in models of human immunodeficiency virus 1-associated neurocognitive disorders. *J Neurosci*, 35(13), 5271–5283. doi: 10.1523/JNEUROSCI.0650-14.2015 [PubMed: 25834052]
- Raybuck JD, Hargus NJ, & Thayer SA (2017). A GluN2B-Selective NMDAR Antagonist Reverses Synapse Loss and Cognitive Impairment Produced by the HIV-1 Protein Tat. *J Neurosci*, 37(33), 7837–7847. doi: 10.1523/JNEUROSCI.0226-17.2017 [PubMed: 28716964]
- Reboul J, Schuller E, Pialoux G, Rey MA, Lebon P, Allinquant B, & Brun-Vezinet F (1989). Immunoglobulins and complement components in 37 patients infected by HIV-1 virus: comparison of general (systemic) and intrathecal immunity. *J Neurol Sci*, 89(2–3), 243–252. [PubMed: 2926452]
- Rozek W, Ricardo-Dukelow M, Holloway S, Gendelman HE, Wojna V, Melendez LM, & Ciborowski P (2007). Cerebrospinal fluid proteomic profiling of HIV-1-infected patients with cognitive impairment. *J Proteome Res*, 6(11), 4189–4199. doi: 10.1021/pr070220c [PubMed: 17929958]
- Ru W, & Tang SJ (2017). HIV-associated synaptic degeneration. *Mol Brain*, 10(1), 40. doi: 10.1186/s13041-017-0321-z [PubMed: 28851400]
- Sa MJ, Madeira MD, Ruela C, Volk B, Mota-Miranda A, & Paula-Barbosa MM (2004). Dendritic changes in the hippocampal formation of AIDS patients: a quantitative Golgi study. *Acta Neuropathol*, 107(2), 97–110. doi: 10.1007/s00401-003-0781-3 [PubMed: 14605830]
- Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, ... Stevens B (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron*, 74(4), 691–705. doi: 10.1016/j.neuron.2012.03.026 [PubMed: 22632727]
- Schafer DP, & Stevens B (2010). Synapse elimination during development and disease: immune molecules take centre stage. *Biochem Soc Trans*, 38(2), 476–481. doi: 10.1042/BST0380476 [PubMed: 20298206]
- Schier CJ, Marks WD, Paris JJ, Barbour AJ, McLane VD, Maragos WF, ... Hauser KF (2017). Selective Vulnerability of Striatal D2 versus D1 Dopamine Receptor-Expressing Medium Spiny Neurons in HIV-1 Tat Transgenic Male Mice. *J Neurosci*, 37(23), 5758–5769. doi: 10.1523/JNEUROSCI.0622-17.2017 [PubMed: 28473642]

- Schraufstatter IU, Khaldoyanidi SK, & DiScipio RG (2015). Complement activation in the context of stem cells and tissue repair. *World J Stem Cells*, 7(8), 1090–1108. doi: 10.4252/wjsc.v7.i8.1090 [PubMed: 26435769]
- Sekar A, Bialas AR, de Rivera H, Davis A, Hammond TR, Kamitaki N, ... McCarroll SA (2016). Schizophrenia risk from complex variation of complement component 4. *Nature*, 530(7589), 177–183. doi: 10.1038/nature16549 [PubMed: 26814963]
- Shi Q, Colodner KJ, Matousek SB, Merry K, Hong S, Kenison JE, ... Lemere CA (2015). Complement C3-Deficient Mice Fail to Display Age-Related Hippocampal Decline. *J Neurosci*, 35(38), 13029–13042. doi: 10.1523/JNEUROSCI.1698-15.2015 [PubMed: 26400934]
- Spudis S (2013). HIV and neurocognitive dysfunction. *Curr HIV/AIDS Rep*, 10(3), 235–243. doi: 10.1007/s11904-013-0171-y [PubMed: 23860944]
- Stephan AH, Madison DV, Mateos JM, Fraser DA, Lovelett EA, Coutellier L, ... Barres BA (2013). A dramatic increase of C1q protein in the CNS during normal aging. *J Neurosci*, 33(33), 13460–13474. doi: 10.1523/JNEUROSCI.1333-13.2013 [PubMed: 23946404]
- Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, ... Barres BA (2007). The classical complement cascade mediates CNS synapse elimination. *Cell*, 131(6), 1164–1178. doi: 10.1016/j.cell.2007.10.036 [PubMed: 18083105]
- Thielens NM, Tedesco F, Bohlson SS, Gaboriaud C, & Tenner AJ (2017). C1q: A fresh look upon an old molecule. *Mol Immunol*, 89, 73–83. doi: 10.1016/j.molimm.2017.05.025 [PubMed: 28601358]
- Tran MTN, Hamada M, Jeon H, Shiraishi R, Asano K, Hattori M, ... Takahashi S (2017). MafB is a critical regulator of complement component C1q. *Nat Commun*, 8(1), 1700. doi: 10.1038/s41467-017-01711-0 [PubMed: 29167450]
- Tremblay ME, Marker DF, Puccini JM, Muly EC, Lu SM, & Gelbard HA (2013). Ultrastructure of microglia-synapse interactions in the HIV-1 Tat-injected murine central nervous system. *Commun Integr Biol*, 6(6), e27670. doi: 10.4161/cib.27670 [PubMed: 24563721]
- Vasek MJ, Garber C, Dorsey D, Durrant DM, Bollman B, Soung A, ... Klein RS (2016). A complement-microglial axis drives synapse loss during virus-induced memory impairment. *Nature*, 534(7608), 538–543. doi: 10.1038/nature18283 [PubMed: 27337340]
- Williams R, Yao H, Peng F, Yang Y, Bethel-Brown C, & Buch S (2010). Cooperative induction of CXCL10 involves NADPH oxidase: Implications for HIV dementia. *Glia*, 58(5), 611–621. doi: 10.1002/glia.20949 [PubMed: 19941336]
- Youn GS, Ju SM, Choi SY, & Park J (2015). HDAC6 mediates HIV-1 tat-induced proinflammatory responses by regulating MAPK-NF-kappaB/AP-1 pathways in astrocytes. *Glia*, 63(11), 1953–1965. doi: 10.1002/glia.22865 [PubMed: 26031809]

Figure 1:

Complement deposition occurs in response to cortical HIV-1 Tat injection. In order to visualize peripheral monocyte infiltration into the brain in response to HIV-1 Tat, bone marrow aspirates from CX3CR1-GFP^{+/±} mice were transplanted into CD45.1 mice irradiated to achieve complete myelosuppression. After 4 weeks of recovery, chimeric animals were stereotactically injected in the cortex with Tat or saline and mice were processed for IHC 28 days later. A) Serial brain sections surrounding a Saline (top) or Tat (bottom) injection site immuno-stained for C1q (turquoise/blue), C3 (red), or GFP (green). 4× magnification. B) Increased magnification (10×) of injection sites highlighted by white boxes in Panel A. Scale bars = 60μm. C) C1q and C3 are secreted proteins and generally have a diffuse staining pattern near the injection site (60× magnification; scale bars = 10μm). A few cells in the field of view have intense C3 labeling (white arrowheads), but these cells are not infiltrating, CX3CR1-GFP positive cells. CX3CR1-GFP^{+/±} cells do occasionally have more intense C1q (yellow arrowheads). D) Quantification of C1q, C3, and infiltrating CX3CR1-GFP⁺ cells fluorescence intensity at the injection site or 500μm lateral from the injection site normalized to saline injected controls. Statistical analysis: Student's t-test; n=5–7 animals per group; p<0.1=*, p<0.05=**.

Figure 2:

C1qa KO animals are not protected from HIV1-Tat induced synapse loss or microgliosis and monocyte cell infiltration. WT and C1qa KO animals were injected with saline or HIV1-Tat. After 28 days, brains were processed for IHC using antibodies against piccolo and IBA1 to label presynaptic terminals and microglia/monocytes, respectively. A) Images at 20× magnification show microgliosis/monocyte infiltration and loss of piccolo staining at the injection site. Scale = 32μm. Boxes indicate regions shown at higher magnification in Panel D. Solid line boxes = Injection site; Dashed line boxes= 150μm lateral. B) Quantification of the IBA1+ cell volume per 60× FOV at the injection site, 150μm lateral, or 300μm lateral. C) Quantification of the surface area to volume ratio of IBA1+ cells at the injection site, 150μm lateral, or 300μm lateral. D) Tat exposure results in significant loss of piccolo puncta at the injection site and a smaller loss of puncta at 150μm lateral from the injection site. 60× magnification. Scale bars = 2.7μm. E) Quantification of synapse number at the injection site, 150μm lateral, or 300μm lateral measured as the number of piccolo puncta per 60× FOV. F) Quantification of the volume occupied by piccolo aggregates per 60× FOV at the injection site, 150μm lateral, or 300μm lateral. These bright piccolo puncta were identified as having fluorescence values 7× the standard deviation. Statistical analysis for all graphs: Two-Way ANOVAs were performed at each location with n=6–7 animals per group using genotype and injection type as the independent variables. Significant main effects were found for the Tat vs Saline injection ($p < 0.05$ - grey #). There were no significant main effects due to genotype and no significant interactions between the injection type and genotype. The ANOVAs were followed by Holmes-Sidak multiple comparisons tests. $p < 0.05 = *$, $p < 0.01 = **$. G) IHC with antibodies to C1q on brain sections from Tat- or saline-injected mice show that no C1q is expressed in C1qa KO mice compared to WT mice. Images show cortex and hippocampus. 4× magnification; Scale bars = 60μm.

Figure 3:

Tat exposure leads to a decrease in multiple synaptic proteins and an increase in inflammatory markers in both WT and C1qa KO animals 7 days after HIV-Tat injection. A-F) Cortical biopsies surrounding the HIV1-Tat or control injection site of WT or C1qa KO animals were collected and lysates were probed for complement, synaptic, or inflammatory proteins. A) Representative western blots for C1q and GAPDH loading control. B) Quantification of C1q blot shown in Panel A with band densities normalized to WT controls (n=2). C) Representative western blots of synaptic proteins: PSD95 (postsynaptic), vGlut1 (excitatory, presynaptic), vGAT (inhibitory, presynaptic), Synapsin (presynaptic), and Synaptophysin (presynaptic) as well as the loading control protein, actin. D) Quantification of synaptic protein western blots (n = 4–6 combined from 2 independent experiments of 2–3 animals each (only 1 experiment shown in blots in Panel C). Band densities were normalized to WT-Control. E) Representative western blots of inflammatory markers: IBA1, Arg1,

IL-10, IL-1 β . F) Quantification of western blots in Panel E with band densities normalized to WT-Controls. n=4. Statistical Analysis for all graphs: One-way ANOVA for each protein followed by Tukey's multiple comparisons test. p<0.05=*, p<0.01**.

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Figure 4:

C1q is not required for microglia/monocytes to contact and/or engulf synapses in response to HIV1-Tat. Brains from WT or C1qa KO mice injected with Saline or HIV1-Tat were immunostained for IBA1 (red or grey) and Piccolo (green). A and B) Maximum intensity projections at the injection site (A) or 300µm lateral of the injection site (B). Images labeled “Piccolo contacting IBA” were generated by subtracting all piccolo fluorescence not co-localizing with IBA1. Green piccolo puncta are shown against grey IBA1. Scale= 5µm. C) Quantification of the % of piccolo puncta per field of view contacting IBA1+ cells. D) Quantification of the number of piccolo puncta co-localizing with IBA1 per µm³ of IBA1 volume. E) Quantification of the % of large piccolo aggregates per field of view contacting IBA1+ cells. Piccolo aggregates were defined as having fluorescence values 7× the standard deviation. Statistical analysis for all graphs: Two-Way ANOVAs were performed at each location with n = 6–7 animals per group using genotype and injection type as the independent variables. Significant main effects were found for the Tat vs Saline injection ($p < 0.05$ grey #). There were no significant main effects due to genotype and no significant interactions between the injection type and genotype. The ANOVAs were followed by Holmes-Sidak multiple comparisons tests. $p < 0.05 = *$, $p < 0.01 = **$.

Figure 5:

WT animals have elevated C3 activation in response to TAT exposure compared to mice with C1qa KO. Saline or HIV1-Tat was injected into the cortex of WT or C1qa-KO animals and the animals were processed for IHC 28 days later using anti-C3 antibodies. Images were taken at the injection site. Scale = 60 μ m. B) Quantification of C3 fluorescence intensity. Statistical Analysis: Two-Way ANOVA performed with independent variables: Injection treatment (Tat vs Saline; $F_{(1,22)}=11.56$; $p=0.003$ - grey #) and Genotype (WT vs C1qa KO; $F_{(1,22)}=1.58$; $p=0.222$). $n=6-7$ animals per group. No significant interaction between injection treatment and genotype was found ($F_{(1,22)}=1.55$; $p=0.226$). ANOVA was followed by Holm's-Sidak multiple comparison test ($p<0.05$ = *). C) Diagram of C3 cleavage products. C3 is composed of an α and β chain connected by multiple disulfide bonds. The α chain is cleaved at multiple points to activate and then de-activate C3. The C3d region of the α chain is highlighted with a striped dark grey box to indicate C3 peptides recognized by the C3d antibody. C3d fragment levels can be used to indicate the amount of chronic complement pathway activation. D and E) Cortical biopsies surrounding the HIV1-Tat or control injection site of WT or C1qa KO animals were collected 7 days post injection and lysates were probed by western blot with C3, C3d, or actin antibodies. D) Representative western blot for C3, C3d, and actin. E) Quantification of blots in Panel D with band densities normalized to WT controls. $N=4$. Statistical Analysis: One-way ANOVA followed by Tukey's multiple comparisons tests. $p<0.05$ =*, $p<0.01$ **.