IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Enteroviral Infection Leads to Transactive Response DNA-Binding Protein 43 Pathology *in Vivo*

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that primarily affects motor neurons in the cerebral cortex, brainstem, and spinal cord, leading to progressive paralysis and eventual death. Approximately 95% of all ALS cases are sporadic without known causes. Enteroviruses have been suspected to play a role in ALS because of their ability to target motor neurons and to cause muscle weakness and paralysis. *In vitro* enteroviral infection results in cytoplasmic aggregation and cleavage of transactive response DNA binding protein-43, a pathologic hallmark of ALS. However, whether enteroviral infection can induce ALS-like pathologies *in vivo* remains to be characterized. In this study, neonatal BALB/C mice were intracranially inoculated with either a recombinant coxsackievirus B3 expressing enhanced green fluorescent protein or mock-infected for 2, 5, 10, 30, and 90 days. Histologic and immunohistochemical analysis of brain tissues demonstrated sustained inflammation (microglia and astrogliosis) and lesions in multiple regions of the brain (hippocampus, cerebral cortex, striatum, olfactory bulb, and putamen) in parallel with virus detection as early as 2 days for up to 90 days after infection. Most notably, ALS-like pathologies, including cytoplasmic mislocalization of transactive response DNA binding protein-43, p62-, and ubiquitin-positive inclusions, were observed in the areas of infection. These data provide the first pathologic evidence to support a possible link between enteroviral infection and ALS. (*Am J Pathol* 2018; 188: 2853–2862; <https://doi.org/10.1016/j.ajpath.2018.08.013>)

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that primarily targets motor neurons of the central nervous system (CNS), but it may also result in secondary effects, such as muscle weakness and cognitive impairment.^{1–3} The prevalence of ALS in both Europe and North America is around three to five cases per 100,000 individuals each year, with the overall lifetime risk increasing with age and being higher in men than in women (1:350 versus 1:400).^{1,3} Currently, there is no cure for ALS. Two treatments approved by the Food and Drug Administration can only modestly delay disease progression and prolong the lifespan of the patients.⁴

ALS is typically grouped into two categories: familial and sporadic ALS. Familial ALS is responsible for

approximately 5% of all cases and is associated with mutations in >30 genes that encode proteins involved in various cellular functions, including vesicle trafficking, RNA processing, and axonal integrity.^{5,6} Sporadic ALS, however, accounts for the majority of ALS cases (approximately 95%)

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without clear cause. A gene–environment interaction model that involves both genetic mutations and environmental risk factors, such as viral exposure, physical activity, smoking, heavy metals, and pesticides and chemicals, has been proposed to contribute to the development and progression of sporadic ALS.⁷

Two widely studied viruses in the context of ALS are human endogenous retrovirus (HERV) and enterovirus (EV). Increased gene expression of HERV-K and reverse transcriptase activity have been found in the blood and brain samples of ALS patients.⁸ Moreover, transgenic mouse study reveals that expression of HERV-K in the neurons can lead to motor dysfunction, suggesting a role of HERV infection in ALS.⁹ However, despite great efforts for the discovery of EV genome in ALS samples, the clinical data are controversial,^{10–16} probably because of the differences in viral detection techniques and/or in the disease stage of sample collection, as well as the potential virus-triggered prion-like mechanism, in which an active viral infection may not be required for ALS progression.¹⁷

EVs are a family of positive, single-stranded RNA viruses, which include poliovirus, coxsackievirus, echovirus, and EV (eg, EV-A71 and EV-D68).¹⁷ EVs are known to infect the CNS and are responsible for various neurologic diseases, such as poliomyelitis, meningitis, encephalitis, and non-polio flaccid paralysis.^{18,19} In addition to acute infection, EVs can also cause a persistent infection within the CNS, which may be reactivated years later.^{20–22} Although a causal relationship between chronic EV infection and ALS development remains to be established, our previous research in cultured cells demonstrates that EV infection induces ALS-like transactive response DNA-binding protein-43 (TDP-43) pathology, that is, cytoplasmic mislocalization, aggregation, and cleavage of TDP-43,²³ indicating a possible involvement of EVs in ALS pathogenesis.

Here, we investigated the neuropathologic phenotype of a persistent CNS infection of coxsackievirus B3 (CVB3) in mice. Our results reveal that EV infection induces a similar ALS-like pathology *in vivo*.

Materials and Methods

Isolation and Production of Recombinant Coxsackievirus

Recombinant CVB3 expressing enhanced green fluorescent protein (eGFP-CVB3) was generated as described previously.²⁴ In brief, eGFP (Clontech, Palo Alto, CA) was inserted into pMKS1, a plasmid that includes a unique SfiI restriction enzyme site in the backbone of CVB3 clone pH3. The new pMKS1 was transfected into COS cells for 48 hours, and the cell culture was subjected to three cycles of freeze-thaw on dry ice to release viruses. After centrifugation, the supernatant was harvested and added to HeLa RW cell culture. After 48 hours, eGFP-CVB3 virus was collected and plaque purified.

Mouse Breeding and Viral Inoculation

BALB/c mice were obtained from Harlan Sprague Dawley (Harlan Laboratories, San Diego, CA). The breeding pairs were monitored daily to identify pups within 24 hours of birth. The neonates at 2 to 3 days old were intracranially inoculated with either a nonlethal dose of eGFP-CVB3 (1×10^6 plaque-forming units) or equal volume (25 μ L) of Dulbecco's modified Eagle's medium (mock infection) as previously reported.²⁰ All mice survived except for a few who died from the procedure of intracranial injection, independent of viral infection. All animal procedures were approved by the San Diego State University Institutional Animal Care and Use Committee and are in strict accordance with the Public Health Service Policy and United States Department of Agriculture Animal Welfare Regulations.

Mouse Brain Preparation

At 2, 5, 10, 30, and 90 days after infection, mice were euthanized in a bell jar with isoflurane, followed by immediate decapitation. Mouse brains were harvested and fixed with 4% formaldehyde.

Immunohistochemistry and Histochemical Staining

Paraffin-embedded sections (4- μ m thick) were deparaffinized first through xylene and then with a gradually decreasing concentration of isopropanol (100%, 90%, and 70%). Hematoxylin and eosin staining was conducted to evaluate virus-induced brain damage. The sections were also subjected to immunohistochemical (IHC) staining. Briefly, antigen retrieval was performed by heating the sections in citrate buffer pH 6.0 (005000; Life Technologies Carlsbad, CA) for 25 minutes at 121°C. Slides then underwent peroxidase blocking by using hydrogen peroxide (30 mg/mL), followed by washes with 1 $\times$ tris-buffered saline (0.05 mol/L Tris, 0.155 mol/L NaCl, pH 7.6). After blocking, sections were incubated with primary antibodies overnight at 4°C. The MACH4 Universal HRP-Polymer Detection System (BRI4012H; Biocare Medical, Pacheco CA) was then used to detect the staining according to the manufacture's procedure. At the end of the procedure, all slides were also counterstained with hematoxylin solution Gill II (GHS232; Sigma-Aldrich, St. Louis, MO).

Mouse brain tissues were immunostained by using the follow primary antibodies diluted in tris-buffered saline/phosphate-buffered saline (1% bovine serum albumin, 1.5 mol/L; NaCl, 0.5 mol/L; pH 7.6): double-stranded RNA (dilution 1:1000; J2 mouse IgG2a monoclonal antibody; 10010200; Scicon, Szirák, Hungary), GFP (dilution 1:200; mouse monoclonal antibody; sc-9996; Santa Cruz Biotechnology, Santa Cruz, Dallas, TX), glial fibrillary acidic protein (GFAP; dilution 1:200; mouse monoclonal antibody; SMC-441; StressMarq, Victoria, BC, Canada), ionized calcium binding adaptor molecule 1 (Iba1; dilution

1:200; mouse monoclonal antibody; sc-32725; Santa Cruz Biotechnology), pSTAT3 (dilution 1:200; mouse monoclonal antibody; sc-8059; Santa Cruz Biotechnology), choline acetyltransferase (ChAT; dilution 1:500; mouse monoclonal antibody; sc-55557; Santa Cruz Biotechnology), RZ3 (pThr-231 Tau; dilution 1:300; mouse monoclonal antibody; courtesy of Dr. Peter Davies at the Feinstein Institute for Medical Research, New York, NY), TDP-43 (dilution 1:1000; rabbit polyclonal antibody; 10782-2-AP; Proteintech, Rosemont, IL), p62/sequestosome-1 (SQSTM1; dilution 1:200; mouse monoclonal antibody; sc-28359; Santa Cruz Biotechnology), and ubiquitin (dilution 1:2000; mouse monoclonal antibody; sc-8017; Santa Cruz Biotechnology).

Images were taken by using the Aperio ScanScope AT (Digital slide scanner; Leica Biosystems Inc., Buffalo Grove, IL) or a Nikon Eclipse E600 microscope equipped with a SPOT Flex Model 15.2 64 Mp Shifting Pixel camera (Diagnostic Instrument Inc., Sterling Heights, MI).

Quantification and Statistical Analysis

Quantification of immunohistochemistry images was performed by using ImageJ version 1.0 (NIH, Bethesda, MD) with the combination of Color Deconvolution Plugin version 1.5 (<http://www.mecourse.com/landinig/software/cdeconv/cdeconv.html>) to generate the optical density value based on the intensity of the staining as described.²⁵ The quantified results are presented as means $\pm$ SD for two separate brain regions (hippocampus and cerebral cortex) at the indicated time points after infection. Statistical analysis was conducted by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test by using GraphPad Prism software version 6.0c (GraphPad Software, La Jolla, CA). *P* value of 0.05 was considered to be statistically significant.

Results

Establishment of a Persistent Viral Infection within the Mouse Brain

To determine the *in vivo* impacts of EV infection on the neuropathology, the neonates of BALB/c mice, a EV-susceptible strain, were intracranially inoculated with a nonlethal dose of eGFP-CVB3 or mock-infected with Dulbecco's modified Eagle's medium for 2, 5, 10, 30, or 90 days. Mouse brains were harvested for viral detection by IHC staining by using antibodies against GFP and double-stranded RNA, an intermediate form of the replicating viral genomic RNA. IHC staining of GFP (virally expressed protein) demonstrated focal infections located mainly in the hippocampus region, with the most active infection occurring between day 2 and day 5 post-infection (PI) (Figure 1). The GFP levels gradually decreased but remained detectable up to day 90 PI. The IHC signal for double-stranded RNA

peaked at day 5 PI and then markedly reduced with only faint detection on days 30 and 90 PI. These results were consistent with the previous reports that viral RNA that peaked at day 2 to 5 PI can still be detected by real-time RT-PCR up to day 90 PI,²⁶ suggesting a persistent viral infection within the brain. In addition to the hippocampus, viral infections in other areas of the brain, that is, cerebral cortex (especially the neocortex) (Figure 1), olfactory bulb, striatum, and putamen (data not shown), were also observed. Corresponding to the peak infection time and areas, histologic examination by hematoxylin and eosin staining revealed massive immune cell infiltrations and substantial tissue damages between day 2 and day 10 PI within the regions of viral infection (Figure 2). Tissue vacuolization was observed at the later times (Figure 2). These results supported previous findings that CVB3 has a high tendency to infect actively replicating cells that usually reside within the neurogenic regions, such as the hippocampus, olfactory bulb, and lateral ventral regions of the CNS to establish persistent viral infections.^{27–29}

Increased CNS Immune Responses after Infection

Because neuroinflammation plays an important role in ALS pathogenesis,³⁰ the effects of EV exposure on the pattern and extent of brain inflammation were studied. IHC staining was conducted to assess the level of astrocyte reactivity with anti-GFAP and anti-pSTAT3 antibodies, microglial activation using anti-Iba1 antibody, and natural killer (NK) cell maturation and neurogenesis with anti-ChAT antibody.

Compared with mock infection, CVB3-infected mice at various time points after infection demonstrated increased immune responses. In the hippocampus region, IHC staining of GFAP, a reactive astrocyte marker,³¹ displayed significant increases in expression intensity at day 5 to 10 and day 90 PI (Figure 3, A and B). Likewise, another reactive astrocyte marker, pSTAT3,³² also exhibited enhanced CNS astrocyte activation, in particular at day 5 PI. As a marker of microglia,³³ Iba1 staining revealed a continuous immune response (microgliosis) toward the viral infection with a peak expression at day 5 PI. IHC staining for ChAT, a marker for NK cell maturation and neurogenesis,³⁴ demonstrated a strong reaction at the early phase of viral infection, and the immunoreactivity remained detectable until the end of the experiment. Similarly, significantly increased GFAP staining was observed at day 5 and day 90 PI in the cerebral cortex region (Figure 3C). Together, these data indicated that CVB3 induced microgliosis, astrogliosis, and activation of NK cells in persistently infected mouse brains.

ALS-Related Proteinopathies after Intracranial CVB3 Infection

Familial ALS and sporadic ALS share the pathologic feature of TDP-43, which has been identified in >95% of all ALS cases.^{35,36} Mice with chronic CVB3 infection exhibited

Figure 1 A nonlethal dose of coxsackievirus B3 (CVB3) causes a persistent viral infection in mouse brain. Two- to 3-day-old BALB/C neonatal mice were either infected intracranially with a nonlethal dose of enhanced green fluorescent protein (eGFP)-CVB3 (1×10^6 plaque-forming unit) or mock-infected with Dulbecco's modified Eagle's medium. Mouse brains were harvested at day 2, 5, 10, 30, and 90 post-injection (PI). Immunohistochemical staining with an anti-GFP or anti-double-stranded RNA (dsRNA) antibody (brown) was performed, followed by nuclear counterstaining with hematoxylin. The figure includes the whole-slide view (**left column**; anti-GFP staining) to illustrate the distribution of the infection and the corresponding enlarged images of the infected regions (**all other columns**). The **red arrows** indicate dsRNA-positive staining as focal infections in the tissue. $n = 3$ individual mouse brain samples at each time point. Scale bars: 2 mm (**left column**, GFP mock, GFP Day 10 PI); 3 mm (**left column**, GFP Day 2 PI, GFP Day 5 PI, GFP Day 90 PI); 5 mm (**left column**, GFP Day 30 PI); 60 μ m (**all other columns**). CC, cerebral cortex; HIP, hippocampus.

signs of flaccid tail tone and hind limb dysfunction.²⁰ CVB3 infection resulted in cytoplasmic mislocalization and aggregation of TDP-43 in virus-infected HeLa cells²³ and primarily isolated mouse neuronal cells (Y.C.X. et al., unpublished data). Here, we extended our previous work to examine the distribution and expression pattern of TDP-43 in CVB3-infected mouse brains.²³ To better correlate virus-infected areas with TDP-43 alteration, two serial slides from mock and CVB3-infected mouse brains were stained

for GFP and TDP-43, respectively. TDP-43 was predominantly localized in the nucleus in mock-infected neuronal cells, whereas in day 5 CVB3-infected brain within the hippocampus region, TDP-43 was mostly mislocalized to the cytoplasm, along with nuclear down-regulation (**Figure 4A**). Notably, at days 30 and 90 PI, TDP-43 pathology remained apparent in the survival cells within the infected areas (**Figure 4B**), suggesting a similar pathologic phenotype to ALS.

Figure 2 Intracranial coxsackievirus B3 infection results in brain lesions. Hematoxylin and eosin staining was conducted on the collected mouse brain tissues. The **left column** of the whole tissue images illustrate the distribution of viral infection, and the **middle and right columns** show the corresponding magnified images of the infected regions. Within the area of viral infection, immune cell infiltration and tissue lesions can be observed. $n = 3$ individual mouse brain tissues at each time point post-infection (PI). Scale bars: 2 mm (left column, mock and Day 2 PI); 3 mm (left column, Day 5 PI, Day 10 PI, Day 30 PI, Day 90 PI); 60 μm (**middle and right columns**). CC, cerebral cortex; HIP, hippocampus.

To further characterize the cytoplasmic inclusions, brain tissues were stained for p62/SQSTM1 (a ubiquitin-binding protein), ubiquitin, and phosphorylated Tau (a microtubule-associated protein). Both p62 and ubiquitin were associated with protein aggregates detected in ALS.³⁷ Within the similar infected regions observed in TDP-43 pathology, p62/SQSTM1 and ubiquitin were stained positive at all infection time points, with the exception of the mock and the faintly positive day 2 PI samples (Figure 5). However, the immunoreactivity of phosphorylated Tau, which has been observed in ALS with cognitive impairment,³⁸ was not evident within the infected region until day 90 PI (Figure 5).

Discussion

To properly associate EV infection with the pathology of ALS in infected mouse brains, the areas of viral infection were confirmed, and ALS-associated pathologic

phenotypes within the identified regions of infection were analyzed. CVB3 infections were focally localized in multiple areas of the brain, including the hippocampus and cerebral cortex, and caused massive tissue damages, that is, structural fragmentation and vacuolization at early and late infections, respectively. This pattern of infection is likely because of the susceptibility of migrating neuronal progenitor cells to CVB3 infection and/or the distribution of viral receptors (ie, coxsackievirus and adenovirus receptor, and its co-receptor decay-accelerating factor).^{20,27,28} Virus-mediated damages of the hippocampus could lead to possible behavioral changes, such as poor long-term memory and spatial navigation, whereas impairments to the cerebral cortex, especially the neocortex (the newest evolutionary layer of cerebral cortex), might affect cognition, sensory perception, and motor function.³⁹ Overall, the various possible consequences of EV infection appear to correlate with the symptoms associated with several neurodegenerative diseases, such as ALS and Alzheimer

Figure 3 Coxsackievirus B3 (CVB3) infection caused increased immune responses in the mouse brain. **A:** Mouse brain tissues were harvested, and immunohistochemical staining was performed with antibodies against glial fibrillary acidic protein (GFAP), pSTAT3, ionized calcium binding adaptor molecule 1 (Iba1), and choline acetyltransferase (ChAT) as indicated. **B and C:** CVB3-induced immune responses in the hippocampus (**B**) or cerebral cortex (**C**) region were quantified by optical density as described in the [Materials and Methods](#). Data are expressed as means $\pm$ SD. $n = 3$ to 6 individual mouse brain samples in the hippocampus region at each time point (**A**); $n = 5$ mock samples (**B** and **C**); $n = 3$ samples at day 2 post-infection (PI) (**B** and **C**); $n = 4$ samples at day 5 PI (**B** and **C**); $n = 4$ samples at day 10 PI (**B** and **C**); $n = 6$ samples at day 30 PI (**B** and **C**); $n = 6$ samples at day 90 PI (**B** and **C**). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Scale bars = 60 μ m.

disease. Even though motor dysfunction was seen during the experimentation, more precise and robust behaviors and physiological tests, however, will be required to confidently link EV infection with clinical signs of neurodegenerative diseases.

Within the infected regions, levels of GFAP and pSTAT3, which mark the activation of reactive astrocytes, were markedly increased with a peak at 5 to 10 days and remained high until 90 days PI. This result indicates an elevated reactive astrocyte response. In addition, an

Figure 4 Coxsackievirus B3 infection led to cytoplasmic mislocalization of transactive response DNA binding protein-43 (TDP-43) in the brain. Immunohistochemical staining on mouse brains was conducted as above by using anti-green fluorescent protein (GFP) and anti-TDP-43 antibodies. **A:** Representative images of TDP-43 staining in the region of enhanced (e)GFP-CVB3-infected hippocampus at day 5 post-infection (PI). **Red boxes** indicate the location of the enlarged images; **red arrows**, to the observed TDP-43 cytoplasmic mislocalization. **B:** Immunohistochemical staining images of TDP-43 in the hippocampus region at different time points after infection. **Boxed areas** are shown at higher magnification in the **insets**. $n = 3$ individual mouse brain samples at each time point. Scale bars: 60 μm (**A**, **top** and **middle row**, and **B**); 30 μm (**A**, **bottom row**).

increase in infiltration/activation of microglia/macrophage within the infected regions, in particular the hippocampus region at early time points PI, was also observed through Iba1 immunostaining. Collectively, these data suggest a peak immune response toward an acute virus infection in the brain, and then a sustained low level of microglia/macrophage response to a chronic infection that is likely led by autoreactive T cells.⁴⁰ Of more interest, elevated stimulations were also observed in both NK cell maturation and neurogenesis at day 5 and day 90 PI, specifically in the hippocampus region, by ChAT staining. Activation of

microglia/macrophages in this context could stimulate circulating NK cells and excite the release of cytokines and chemokines, such as IL-15 and IL-12, which are coincidentally elevated in both the serum and cerebral spinal fluid from EV-infected patients and ALS patients.^{19,41} Building on previous findings that active immune systems are likely the promoters for ALS-like neurodegeneration,^{1–3} we speculate that in addition to virus-induced direct damages to the CNS, injury secondary to host immune responses may drive the development of the disease even further.

Figure 5 Coxsackievirus B3 infection induced the accumulation of p62- and ubiquitin-positive inclusions in the brain. Hippocampus regions were immunostained by using antibodies against p62/sequestosome-1 (SQSTM1), ubiquitin, and phosphorylated Tau (pTau) as indicated. **Red arrows** point to the positive staining within the illustrated tissues. $n = 3$ individual mouse brain tissues at each time point. Scale bars = 60 μm . PI, post-infection.

Intracellular protein inclusions are a pathologic hallmark of ALS neurodegeneration. TDP-43 inclusion is particularly important because it is identified in most ALS cases, contributing significantly to the pathogenesis of ALS.⁴² Within EV-infected regions, typical TDP-43 pathology in the form of nuclear-to-cytoplasmic mislocalization could be seen. The degree/frequency of TDP-43 mislocalization appeared to be correlated well with the severity of the infection, in which day 5 PI, the peak of viral infection, has a larger area of TDP-43 abnormalities than later time points. This is likely because virus-infected cells die as a result of productive viral replication after the acute viremia period. However, the remaining survival cells in the infected areas retained abnormal TDP-43 phenotype. In addition to TDP-43, EV-infected regions showed strong positive staining for p62/SQSTM1 and ubiquitin, two proteins also known as major components of ALS-related inclusions.³⁷ Of interest, phosphorylation of tau protein was observed only at day 90 PI. Phosphorylated tau is not generally associated with ALS except for individuals with impaired

cognition.³⁸ The mechanisms by which EV infection induces ALS-like proteinopathies are unclear. Expression of CVB3-encoded proteinase 2A causes cytoplasmic redistribution of TDP-43,²³ suggesting a mechanism of EV-induced TDP-43 pathology through the disruption of nucleocytoplasmic trafficking by viral proteinase cleavage of nucleoporin proteins.⁴³ Moreover, recent findings that EVs, including CVB3, poliovirus, and EV-D68, inhibit autophagic flux by preventing the formation of the soluble N-ethylmaleimide-sensitive factor activating protein receptor complex indicate that enhanced accumulation of p62 and ubiquitin may be a consequence of defective autophagy.^{44,45}

Conclusions

The present study demonstrates that CVB3 infection induces characteristics of TDP-43 pathology in virus-infected neural cells, accompanied by the presence of CNS lesions, microgliosis, and astrogliosis in persistently infected mouse

brains. Even though human studies had failed to establish a conclusive relationship between ALS and EV infection,¹⁷ animal models, unlike human models in which many external and internal factors cannot be controlled, will confirm the likelihood of this connection in a more controlled manner. Therefore, although animal studies like the one presented here may not lead to a solid conclusion, they can prompt more robust and specific clinical studies in the causal relationship between ALS patients and EV infection in which a more decisive result could be discovered.

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Supplemental Data

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