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ERVK activity in CD8⁺ T cell immune cell compartment in patients with ALS

Snigdha Sharma¹, Claudia Cortés-Pérez¹, Sasha Bird¹, Domenico Di Curzio¹, Alex Vandenakker¹, Kerri Schellenberg³ and Renée N. Douville^{1,2*} 

Abstract

Endogenous retrovirus-K (ERVK) expression has been associated with Amyotrophic Lateral Sclerosis (ALS), and its viral proteins can be detected in affected brain and spinal cord tissues. Despite confirmation of ERVK load in the blood of patients with ALS, few studies have examined ERVK protein expression in immune cells. ERVK produces an enzyme called integrase (IN), which can cause DNA damage during the integration of viral DNA into the host genome. Given that genomic instability is a hallmark of ALS, we hypothesized that the ERVK IN enzyme may also be expressed in lymphoid and myeloid-derived immune cells of patients. Peripheral blood mononuclear cells (PBMC) were isolated from blood specimens using Ficoll isolation, and either flash-frozen for western blot analyses or affixed onto slides using the cytospin technique for subsequent confocal microscopy analysis. Image analysis of confocal micrographs revealed that ERVK IN expression was significantly elevated in CD3⁺CD8⁺ T cells from a subset of patients with ALS as compared to controls. CD3⁺CD8⁺ T cells in ALS exhibit enhanced number and size of ERVK IN puncta within the nucleus and at the plasma membrane. The DNA damage load, as measured by marker γH2AX, was strongly associated with ERVK IN levels in both controls and patients with ALS. Stratification of molecular data based on clinical parameters showed an association of elevated ERVK IN load in CD8⁺ T cells from patients with ALS with lower ALSFRS-R and higher King's scores, as well as a significant decline in lung function metrics. In bulk PBMC from patients with ALS, ERVK IN was associated with expression of immune checkpoint marker PD-1, but not T cell exhaustion marker TOX. ERVK IN in CD14⁺CD11b⁺ myeloid cells was also elevated, with ERVK⁺ cells exhibiting notable membrane ruffling typical of immune cell activation and increased expression of HLA-DR. However, ERVK IN expression in monocytes was not correlated with clinical metrics in patients with ALS. This work points to the use of ERVK IN in CD8⁺ cytotoxic T cells as a blood biomarker for ALS clinical trials, especially those focused on testing the efficacy of antivirals as a therapeutic strategy for ALS.

Keywords Endogenous retrovirus-K (ERVK/HERV-K), Integrase (IN), Amyotrophic Lateral Sclerosis (ALS), Peripheral blood mononuclear cells (PBMC), T cells, Monocytes, Inflammation, DNA damage

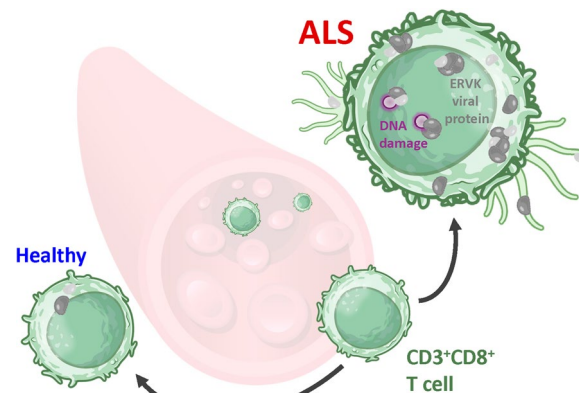
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Graphical Abstract



Background

Proteinopathies, such as the motor neuron disease Amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Alzheimer's disease (AD), lead to neuronal death. A root cause of this neuronal demise is neuroinflammation, with pathology mediated by both resident immune sentinel cells in the central nervous system (CNS) and circulating peripheral immune cells. A challenge has been identifying triggers that activate immune cells in neurodegenerative disease, as well as the root causes of immune imbalances.

Several groups have reported significant alterations in both monocyte and cytotoxic T cell profiles in ALS, which are closely tied to disease advancement. Changes in peripheral monocyte counts and subtypes have been documented in individuals with ALS [1–4], although a decrease in total blood monocyte numbers has also been reported [5]. Monocyte immune activation markers have been shown to increase with disease duration and severity in ALS [3], alongside a shift towards a pro-inflammatory phenotype [3, 6], and increased migratory trafficking into the CNS [1].

Another immune cell compartment with notable changes in neurodegenerative disease is cytotoxic CD8⁺ T cells. Enhanced CD8⁺ T cell activation in ALS occurs both in the circulation and the cerebrospinal fluid [7]. There is an increased frequency of CD8⁺ T cell effectors in ALS [8, 9], with the discovery that clonally expanded cells exhibit unique transcriptomic profiles [10, 11]. In ALS, loss of TDP-43 functionality leads to aberrant cryptic splicing and generation of neo-epitopes recognized by clonally expanded and functionally differentiated CD8⁺ T cells [12]. Recently, Kim et al. identified the proliferation of blood and cerebrospinal fluid effector memory CD8⁺ T cell clones in sporadic ALS when compared to control cases, noting an overrepresentation of virus-associated T cell receptor (TCR) motifs in these cells [9]. A nominal

decrease in both CD8⁺CD57⁺ and CD8⁺PD-1⁺ blood-derived T cell subtypes was associated with ALS risk in participants from the Berlin Aging Study II [13], suggesting depletion from circulation or migration into tissues. A low level of CD3 on central memory CD8⁺ T cells is also an ALS risk factor [14]. Several neuropathological studies have confirmed CD8⁺ T cell infiltration into the CNS of patients with ALS [15–17]. Together, this evidence aligns with the increasing understanding that ALS is a systemic disease, with a clear role of the immune system in disease progression [18–21].

Given the distinct immune profiles in ALS, one could postulate that these changes were associated with antiviral immunity. One virus that has been consistently associated with proteinopathy and neuroinflammation is endogenous retrovirus-K (ERV/K/HERV-K). Endogenous retroviruses (ERVs) are remnants of past retroviral infections where the virus's genetic material has integrated into the host's DNA, often appearing as multicopy viral symbionts [22]. ERVs are physiologically repressed (reviewed in [23]), but their basal expression can be heightened by various factors, including medications, hypoxia, infections, inflammation, and specific mutations [23–26]. The expression of ERVK has been associated with several neurological inflammatory diseases, including ALS, FTD, and AD [27–31].

A recurring theme associated with ERVK-related diseases is genomic instability [32]. DNA damage in neurons is a key feature of ALS [33, 34]. A retroviral enzyme that is known to inflict DNA damage is integrase (IN) [35]. During the retroviral life cycle, the integration of viral DNA into the host's DNA involves an IN-mediated strand-transfer reaction, leading to DNA breaks in the host genome [35]. These integration sites are frequently identified by nuclear phosphorylated γH2AX foci [36, 37], which correlate positively with the occurrence of double-stranded DNA breaks (DSB) in mammalian cells

Table 1 Cohort demographic data[illegible]

Table 1 (continued)

Diagnosis	Specimen ID	Age	Sex	PBMC (cells/ml blood)	Clinical parameters								Notes
					ALS-FRS-R (x/48)	Symptom onset	FVC (%)	PCF (L/min)	Ventilation	Feeding tube	Riluzole	Edaravone	
	C11	88	F	N/A									Failed PBMC isolation
	C12	69	F	7.57 x 105									
	C13	51	M	7.05 x 105									
	C14	70	M	7.50 x 105									

N.B. ALS clinical assessments were not performed in control cases

[38, 39]. Given that endogenous retroviruses can reintegrate into new locations within the parental genome, ERVK re-entry could lead to the buildup of DNA breaks and genomic instability, triggering cellular stress responses and inflammation [40].

Given that ERVK activation is linked to inflammatory signalling [24], we investigated whether an ERVK signature can be found in immune cells from patients with ALS, and if viral protein expression was associated with indicators of immune dysregulation or clinical metrics of disease severity.

Materials and methods

Ethics statement

All research involving human-derived samples was approved under a multi-site protocol by the University of Winnipeg Human Research Ethics Board (14921), St. Boniface Hospital (RRC/2021/2050), and the University of Saskatchewan Biomedical Research Ethics Board under protocol 1885. All participants consented to blood donation, and samples were deidentified by clinician Dr. Kerri Schellenberg before specimens were processed by the Douville lab.

Diagnosis and demographics of participant samples

Clinical examination was used to confirm the clinical diagnosis of ALS. Table 1 indicates the individual patient diagnosis and sample descriptors used in this study.

PBMC isolation and specimen preparation

Whole blood samples were diluted in PBS solution and processed on a ficoll gradient (Cytiva #17144002) as previously described [41]. The time from collection to processing for all patient samples was < 24 h. Extracted ex vivo peripheral blood mononuclear cells (PBMC) were counted and aliquoted into 2 × 10⁶ cells per dry pellet and frozen until subsequent western blot analysis. Alternatively, 1 × 10⁵ cells were spun onto slides (Fisher #12-550-15) using a StatSpin cytofuge (800 rpm, 4 min)

(Figure S1). Cytospin specimens were fixed in 4% PFA for 5 minutes, washed with PBS, dried, and stored at -80 °C until use.

Immunostaining

To determine the ERVK viral protein loads in select immune cells of PBMC specimens, immunohistochemistry was performed to detect the levels and localization of these target proteins in PBMC. PBMC were washed in triplicate with wash buffer (TBS + 0.05% TritonX100; TBS-T) then were stained with 100 µL of primary antibodies as described in Table S1 to then be incubated at room temperature for 2 h. Following, PBMC were triple washed with wash buffer and then were counterstained with 100 µL of secondary antibodies as described in Table S1 at room temperature for 1 h. After, they were rinsed, stained with DAPI (Life Technologies, #D1306), coverslipped, and mounted using ProLong Gold anti-fade reagent (Invitrogen, #P10144). All samples were batch stained with case-control matched slides.

Confocal microscopy and image analysis

Confocal microscopy was used to assess and colocalize the expression of ERVK proteins and cellular markers in immune cells. Immunostained slides were imaged with Olympus FV1200 laser scanning confocal microscope (Olympus, Japan) fitted with the Olympus Fluoview version 4.0B software suite. All images were taken at 1.0 or 2.5X magnification. CellSens software 4.2 software was used to deconvolute the confocal micrographs for accurate determination of the cellular staining patterns. CellSens was also used to process images, create region of interest (ROI) masks and counts, measure staining intensity within ROIs, and create orthogonal image views. For each marker, ROIs were mapped to each cell type of interest and the sum gray intensity value for each staining channel was measured in a given cell or puncta. Image J (Fiji for Windows x86-64) was used to generate intensity profiles of markers across arbitrary lines in individual cells.

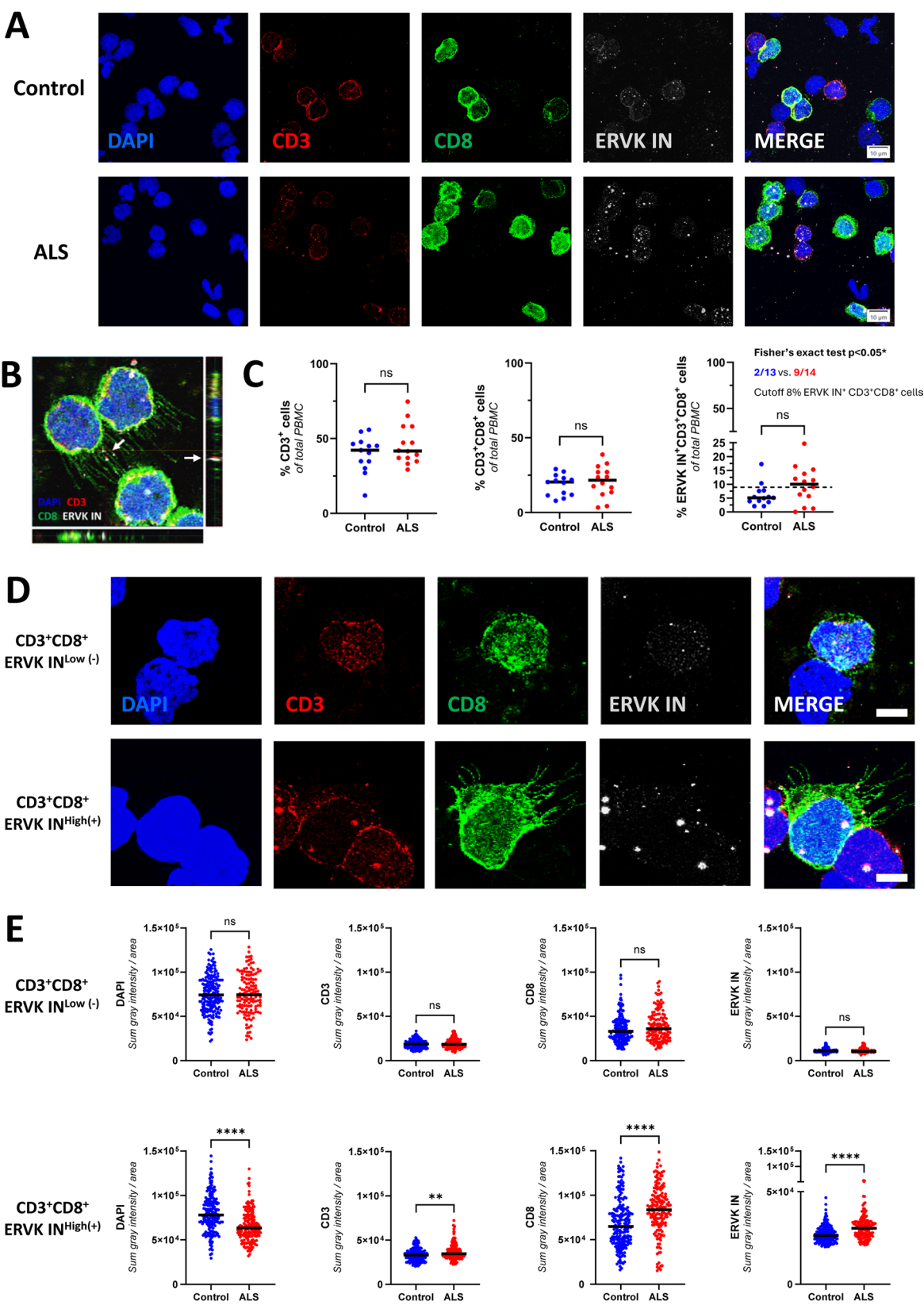


Fig. 1 (See legend on next page.)

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Fig. 1 Patients with ALS exhibit elevated ERVK integrase in their CD3⁺CD8⁺ T cells. ERVK integrase (IN) was quantified in CD3⁺CD8⁺ cells from patients with ALS and controls. **A** Representative micrographs of ERVK integrase (IN) expression in CD3⁺CD8⁺ T cells from control and ALS PBMC specimens. Scale bar is 10 μ m. **B** Frequently in ALS specimens, CD3⁺CD8⁺ T cells exhibited filopodia extensions, with ERVK staining along projections (arrows). **C** No difference in CD3⁺ T cell counts or CD3⁺CD8⁺ T cell counts between control and ALS specimens; however, patients with ALS (9/14) more frequently expressed ERVK IN in their CD3⁺CD8⁺ T cells as compared to controls (2/13), with a cutoff of > 8% ERVK IN⁺ CD3⁺CD8⁺ T cells. **D** Representative micrographs of ERVK IN^{Low(-)} and ERVK IN^{High(+)} CD3⁺CD8⁺ T cells in controls and patients with ALS, respectively. Scale bar (white) is 5 μ m. **E** For a given CD3⁺CD8⁺ T cell, an ROI was drawn to encompass the cell boundary (area), and the total sum intensity of immunostaining per marker was measured (sum gray intensity). Among ERVK IN^{High(+)} CD3⁺CD8⁺ T cells, specimens from ALS patients exhibited a decrease in nuclear DAPI staining with concomitant elevation in CD3 (T cell marker), CD8 (cytotoxic T cell marker), and ERVK IN expression as compared to controls. No differences in T cell markers or ERVK IN expression were apparent in ERVK IN^{Low(-)} CD3⁺CD8⁺ T cells between clinical groups. Mann-Whitney non-parametric t-tests, ** $p < 0.01$, **** $p < 0.0001$, n.s. = non-significant

Western blots

PBMC aliquots were lysed on ice with 50 μ l of in-house RIPA lysis buffer (1% SDS, 0.5% DOC and 1% NP-40 in 1X TBS) to extract proteins. The RIPA buffer was supplemented with 1x HALT protease and phosphatase inhibitor cocktail (Thermo Scientific #78442). BCA assay (Thermo Scientific #PI23227) was used to determine the protein content of each sample as per the manufacturer's instructions. Cell lysates were prepared for SDS-PAGE and heated at 95 °C for 10 min. Proteins (15–30 μ g per lane) were separated by SDS-PAGE using a 10% Quick Cast TGX gel (#161–0173) or 4–20% pre-cast TGX gradient gel (BioRad #456–8096), subsequently gels were imaged for total protein quantification, and then gels were transferred onto a PVDF membrane (eBLOT #L00735). The membrane was blocked in 3% BSA in TBS-T blocking buffer for 1 h and probed with the desired primary antibody overnight at 4 °C, followed by incubation at room temperature for 2 h. Details of primary and secondary antibodies used are listed in Table S1. Membranes were imaged using a BioRad ChemiDoc chemiluminescent imager. BioRad Image Lab software was used to determine the molecular weight and relative density (normalized to total protein) of each band.

Statistical analysis

Statistical analysis and graphing were performed using GraphPad Prism Software Version 10.6.1. Descriptive statistics and normality were performed to identify parametric and non-parametric data sets. As appropriate, groups were compared using parametric (Welch's) and non-parametric (Mann-Whitney) t-tests, as specified in the figure legends. Correlation tests (Pearson's for normally distributed datasets and Spearman's for non-parametric data) and linear regression lines were applied to XY graphs to determine the relationship between markers.

Results

PBMC specimens were purified from whole blood and ex vivo immune cells placed on slides for confocal microscopy analysis or fresh-frozen for western blot analysis, from age and sex-matched controls ($n = 13$) or patients with ALS ($n = 14$) (Fig. S1). The corresponding

deidentified clinical information was correlated with biological markers of ERVK activity in specific cell types.

CD3⁺CD8⁺ T cells in ALS exhibit enhanced ERVK IN expression

Confocal imaging was used to assess ERVK IN load in immune cells. Punctate ERVK IN staining was observed in CD3⁺CD8⁺ T cells from patients with ALS but rarely in controls (2/13 cases with more than 20 ERVK IN⁺ puncta per cell). This was accompanied by CD3⁺CD8⁺ T cells exhibiting morphological changes, such as membrane ruffling (Fig. 1A) and filopodia extensions (Fig. 1B), in ALS specimens. This aggregation of viral proteins at the immune cell membrane and within filopodia membrane extensions is consistent with the formation of virological synapses and filopodia-mediated virus transmission [42, 43]. No differences were observed in the percentage of CD3⁺ or CD3⁺CD8⁺ T cells between patients with ALS and healthy controls (Fig. 1C). Within this cohort, patients with ALS carry 2-fold more ERVK IN⁺CD3⁺CD8⁺ T cells than controls (median, range 2–17.3% controls versus 0–24.6% ALS, Mann-Whitney test $p = 0.1080$), with the highest abundance measured at 24.6% of total PBMC (case S07) (Fig. 1C). The frequency of ALS patients exhibiting an increased burden of ERVK IN⁺ cytotoxic CD3⁺CD8⁺ T cells in PBMC was significantly elevated compared to control specimens (Fisher's exact test, ERVK IN⁺ > 8% CD3⁺CD8⁺ T cells, $p < 0.05$, 2/13 controls vs. 9/14 patients with ALS, Fig. 1C). This ERVK IN^{High(+)} (cutoff > 25 puncta/cell, Fig. 1D) subset of cytotoxic CD3⁺CD8⁺ T exhibited elevation in cellular CD3 and CD8 marker levels in ALS, with a decrease in nuclear staining as measured by DAPI (Fig. 1E). No statistical differences in T cell markers or ERVK IN expression were apparent in ERVK IN^{Low(-)} CD3⁺CD8⁺ T cells between controls and patients with ALS. Additionally, when examining ERVK IN load in CD3⁺CD8⁻ cells (likely regulatory T cells), there were no differences in ERVK expression between control and ALS cases, despite a clear increase in CD3 expression in ALS specimens (Fig. S2).

In addition, blood-borne CD14⁺CD11b⁺ monocytes exhibited a statistically higher ERVK load in ALS as compared to controls (Fig. S3), yet the sum grey staining

intensity/area was 10-fold lower than in CD8⁺ T cells (Fig. 1). Nonetheless, notable morphological changes were apparent in ERVK IN⁺ CD14⁺CD11b⁺ monocytes within a subset of ALS patients (9 of 14), with evidence of membrane ruffling (Fig. S3A, C) and increased expression of CD11b and HLA-DR (Fig. S3D). Moreover, ERVK IN expression in monocytes was strongly correlated with the tetraspanin CD53 (Fig. S4), a known marker for intracellular compartments containing retroviral assemblies beneath the cell membrane during viral egress [44]. Thus, despite a moderate abundance of viral proteins with an ERVK IN epitope in monocytic cells, the mobilization of ERVK proteins may differ between controls and patients with ALS, impacting cellular activation and viral assembly.

CD3⁺CD8⁺ T cells in ALS exhibit enhanced number and size of ERVK IN aggregates

Viroplasm (aka. viral factories or inclusions) are membraneless condensates within a host cell where viral replication and assembly occur. Similar to virological synapses in HIV infection [45], we observed polysynaptic ERVK IN⁺ puncta in cytotoxic T cells from select ALS cases, in greater number ($p < 0.01$) and frequency (Fisher's exact test, $p < 0.05$) among patients with ALS compared with controls (Fig. 2A). The number of ERVK IN puncta per cell ($p < 0.0001$, range 1–58) and overall puncta size ($p < 0.05$, range 0.33–20.25 μm^2) were consistently elevated in CD8⁺ T cells from patients with ALS (Fig. 2B). The staining intensity of T cell markers was also elevated within ERVK IN aggregates in ALS. Within these viral protein

condensates, ALS patients exhibited an immunological signature of increased CD3 and CD8 (Figs. 2C and 3). Representative intensity profiles are a useful tool to assess viral deposition and colocalization with cellular partners [46]. Within individual representative cells, an arbitrary line through each cell was used to quantify the degree of dispersion and colocalization of CD3, CD8, and ERVK IN signal intensity (Fig. 3). This analysis approach demonstrated that ERVK IN⁺ puncta accumulated on the plasma membrane of CD3⁺CD8⁺ T cells in controls, but in ALS-derived cytotoxic cells, ERVK IN deposits were evident in the nucleus (Fig. 3C, blue arrow) as well as more abundantly at the cell surface (Fig. 3C, green arrow). Notably, CD3 relocation (red line) from the membrane to ERVK IN⁺ intracellular deposits (gray line) was also evident in CD3⁺CD8⁺ T cells in patients with ALS, but not in controls (Fig. 3C, red arrow).

ERVK IN load in CD3⁺CD8⁺ T cells is correlated with DNA damage

An overall increase in the expression of γH2AX , a DNA damage marker, is apparent in bulk PBMC from patients with ALS compared to controls ($p < 0.05$, Fig. 4AB, Fig. S6). As measured by western blot analysis, this suggests that a greater burden of DNA damage is carried within immune cells of patients with ALS, but this technique cannot identify the impacted cell subset. Microscopy revealed that ERVK IN expression was strongly correlated with the extent of DNA damage (as measured by γH2AX) in CD8⁺ T cells of both controls and patients with ALS (Fig. 4C–E), indicating that the presence of

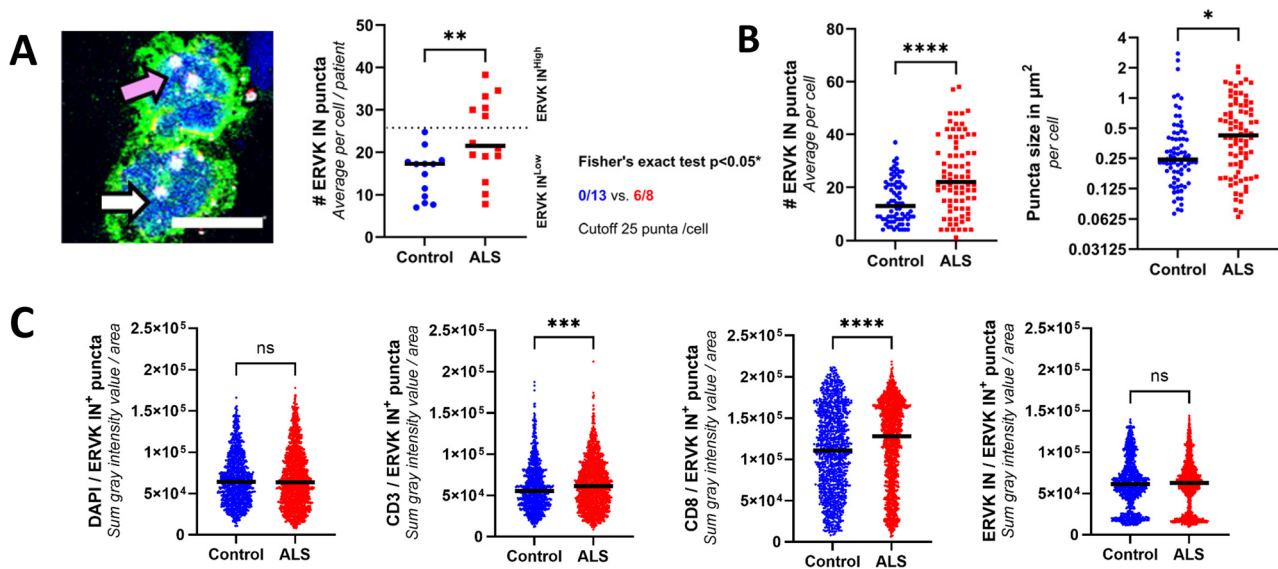


Fig. 2 Cytotoxic CD3⁺CD8⁺ T cells in ALS harbour viroplasm with ERVK integrase deposition. **A** Representative micrograph of ERVK integrase (IN) puncta in the nucleus (white arrow) and at the cell surface (pink arrow). Scale bar is 10 μm . The average number of ERVK IN puncta is elevated in cells from patients with ALS as compared to controls. **B** The number and size of puncta are greater in ALS patient cells as compared to controls. **C** ERVK IN puncta exhibit evidence of virological synapse formation ($\uparrow\text{CD3e}$ & CD8 clustering). Mann-Whitney non-parametric t-tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. = non-significant

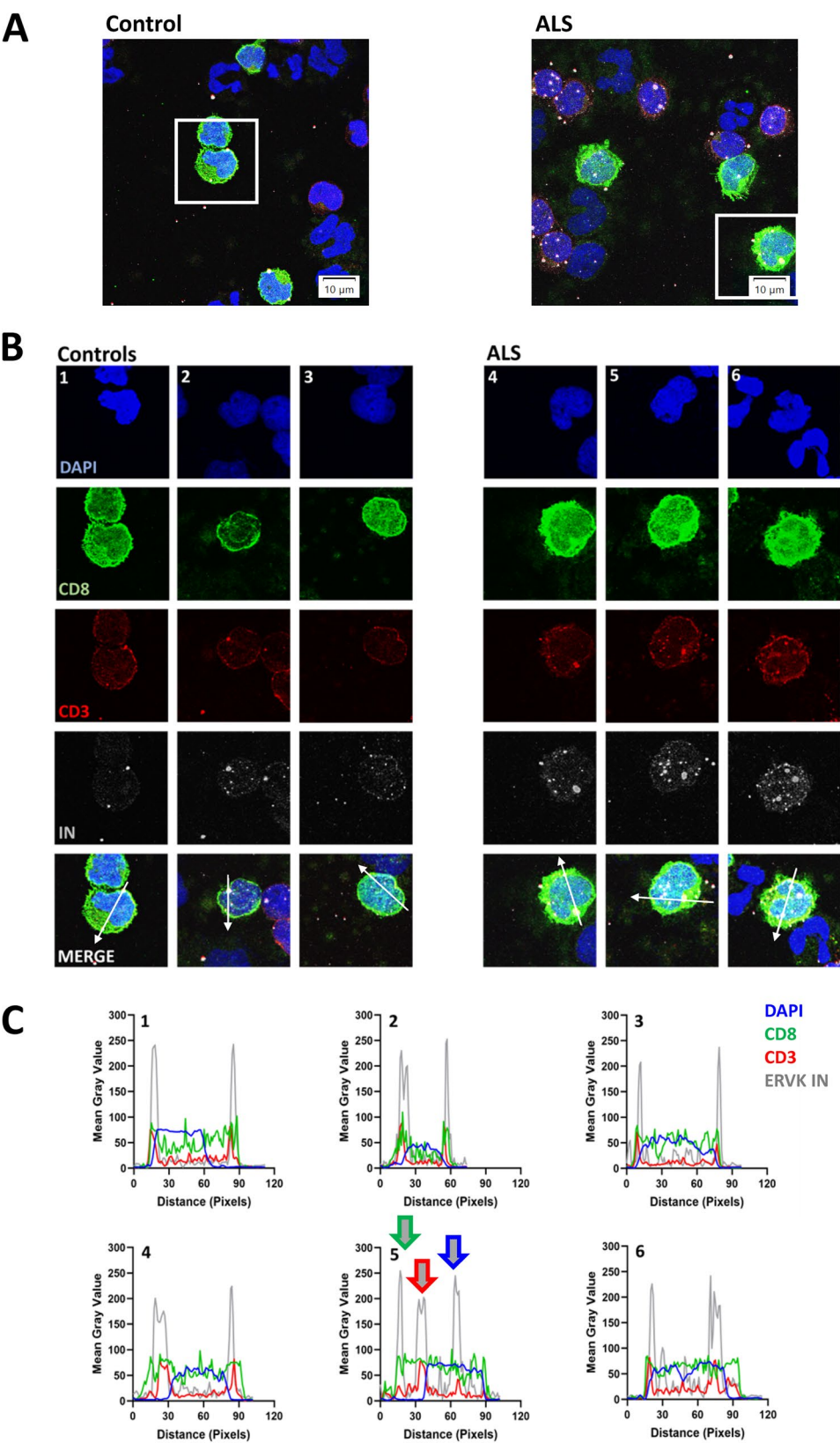


Fig. 3 (See legend on next page.)

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Fig. 3 Accumulation of ERVK IN puncta in nucleus and plasma membrane of CD3⁺CD8⁺ T cells in patients with ALS. **A** Representative micrographs of ERVK integrase (IN) in CD3⁺CD8⁺ T cells from controls and patients with ALS. Scale bar is 10 μ m. **B** Representative magnified images depict ERVK integrase (IN) expression in CD3⁺CD8⁺ T cells obtained from control (1–3) and ALS (4–6) specimens. Arbitrary white arrows (directionality of analysis) were drawn for intensity profile analyses. **C** Intensity profiles (mean gray value of staining) for markers CD3 (red), CD8 (green), ERVK IN (gray) and DAPI (blue) across CD3⁺CD8⁺ T cells, with distance in pixels along the white arrow depicted in merged images from panel A. In panel C5, grey arrows indicate ERVK IN hot spots, with border colour representing co-localization with CD8 (green border), CD3 (red border), and DAPI (blue border)

this viral enzyme has pathological consequences for the immune system. Whether examining this association at the individual cell level (Fig. 4D) or based on average expression patterns within individual PBMC donors (Fig. 4E), ERVK IN load was strongly correlated with the measurement of γ H2AX. Within individual representative CD8⁺ T cells, an arbitrary line through each cell was used to quantify the degree of dispersion and colocalization of CD8, ERVK IN, and γ H2AX signal intensity (Fig. 5). Nuclear ERVK IN⁺ puncta colocalized with peak γ H2AX signal (magenta line) in CD8⁺ T cells, most evident in ALS patient specimens (Fig. 5C). This indicates that ERVK IN is found at sites of nuclear DNA damage in CD8⁺ T cells, and that this occurrence is more frequent in ALS.

Age and sex-specific differences in ERVK burden in CD3⁺CD8⁺ T cells

Microscopy data of CD3⁺CD8⁺ T cells from Fig. 1 were stratified by age and sex of the PBMC donors. In the control group, ERVK load decreased ($r = -0.62$, $p < 0.05$) with age in cytotoxic T cells (Fig. 6A). In contrast, Fig. 6A shows that patients with ALS exhibited an enhancement of ERVK IN expression in their CD3⁺CD8⁺ T cells with increasing age ($r = 0.54$, $p < 0.05$). Moreover, females with ALS exhibited a trend ($p = 0.0635$) towards higher ERVK load than age-matched controls (Fig. 6B), alluding to sex differences in control over ERVK expression in females as compared to males. On average, females with ALS exhibited 1.5-fold higher ERVK IN load than males with ALS, with a tighter coefficient of variation (9.5% vs. 60.6%), suggesting that elevated ERVK is a more consistent finding in females with ALS.

ERVK IN load in CD3⁺CD8⁺ T cells is correlated with ALS severity

The revised ALS functional rating scale (ALSFRS-R) is a clinical rating scale to measure performance in four domains: (1) bulbar (representing speech, swallow, and saliva management), (2) respiratory, (3) upper limb, and (4) lower limb function [47]. The ALSFRS-R score declines with loss of function. Complementarily, the King's staging system refers to the sequential involvement of body regions, with scoring increasing by severity: first body region (stage 1), second and third body regions (stages 2 and 3), and need for gastrostomy or non-invasive ventilation (stage 4) [47]. Elevation of both ERVK IN

load and percentage of ERVK IN⁺ CD3⁺CD8⁺ T cells in ALS was strongly and significantly associated with lower ALS Functional Rating Scale-Revised (ALSFRS-R) scores and higher King's scores (Fig. 7A, B), indicating that patients with higher ERVK activity in cytotoxic T cells exhibit more severe functional decline. Increased DNA damage in cytotoxic T cells was also associated with lower ALSFRS-R scores. Given that the King's score was significantly elevated in patients with higher ERVK IN expression in their CD3⁺CD8⁺ T cells (Fig. 7B), this suggests that ERVK burden in circulating T cells is closely linked to disease severity. In contrast, disease duration was weakly associated with ERVK load in CD3⁺CD8⁺ T cells in patients with ALS (Fig. 7C). Clinical parameters surrounding lung function (Forced Vital Capacity, Peak Cough Flow, and non-invasive ventilation use) showed a significant decline in patients with high ERVK activity in CD8⁺ T cells (Fig. 8).

As a comparator, an association with clinical severity was not observed in monocytic cells (Fig. S5). Together, this indicates that ERVK load in antiviral CD3⁺CD8⁺ T cells is a significant disease severity signature of ALS, especially in females.

ERVK IN is associated with T cell exhaustion markers in ALS

To examine if phenotypic changes were occurring in CD8⁺ T cells from patients with ALS, we performed western blot analysis on protein lysates from ex vivo PBMC specimens (Fig. 9, Fig. S6). No significant difference in ERVK IN load and modest 1.1-fold increase in γ H2AX ($p < 0.05$, Fig. 4A) was observed between bulk PBMC control and ALS specimens as measured by western blot. However, markers of CD8⁺ T cell exhaustion, Programmed cell death protein 1 (PD-1) and Thymocyte selection-associated high mobility group box protein (TOX), were found to be correlated with ERVK IN expression in PBMC (Fig. 9B,D). In both controls and patients with ALS, the levels of PD-1 were correlated with ERVK IN expression in PBMC (Fig. 9B), but not γ H2AX (Fig. 9C). In contrast, ERVK IN expression was associated with TOX abundance in controls (Pearson $r = 0.59$, $p < 0.05$), but negatively associated in ALS specimens (Pearson $r = -0.42$, $p = \text{n.s.}$) (Fig. 9D). No correlation between TOX and DNA damage was observed in this patient cohort (Fig. 9E). Together, this shows that a subset of patients with ALS exhibit an ERVK viral signature in CD8⁺ T cells that is accompanied by T cell exhaustion

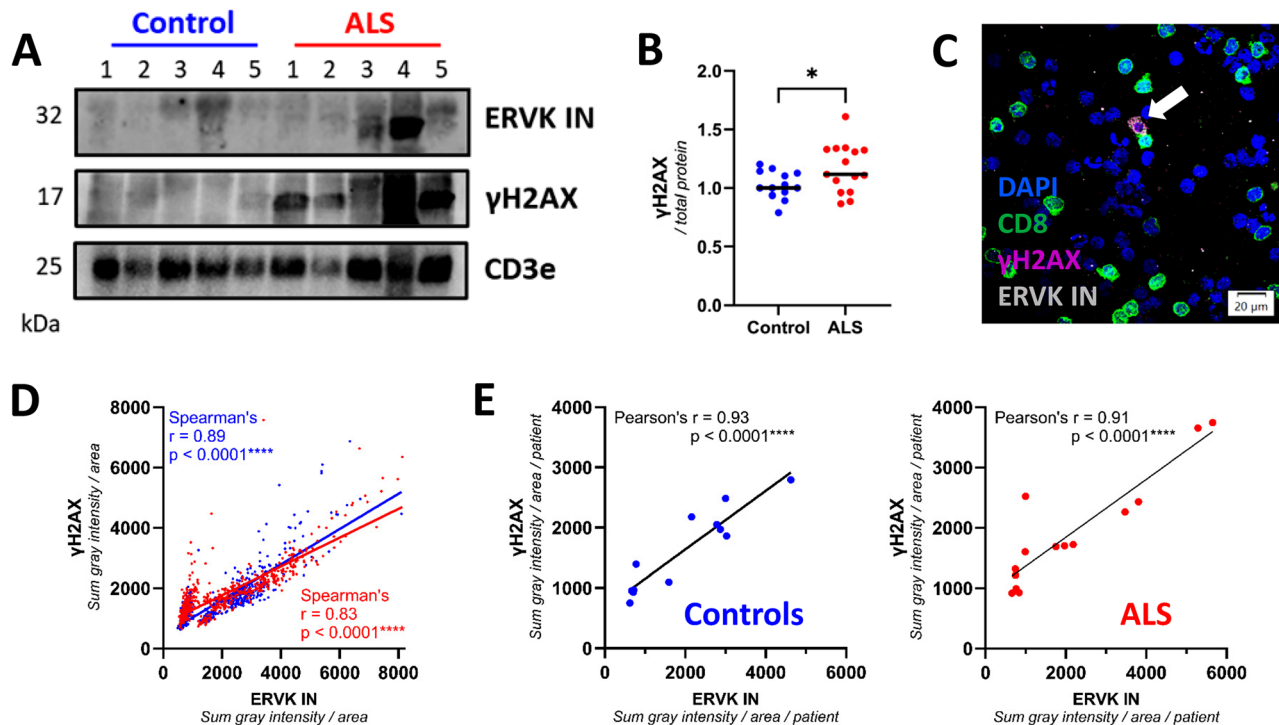


Fig. 4 ERVK IN expression is strongly correlated with the extent of DNA damage in CD8⁺ T cells from controls and patients with ALS. **A** Representative western blot of ERVK integrase (IN), γH2AX, and CD3e levels in bulk PBMC from controls and patients with ALS. **B** Expression of DNA damage marker γH2AX is significantly elevated in bulk PBMC from patients with ALS compared to controls, as measured by western blot and indicated by Welch's t-test, $p < 0.05$ *. **C** Confocal image of a CD8⁺ T cell with elevated ERVK IN and γH2AX expression. Scale bar is 20 μm. **D** Expression of ERVK IN is strongly correlated with DNA damage marker γH2AX in CD8⁺ T cells from both healthy controls and patients with ALS, as measured by confocal microscopy analysis and indicated by Spearman's correlations, $p < 0.0001$ ****. **E** Individual healthy controls and ALS patients exhibit increased DNA damage in CD8⁺ T cells with increasing ERVK IN load (average signal per cell per patient), as indicated by Pearson's correlation, $p < 0.0001$ ****

markers, suggesting that chronic viral antigenic stimulation may impact immune competence in some patients with ALS.

Conclusions

In accordance with our findings, many groups have also reported substantive changes in both monocytes and cytotoxic T cell profiles in ALS, which have been closely linked with disease progression. We detected polysynaptic ERVK IN⁺ puncta in cytotoxic T cells from specific ALS cases, which displayed an immunological profile characterized by increased CD3 and CD8 and filopodia-mediated viral transmission. Conversely, blood-borne monocytes showed a comparable ERVK load in both controls and ALS patients, despite morphological changes in ALS. The expression of ERVK IN was also found to be significantly linked to the degree of DNA damage (evaluated by γH2AX) in CD8⁺ T cells from both controls and ALS patients, signifying that the presence of this viral enzyme has detrimental effects on the immune system via genotoxicity. Together, this suggests that ERVK activity occurs in select immune cells in the peripheral circulation of a subset of ALS patients exhibiting viral proteinopathy, and that these cells may facilitate viral

transfer throughout the body and/or initiate neuropathological lesions, given the association with disease severity markers like ALSFRS-R and King's score [48]. This demonstrates that ERVK can serve as a blood biomarker, carrying therapeutic implications for clinically profiling patients with viral activity signatures and identifying those who may benefit from antiviral treatments.

In addition to neuron degeneration, neuroinflammation mediated by infiltrating immune cells contributes to disease progression in ALS and dementias. Yet, a conundrum is that ALS pathogenesis exhibits simultaneous and opposing immune dysfunctions, such as excessive inflammation and features of autoimmunity coupled with inefficient immune responses. If viewed from an infectious disease stance, the relationship is blurred as viruses can drive immunodeficient states that manifest as hyperinflammation due to the host's inability to contain pathogens and cope with tissue damage [49, 50]. Thus, exploring ERVK as a pathological driver and biomarker of sporadic ALS and FTD may be quintessential to understanding why aspects of the disease are repeatedly associated with virus infection [51, 52]. This study points to CD8⁺ T cells (and to a lesser extent monocytes) as being an important reservoir for ERVK activity in ALS.

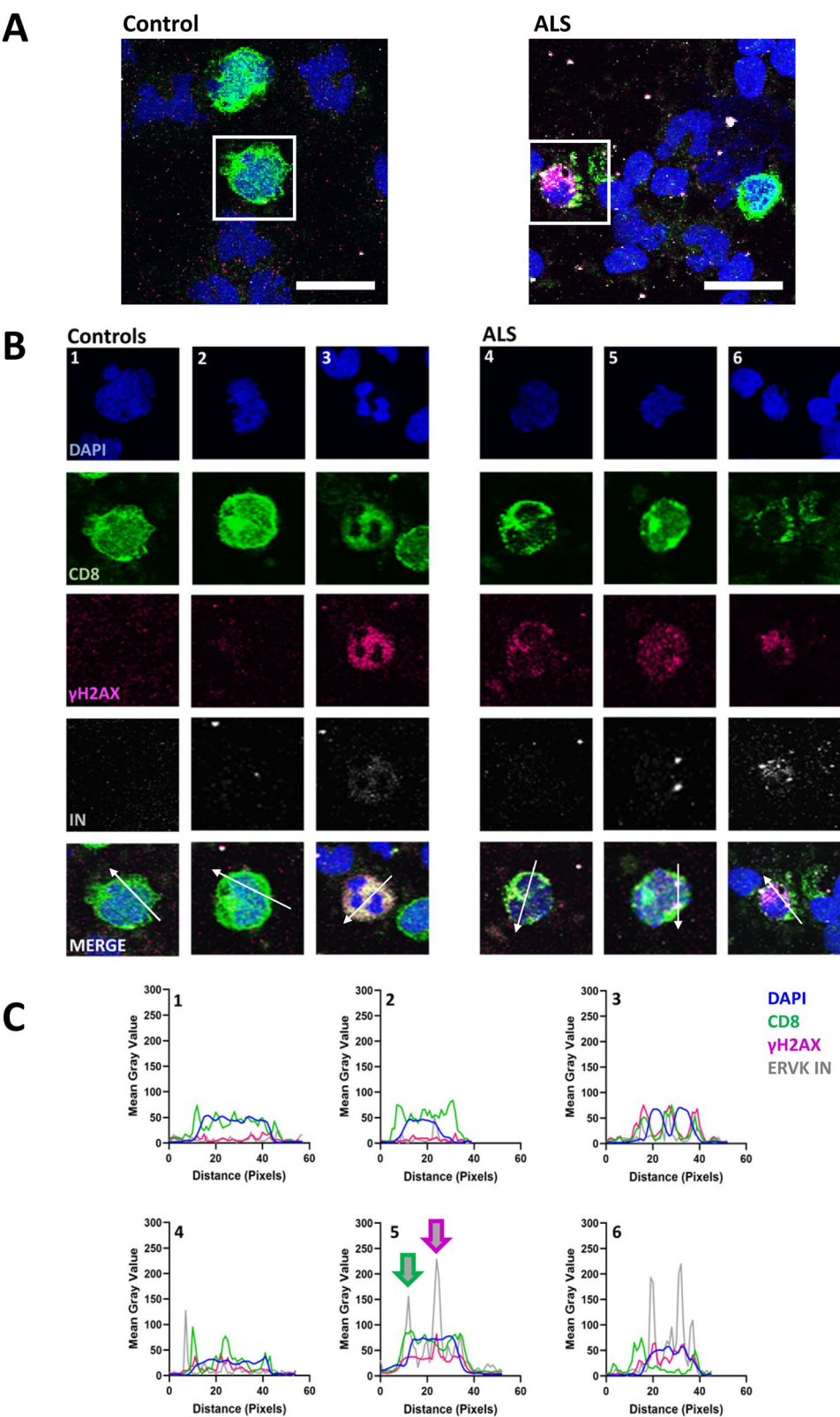


Fig. 5 (See legend on next page.)

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Fig. 5 ERVK IN colocalizes with DNA damage foci in the nucleus CD8⁺ T cells from patients with ALS. **A** Representative micrographs of ERVK integrase (IN) and DNA damage marker γ H2AX in CD8⁺ T cells from controls and patients with ALS. Scale bar is 20 μ m. **B** Representative magnified images depict ERVK integrase (IN) and γ H2AX expression in CD8⁺ T cells obtained from control (1–3) and ALS (4–6) specimens. Arbitrary white arrows (directionality of analysis) were drawn for intensity profile analyses. **C** Intensity profiles (mean gray value of staining) for markers CD8 (green), ERVK IN (gray), γ H2AX (magenta) and DAPI (blue) across CD8⁺ T cells, with distance in pixels along the white arrow depicted in merged images from panel A. In panel C5, grey arrows indicate ERVK IN hot spots, with border colour representing co-localization with CD8 (green border) and γ H2AX (magenta border)

This may be possible in a subset of patients with ALS because ERVK is already endogenated into the target cell genome, thus bypassing cellular tropism, and these individuals may carry mutations in ALS-risk genes related to the antiviral network (such as *TBK1*, *OPTN*, *SETX*, *TARDBP*, *UBQLN2*, etc [18]), that normally restricts viral replication. ERVK IN-expressing CD8⁺ T cells exhibited morphological changes associated with productive virion production [42, 43], such as aggregation of ERVK viral proteins at the immune cell membrane resembling virological synapses and viral protein deposits associated with filopodia membrane extensions. In some ALS cases, ERVK activity in monocytes was further linked to immune activation (membrane ruffling and heightened HLA-DR) and viral egress (association of ERVK proteins with tetraspanin CD53). This suggests that ERVK activity occurs in immune cells in the peripheral circulation of select ALS patients and that these cells may facilitate viral transfer throughout the body [48]. Conceptually, this is not dissimilar to how other human retroviruses spread from the periphery into the CNS [53].

Unlike monocytic cells, CD8⁺ T cells are not typically permissive host cells for viral replication, but there are reported exceptions in humans [54, 55]. HIV can also infect CD8⁺ T cells, albeit not as a primary reservoir [56, 57]. Albalawi and colleagues have demonstrated that HIV infects a select subset of CD8⁺ T cells, that when activated by pathogens will begin to express low levels of CD4 on their cell surface, termed CD4^{dim}CD8^{bright} T cells [58]. Due to their hybrid nature, CD4^{dim}CD8^{bright} T cells support HIV infection and exhibit strong anti-HIV responses [58]. In addition, CD4^{dim}CD8^{bright} T cells mediate HIV neuroinvasion in vivo, as shown in a humanized NOD SCID IL-2R $\gamma^{-/-}$ mouse model reconstituted with

human CD8⁺ T cells, followed by peripheral HIV infection [58]. This creates a productive viral reservoir in the brain and promote anti-HIV immune responses that contribute to neuroinflammation and neuronal injury. Therefore, it is possible that peripheral ERVK activity in CD8⁺ T cells serves as a vehicle to disperse virus in the brain of select patients with ALS, who seem able to support robust viral protein production in their CD8⁺ T cells.

Viruses that cause persistent infections can drive continuous immune activation, leading to CD8⁺ T cell exhaustion, a non-responsive state where they lose their ability to eliminate the virus. CD8⁺ T cells that have reached T cell exhaustion due to chronic antigen stimulation begin to express the transcription factor TOX [59]. The activity of TOX helps maintain the dysfunctional state of these CD8⁺ T cells through epigenetic reprogramming, which leads to the upregulation of inhibitory receptors (like PD-1) and dampened T cell function [59]. We measured both TOX and PD-1 expression in the PBMC compartment in this cohort and observed a correlation between ERVK IN load and PD-1 in both controls and patients with ALS. In contrast, only controls exhibited a positive association between TOX abundance and ERVK IN, as in ALS there was a negative correlation between these two markers. An elevation in soluble PD-1 in sera and an association between PD-1⁺CD4⁺ T cells and ALSFRS-R severity have previously been described in patients with ALS [60]. An altered CD8⁺ T cell immune phenotype has also been previously described in ALS4, a form of juvenile-onset motor neuron disease with slow progression [11]. ALS4 is characterized by a mutation in *SETX*, which encodes Senataxin, a nuclear ATP-dependent DNA/RNA helicase with cellular roles in RNA metabolism and antiviral immunity

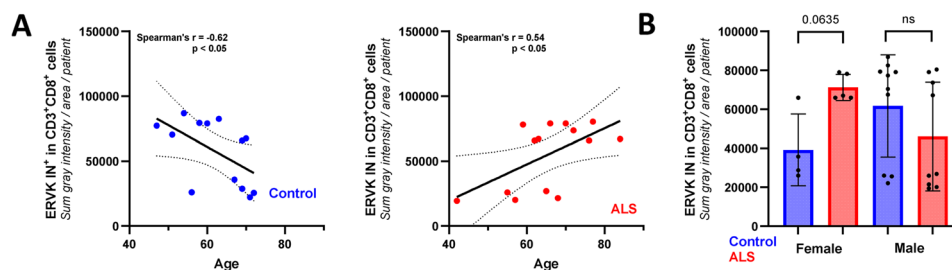


Fig. 6 Age and sex-specific differences in ERVK integrase expression in CD3⁺CD8⁺ T cells. **A** Microscopy data from Fig. 1 were re-analysed based on the age and sex of the PBMC donors. Expression of ERVK integrase (IN) decreases with age (in years) in CD8⁺ T cells from healthy controls, whereas it significantly increases with age in patients with ALS. **B** Females with ALS tend to express higher levels of ERVK IN in their CD8⁺ T cells compared with age-matched controls. Spearman's correlations and Mann-Whitney non-parametric t-tests, n.s. = non-significant

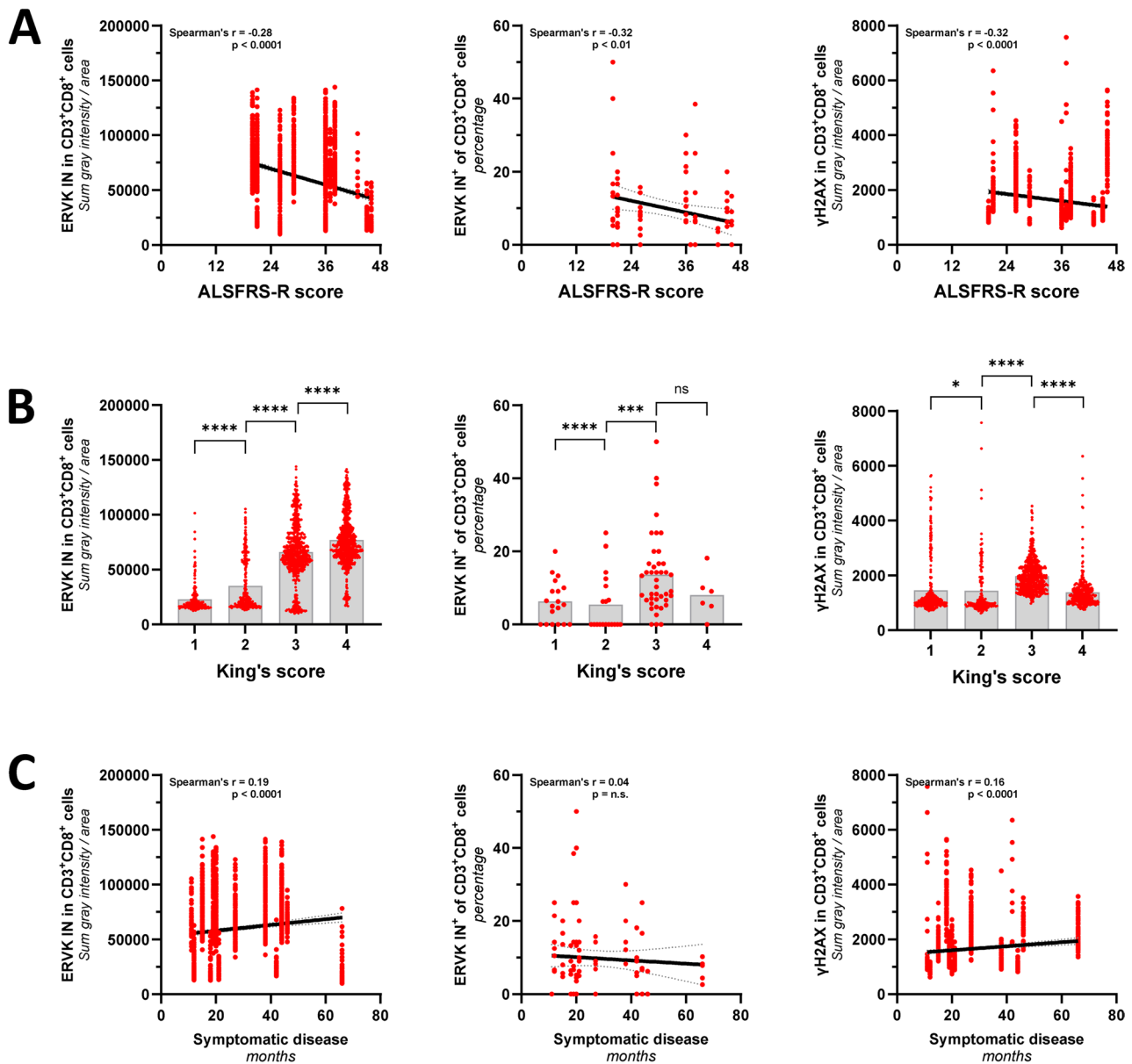


Fig. 7 ERVK integrase expression in cytotoxic CD3⁺CD8⁺ T cells is associated with clinical disease severity in ALS. **A** The severity of Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R score, lower score is more severe disease status) is associated with elevated ERVK integrase (IN) levels in CD3⁺CD8⁺ T cells, increased number of circulating ERVK IN⁺ CD3⁺CD8⁺ T cells in blood (percentage of cells per micrograph), and nuclear DNA damage (yH2AX) within cytotoxic T cells from patients with ALS. **B** King's score measures disease progression as related to anatomical spread and exhibits a strong association with ERVK load in CD3⁺CD8⁺ T cells. **C** Disease duration is weakly associated with ERVK levels in CD3⁺CD8⁺ T cells. Spearman's correlations and Mann-Whitney non-parametric t-tests, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, n.s. = non-significant

[61, 62]. CD45RA⁺ CD28⁻ CD27⁻ terminally differentiated effector memory (TEMRA) CD8⁺ T cells in ALS4 patients exhibited elevated TOX, but an inconsistency in PD-1 expression [11]. TCR profiling suggested expansion of autoreactive TEMRA CD8⁺ T cells in ALS4 [11]. This autoreactive profile may be consistent with a response to a genome-derived virus, like ERVK. However, our results indicate a complex cytotoxic T cell exhaustion pattern, with only PD-1 levels, but not TOX, associated with ERVK activity in ALS. Nonetheless, further analysis of

immune checkpoint markers and proteomic profiling of ERVK⁺ CD8⁺ T cells is warranted.

ERVK profiling in bulk PBMC from patients with ALS has previously been described by Arru et al. (2021) [63], albeit with methodological differences from this work. In their study, immune cell subsets were screened for expression of the ERVK envelope (Env) protein using flow cytometry, where they observed a greater percentage of B cells and NK cells expressing the viral protein in patients with ALS as compared to healthy controls [63].

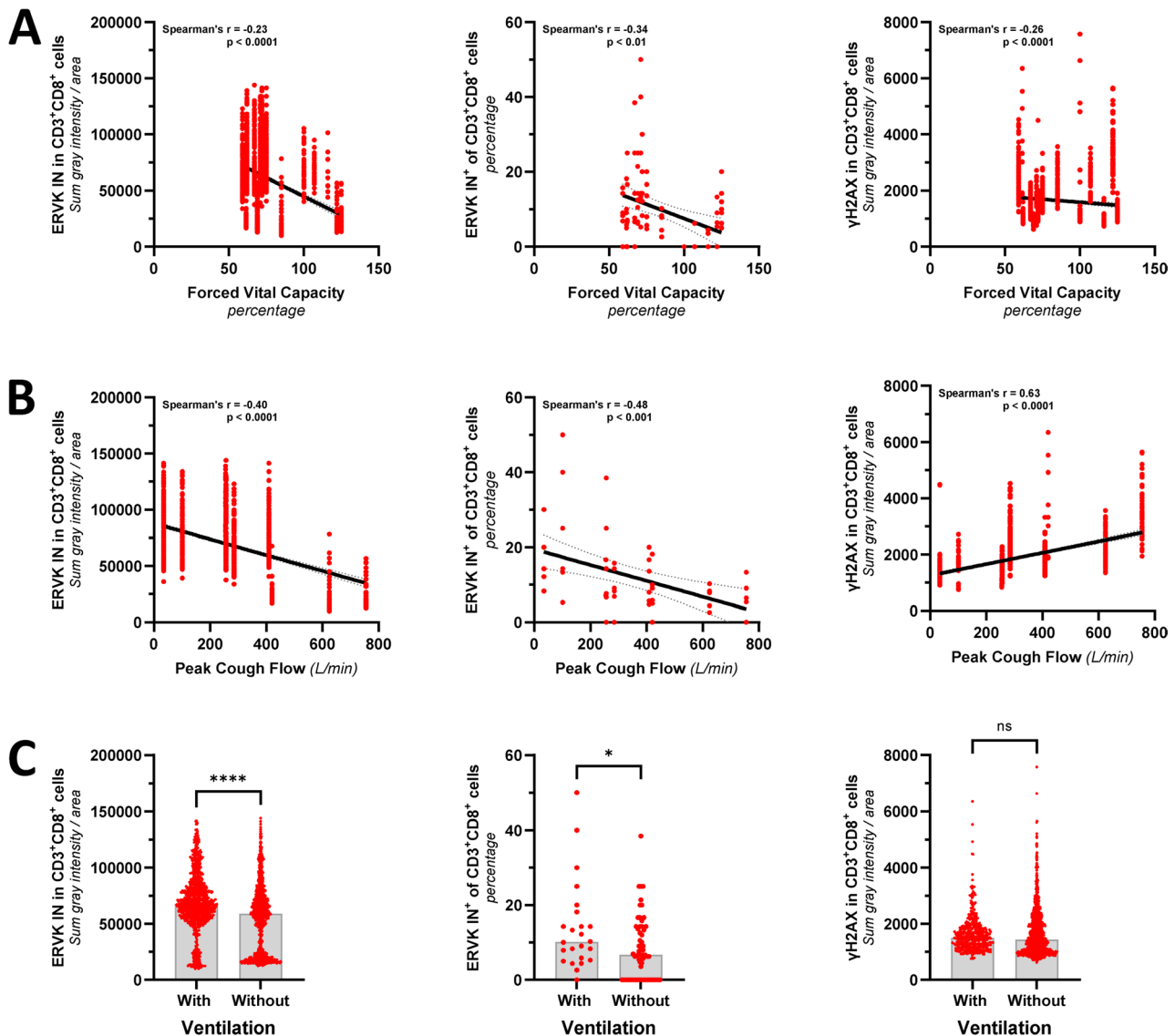


Fig. 8 ERVK integrase expression in CD3⁺CD8⁺ T cells is associated with impaired respiratory function in ALS. The load of ERVK integrase (IN) in CD3⁺CD8⁺ T cells and the frequency of ERVK IN⁺ cells (percentage of cells per micrograph) in patients with ALS are significantly negatively correlated with lung function metrics, such as Forced Vital Capacity (FVC, **A**), Peak Cough Flow (PCF, **B**), and whether the patient requires non-invasive ventilation tube usage or not (**C**). Spearman's correlations and Mann-Whitney non-parametric t-tests, * $p < 0.05$, **** $p < 0.0001$, n.s. = non-significant

Conversely, monocytes from patients with ALS exhibited reduced ERVK Env levels compared to controls [63]. No ERVK Env was detected on CD4⁺ or CD8⁺ T cells with this technique, although these cells produce cytokines and chemokines in response to stimulation with ERVK Env peptides [63]. These divergent datasets may be the result of differential detection of ERVK viral proteins (IN vs. Env), methodological approach, and/or patient cohorts. An improvement on both studies would be to isolate individual immune cell subsets and characterize them independently, with additional cell markers and expression patterns confirmed with proteomics profiling.

This study has additional limitations. The findings in this pilot study will need to be expanded into larger

cohorts of patients with ALS and FTD. In addition, alternate modes of screening specimens for ERVK activity in CD8⁺ T cells are warranted, such as flow cytometry or mass cytometry [64]. While the idea of virus-driven modulation of immune cells and their cargoes has yet to be adopted into the mainstream view of ALS blood biomarkers [65], it has diagnostic and therapeutic implications derived from the field of infectious disease. The production of extracellular vesicles (EVs) from ERVK⁺ immune cells, through either virion or exosome secretion [66], may allow these cells to propagate pathological proteins in ALS / FTD, such as viral proteins, TDP-43, and Tau, ultimately harming neurons [67–70]. Further exploration into this area is needed to comprehend the

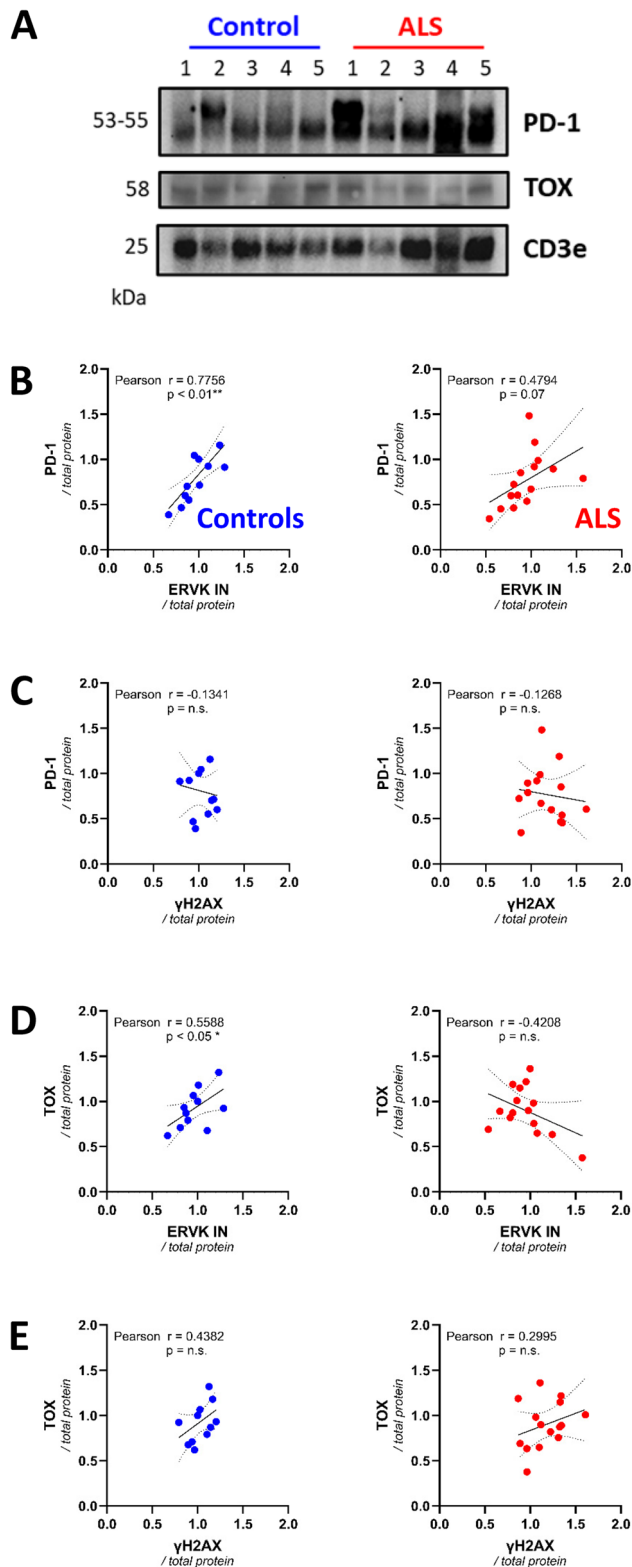


Fig. 9 ERVK IN expression is correlated with immune checkpoint inhibitor PD-1 in PBMC, but not with T cell exhaustion marker TOX or the extent of DNA damage. **A** Representative western blot of PD-1, TOX, and CD3e levels in bulk PBMC from controls and patients with ALS. **B** ERVK integrase (IN) is strongly correlated with PD-1 levels in controls and is positively associated with PD-1 levels in patients with ALS. **C** Conversely, DNA damage marker γ H2AX in PBMC is not correlated with PD-1. **D** ERVK IN is positively correlated with TOX levels in controls but is negatively associated with TOX in patients with ALS. **E** TOX was not associated with γ H2AX in PBMC from controls and patients with ALS. All cell markers were normalized to total protein

consequences of ERVK activity in CD8⁺ T cells and its relationship with neurodegeneration.

Our work aligns with a growing recognition of heterogeneous molecular underpinnings in ALS and FTD [71]. One repeatedly recognized subtype of ALS and FTD is characterized by the reactivation of ERVK and other retrotransposons. Given that viral mechanisms that may drive neuropathology in these cases (reviewed in [27, 72]), there has been keen interest in the possible use of antivirals to treat ALS and FTD [73, 74]. However, there is no gold standard for identifying which ALS and FTD patients exhibit enhanced viral activity and may benefit from alternative therapeutic choices. A limitation of measuring ERVK genome or RNA transcripts with PCR is that it does not prove that pathological ERVK proteins are being produced [73], only that viral nucleic acids are present (which is to be expected even in healthy individuals [75]). Thus, ALS clinical trials with antivirals may continue to fail without an improved patient-stratification strategy. The global Phase 3 placebo-controlled Lighthouse II study [27, 76] was stopped after interim analysis when it was shown that patients with ALS did not benefit from taking the antiviral combination therapy Triumeq in terms of survival (primary outcome measure), as compared with placebo [77]. While 29 centers recruited 400 participants for the study, the participants were not screened for pre-existing ERVK expression before being placed on treatment or placebo. Based on current knowledge of ERVK viral protein prevalence in ALS (estimated at > 30% in this study and others [28]), this would suggest that 2/3 of participants in the trial were not likely to respond to treatment. This demonstrates the need to first identify ERVK⁺ individuals prior to enrolling participants in clinical trials for antivirals. Our study provides viral and cellular protein biomarkers for the identification of ALS patients with elevated ERVK in blood, and may be useful in future trial designs. Moreover, these biomarkers correlate with clinical severity and may be useful to monitor disease progression and response to therapeutic interventions.

In conclusion, our work reinforces the notion of disease heterogeneity in ALS and points specifically to a subset of ALS patients with ERVK activity in CD8⁺ T cells. This work sets the stage for examining blood-based ERVK biomarkers as a novel approach to clinically characterize patients with viral activity signatures and identify those who may benefit from antiviral therapy.

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALSFRS-R	ALS Functional Rating Scale-Revised
CNS	Central nervous system
DSB	Double-stranded breaks
Env	Envelope protein
ERVK	Endogenous retrovirus-K (aka, HERV-K)
EVs	Extracellular vesicles
FVC	Forced Vital Capacity
FTD	Frontotemporal dementia
γH2AX	Phosphorylated histone 2AX
HIV	Human Immunodeficiency Virus
HLA-DR	Human leukocyte antigen-DR
IN	Integrase
PBMC	Peripheral blood mononuclear cells
PCF	Peak Cough Flow
PD-1	Programmed cell death protein 1
ROI	Region of interest
TCR	T cell receptor
TOX	Thymocyte selection-associated high mobility group box protein

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12974-026-03871-7>.

Supplementary Material 1.

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Dedication

This study is dedicated to patients with ALS—we are working on it!

Authors' contributions

Data curation, methodology, validation, investigation, and formal analysis by SS, CCP, SB, DDC, AV, and RND. Conceptualization, supervision, project administration, resources, and funding acquisition by KS and RND. Visualization and writing original draft preparation by SS and RND, review & editing by all authors.

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Data availability

The datasets generated and analyzed in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All research involving human-derived samples was approved under a multi-site protocol by the University of Winnipeg Human Research Ethics

Board (14921), St. Boniface Hospital (RRC/2021/2050), and the University of Saskatchewan Biomedical Research Ethics Board under protocol 1885.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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