

Thymic involution, a co-morbidity factor in amyotrophic lateral sclerosis

Akop Seksenyan^{a, †}, Noga Ron-Harel^{b, †}, David Azoulay^a, Liora Cahalon^b, Michal Cardon^b, Patricia Rogeri^a, Minhee K. Ko^a, Miguel Weil^c, Shlomo Bulvik^d, Gideon Rechavi^e, Ninette Amariglio^e, Eli Konen^g, Maya Koronyo-Hamaoui^{a, †}, Raz Somech^{e, †, †} and Michal Schwartz^{a, b, †, *}

^a Maxine-Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

^b Department of Neurobiology, the Weizmann Institute of Science, Rehovot, Israel

^c Department of Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

^d Hematology Department, Laniado Hospital, Netanya, Israel

^e Cancer Research Center, Sheba Medical Center, Tel Hashomer and Sackler School of Medicine, Tel Aviv, Israel

^f Pediatric Immunology Service, Safra Children's Hospital, Sheba Medical Center, Tel Hashomer and Sackler School of Medicine, Tel Aviv, Israel

^g Department of Diagnostic Imaging, Sheba Medical Center, Tel Hashomer and Sackler School of Medicine, Tel Aviv, Israel

Received: December 19, 2008; Accepted: July 10, 2009

Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating disease, characterized by extremely rapid loss of motor neurons. Our studies over the last decade have established CD4⁺ T cells as important players in central nervous system maintenance and repair. Those results, together with recent findings that CD4⁺ T cells play a protective role in mouse models of ALS, led us to the current hypothesis that in ALS, a rapid T-cell malfunction may develop in parallel to the motor neuron dysfunction. Here, we tested this hypothesis by assessing thymic function, which serves as a measure of peripheral T-cell availability, in an animal model of ALS (mSOD1 [superoxide dismutase] mice; G93A) and in human patients. We found a significant reduction in thymic progenitor-cell content, and abnormal thymic histology in 3–4-month-old mSOD1 mice. In ALS patients, we found a decline in thymic output, manifested in the reduction in blood levels of T-cell receptor rearrangement excision circles, a non-invasive measure of thymic function, and demonstrated a restricted T-cell repertoire. The morbidity of the peripheral immune cells was also manifested in the increase of pro-apoptotic *BAX/BCXL2* expression ratio in peripheral blood mononuclear cells (PBMCs) of these patients. In addition, gene expression screening in the same PBMCs, revealed in the ALS patients a reduction in key genes known to be associated with T-cell activity, including: *CD80*, *CD86*, *IFNG* and *IL18*. In light of the reported beneficial role of T cells in animal models of ALS, the present observation of thymic dysfunction, both in human patients and in an animal model, might be a co-pathological factor in ALS, regardless of the disease aetiology. These findings may lead to the development of novel therapeutic approaches directed at overcoming the thymic defect and T-cell deficiency.

Keywords: neurodegeneration • immunomodulation • neuroprotection • thymus involution • amyotrophic lateral sclerosis • protective autoimmunity • T cells • immune deficiency

Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a rapidly progressing fatal neurodegenerative

disorder, with death typically occurring within a few years after the first appearance of clinical symptoms [1]. Although the majority of ALS cases are sporadic, 10% of them are inherited, with the most abundant mutation occurring in the superoxide dismutase (SOD1) gene [2]. In both the sporadic and familial forms, disease progression is attributed to selective death of motor neurons in the spinal cord, with evidence for local neuroinflammation and acquisition of a cytotoxic phenotype by the microglia [3–6].

[†]These authors contributed equally to this study.

*Correspondence to: Michal SCHWARTZ, Weizmann Institute of Science, Herzl st., Rehovot, Israel.

Tel.: 972-8-9342467

Fax: 972-8-9346018

E-mail: michal.schwartz@weizmann.ac.il

Evidence accumulated by our group over the last decade suggest that the peripheral immune system (including T lymphocytes and blood-borne monocytes), in concert with the central nervous system (CNS) resident immune cells (microglia), play a pivotal role in CNS maintenance, protection and repair [7–13]. Specifically, it was shown in various models of CNS insults that the outcome of the injury is far more severe in immune deficient mice [14–16] and that passive transfer of T cells recognizing abundant self-antigens in the brain mitigates the ensuing damage to the CNS [8, 15, 17–20]. Additionally, in animal models of chronic neurodegenerative diseases, boosting of T-cell levels by active or passive vaccination attenuates disease symptoms and slows down disease progression [21–24], in part, by modulating the microglial phenotype and local cytokine profile [22].

In the mouse model of ALS, it is becoming clear that the ability of the motor neurons to resist the spread of toxicity from the surrounding damaged cells is greatly influenced by the peripheral T cells. Thus for example, breeding mSOD1 mice with immunodeficient mice shortens their life span [20, 25], and as a corollary, active vaccination in some strains of ALS mice [26], or passive transfer of activated T cells, is able to extend life expectancy [24].

We have recently proposed a working hypothesis suggesting that in chronic neurological diseases, before their clinical onset, there is a period of equilibrium that involves a competition between the immune system and the disease-inducing factors. The symptomatic disease emerges only when the immune system fails to cope with such locally emerging threatening factors [27]. Such an immune failure could be a reflection of increasing levels of disease-causing factors that exceed the ability of the immune system to contain, or of a situation in which immune function deteriorates concomitantly with disease progression, due to factors indirectly or directly related to the disease-causing entity.

The T-cell population is maintained by thymic release of nascent naïve T cells throughout life. Bone marrow derived T-lymphocyte precursors migrate to the thymus, where they mature and differentiate into functional T lymphocytes. T-cell malfunction can be manifested at several levels: deficiency in memory cells specific to certain antigens, increased levels of regulatory T cells that suppress effector T-cell activity or a decrease in thymic output. Transient or chronic manifestations of thymic involution have been demonstrated in several diseases [13, 28–30]. Although not the primary cause of the disease, this phenomenon might contribute to the lack of recovery and to further exacerbation of the disease conditions.

Based on our understanding of the immune system's involvement in CNS maintenance [27, 31], we suggested that thymic involution might occur in ALS, and could explain the aggressive and rapid progression of the disease due to the insufficient T-cell mediated protection.

Here, we describe a thymic malfunction/defect that appears to develop in parallel to the motor neuron dysfunction, both in a mouse model of ALS, mSOD1 (G93A), and in ALS patients.

Materials and methods

Animals

B6SJL-TgN(SOD1-G93A)1Gur transgenic SOD1 mice (mSOD) and age-matched controls were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were maintained in a light- and temperature-controlled room. All animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee of the Weizmann Institute or the Cedars-Sinai Medical Center, and maintained in a pathogen-free environment.

Patients

Fresh blood was collected from ALS patients ($n = 15$) and healthy controls ($n = 12$) who had previously given consent according to the guidelines of the ethics committee of Laniado Hospital, Netanya, Israel, supervised and approved by the Israeli Health Ministry Ethics Committee.

FACS analysis of mouse thymocytes and splenocytes

The thymus/spleen was minced and passed through a 40- μ m cell strainer (BD Biosciences, San Jose, CA, USA). The cells were washed with PBS and incubated with 1 ml ACK red blood cell lysis solution (Invitrogen, Carlsbad, CA, USA), followed by a second wash with PBS. A total of 1×10^6 thymocytes were then stained with the following antibodies: FITC-conjugated anti-CD4, PE-conjugated anti-CD8 α and PE-CY5-conjugated anti-CD3 (BD Pharmingen, San Diego, CA, USA), whereas splenocytes were stained with a selection of antibodies specific to the various T-cell receptor (TCR) V β alleles in combination with APC anti-CD3 (BD Pharmingen). FACS results were analysed using the CyAn ADP (Dako cytometry, Fort Collins, CO, USA) Summit v4.1 (Dako cytometry) software. Data are presented as means \pm S.E.M., unless noted otherwise.

Isolation of mononuclear cells from human peripheral blood

Fresh blood, collected from ALS patients and healthy controls, was diluted 1:1 with PBS, and loaded on a Ficoll gradient (Uni-Sep, Novamed, Jerusalem, Israel). Tubes were centrifuged for 20 min. at $1000 \times g$, at 20°C. The mononuclear cell phase was collected and washed twice with PBS. Cells were used as sources for mRNA and DNA (purified by QIAGEN (Valencia, CA, USA) RNeasy and DNeasy kits, according to the manufacturers' protocols).

Analysis of human TCR gene rearrangement

Analysis was performed with DNA extracted from patients' peripheral blood mononuclear cells (PBMCs). The different recombination events of the TCR genes were quantified by real-time quantitative polymerase chain reaction (Q-PCR), using the ABI PRISM 7500 Sequence Detector *TaqMan* system (Applied Biosystems, Rotkreuz, Switzerland), as previously described [32]. The following TCR recombinations were determined in

Table 1 Human TCR gene rearrangement primers and probe

Target	Forward primers (5'-3')	Reverse primers (5'-3')	Probe (FAM-TAMRA) (5'-3')
TCRA germline	tttcaacctgctgacacct	ggcacattagaatctctcaactga	ctgacattggagccaacactagaggaatc
TCRD-D δ 2- δ 3	caaggaaagggaaaaggaagaa	Tgtcccctgcagttttgtac	atagccacagtgtctacaaaacctacagagacct
TCRD-D δ 2- δ 1	agcgggtggtgatggcaaat	ttagatggaggatgcctaacctta	cccgtgtgactgtggaaccaagtaagtaactc
TREC	cacatcccttcaacctgct	gccagctgcagggttagg	acacctctggtttgtaaaggtgccact

independent PCR reactions: Genomic loss of germline *TCRA* DNA (indicative of immature T cells), Dd2-Dd3 (representative of the early CD4⁻CD8⁻ stage), Dd2-Jd1 rearrangements (representative of the late CD4⁻CD8⁻ stage) and TCR rearrangement excision circle (TREC), when the *TCRD* gene is deleted from the *TCRA/D* gene complex (representative of the late CD4⁺/CD8⁺ stage). Primers (900 nM) and FAM probes (250 nM) for PCR are listed in Table 1. Amplification reactions (25 μ l) contained 0.5 mg of genomic DNA, 12.5 μ l of TaqMan universal PCR master mix (Perkin Elmer Applied Biosystem, Foster City, CA, USA) and the appropriate primers and probes. PCR (2 min. at 50°C followed by 95°C for 10 min., then 40 cycles at 95°C for 15 sec. and 60°C for 1 min.) was carried out in an ABI PRISM 7900 Sequence Detector *TaqMan* system (Applied Biosystems). The number of TRECs in a given sample was estimated by comparing the CT value obtained with a standard curve obtained from PCRs performed with 10-fold serial dilutions of an internal standard provided by Dr. Daniel Douek (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). The dilutions contained 10²–10⁶ copies of signal-joint T-cell receptor excision circles (sjTRECs), in triplicates. The number of copies of other recombinations of TCR genes in a given sample was quantified in a relative manner, because a standard curve for other real-time PCR experiments was not available. Amplification of RNaseP (Applied Biosystems) was used to verify the quantity and presence of genomic DNA. Age matched normal individuals were used as controls.

TCR- γ PCR and fluorescent fragment analysis of ALS patients

TCR- γ rearrangements were amplified by PCR according to the standardized Biomed 2 protocol [33]. For GeneScan analyses, V γ primers were fluorescently labelled and used for PCR, as described in the Biomed 2 protocol [33]. Fluorescence-labelled PCR amplicates (1 μ l of each) was added to a mixture of 12.5 μ l deionized formamide and 0.5 μ l GeneScan 500TM Rox internal lane standard (PE Applied Biosystem, Weiterstadt, Germany).

Thymus imaging

An ECG-gated MRI scan of the chest and anterior mediastinum was obtained using a 1.5 Tesla MRI scanner and double inversion recovery technique.

Immune-related gene expression screening

Pre-designed TaqMan low-density arrays (TLDA; 96 TaqMan[®] Gene Expression assay preconfigured in a 384-well format, Part no. 4370573, microfluidic cards; Applied Biosystems), were used in a two-step RT-PCR process using the ABI Prism 7900HT Sequence Detection System (Applied

Biosystems) with a TaqMan Low-Density Array Upgrade (Applied Biosystems) to test two ALS patients and two age-matched healthy controls. Each cDNA sample (100 μ l) was added to an equal volume of 2 \times TaqMan Universal PCR Master Mix (Applied Biosystems). After gentle mixing, the solution was transferred into a loading port on a TLDA card (Applied Biosystem). Each of the samples was analysed in a single TLDA run with each of two TLDA experiments running on separate cards using an Applied Biosystems 7900 HT Fast-Real-Time PCR System. Thermal cycler conditions were as follows: 2 min. at 50°C, 10 min. at 94.5°C and 30 sec. at 97°C, and 1 min. at 59.7°C for 40 cycles. Data acquisition was done according to the manufacturer's directions. For analysis, expression levels of target genes were normalized to GUSB. Only genes with reproducible amplification curves in duplicate determinations were further verified in a larger group of ALS patients and age-matched healthy controls.

mRNA gene analysis by quantitative RT-PCR

The expression of specific mRNAs was assayed using fluorescence-based Q-PCR with selected gene-specific primer pairs. Q-PCR reactions were performed with Absolute(tm) Q-PCR SYBR[®] Green ROX mix (ABgene). Q-PCR products were detected by SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN, USA), measured in triplicate for each of the cDNA samples using the Rotor-Gene 6 instrument (Corbett Research, Mortlake, Australia), and analysed using Rotor-Gene 6000 software (version 1.7, Corbett). 18S ribosomal RNA (18S) was chosen as a reference gene. The amplification cycle was 95°C for 5 sec., 60°C for 20 sec. and 72°C for 15 sec. At the end of the assay, a melting curve was constructed to evaluate the specificity of the reaction. The primers are listed in Table 2.

Statistical analysis

The JMP statistics package was used to perform statistical analysis of all data. Data were analysed using a two-tailed Student's t-test or a Wilcoxon–Mann–Whitney test, corrected for a large sample approximation, with genetic background (mice) or clinical status (human) as the between-subject factors.

Results

Thymic malfunction in mSOD1 mice

We first tested whether any thymic malfunction spontaneously develops in mSOD1 mice, concomitantly with disease onset and/or

Table 2 Primers for gene expression analysis

Gene	Forward 5'-3'	Reverse 5'-3'
<i>18S</i>	TGTTCAAAGCAGGCCCGAG	CGGAACTACGACGGTATCTGATC
<i>IFNG</i>	GTGTGGAGACCATCAAGGAAGACA	GCGTTGGACATTCAAGTCAGTTACCG
<i>IL18</i>	ACAGCTTCGGGAAGAGAAAGGAA	TGTCTTCTACTGGTTCAGCAGCCA
<i>IL8</i>	TGCCAAGGAGTGCTAAAG	CTCCACAACCCCTCTGCAC
<i>BCL2</i>	AGTACCTGAACCGGCACCT	CAGCCAGGAGAAATCAAACAG
<i>BAX</i>	CTGCAGAGGATGATTGCCG	TGCCACTCGGAAAAAGACCT
<i>CD80</i>	GCCTGACCTACTGCTTTGCC	GGGCGTACACTTTCCCTTCTC
<i>CD86</i>	GCGGCCTCGCAACTCTTATA	TCTCTTTTCTGGTCTGTCTACTCTC

progression. Maturation in the thymus goes through distinct stages defined by the expression of CD4 and CD8 co-receptors. On the basis of the expression of these two markers, thymocytes can be classified as double-negative (DN; CD4⁻CD8⁻), double-positive (DP; CD4⁺CD8⁺) or single-positive (SP; CD4⁺ or CD8⁺) cells [34]. T-cell precursors that enter the thymic cortex are DN. As maturation proceeds, cells move toward the medulla and become DP thymocytes. DP cells continue their maturation by reaching the medulla and becoming mature SP T cells; they then leave the thymus to travel through the bloodstream and reach secondary lymphoid organs [34]. To search for a T-cell deficit that may occur in ALS during the course of disease progression, we first analysed the thymic cellular composition in mSOD1 mice (G93A) during disease progression (120–130-day-old animals), as well as in wild-type age-matched controls. We found a significant reduction in thymic cell content during the course of disease progression in mSOD1 mice (Fig. 1A), compared to their age-matched wild-type controls. In absolute terms, the thymocyte number in mSOD1 mice was significantly lower in all thymocyte populations tested: DN cells, DP cells, and the two populations of SP cells (Fig. 1B–E). This reduction in thymic cellularity correlated with an increased proportion of DN cells, and decreased proportion of DP cells (Fig. 1F and G, respectively). These results suggested that the thymus of ALS mice undergoes accelerated degeneration. To address whether this phenomenon is correlated with disease progression, we tested thymic cellularity in 60-day-old mSOD1 mice, before disease onset. The only difference found between wild-type and mSOD1 mice at this time-point was the elevation in the number of CD8⁺ thymocytes (Fig. 1H). By disease end-stage, the shrinkage of the thymus in the mSOD1 mice could be observed macroscopically (Fig. 1I). Moreover, sections taken through the thymus and stained by haematoxylin and eosin, revealed a complete loss of structure (no clearly delineated cortex and medulla could be discerned) in the 120-day-old mSOD1 mice, relative to 60-day-old mSOD1 or wild-type mice (Fig. 1J). Similar thymic dysplasia is found in severe combined immune deficient (SCID) mice [35], as well as under conditions of stroke and cancer [28, 30]. To test whether the reduction in T-cell numbers was

a general phenomenon or restricted to some but not all clones, we examined the V β T-cell repertoire in the spleen of mSOD1 and control mice. The normal clonal distribution observed in mSOD1 mice in the different stages of the disease (Fig. 1K) suggests that the loss was not selective to a single clone, and that no immune activation of a selective clone took place, as is the case in autoimmune diseases [36]. The thymic dysfunction that developed along with disease progression, was also manifested by a progressive reduction in spleen size, and significant changes in the proportion of CD4⁺/CD3⁺ T cells (reduction of 7%; $P = 0.05$) and CD8⁺/CD3⁺ cells (increase of 12.5%; $P = 0.01$) in the blood, from day 110 onward.

Thymic output deficit in ALS patients

In extending the findings to ALS patients, we quantified sjTRECs, as a measure of thymic output, in the PBMCs. sjTRECs are stable DNA episomes that form during TCR rearrangement. Because sjTRECs are not replicated during chromosomal DNA replication, they are diluted out during cell division, which takes place both as part of the priming of recent thymic emigrants (naïve T cells) to become memory T cells, and as part of homeostatic cell division of naive T cells. The level of sjTRECs in peripheral blood T cells is therefore an accepted measure of thymic function [13]. For example, a reduction in the sjTREC numbers within the CD4⁺ T-cell population occurs during normal aging [13, 37–39], as a result of thymic involution. We therefore first quantified sjTREC in the PBMCs of 11 ALS patients (30–62 years-old) and 12 age-matched controls (25–63 years-old). The 11 patients included both genders ($n = 2$ females, $n = 9$ males), and were randomly recruited. Thus, this population included sporadic, and possibly also familial, ALS patients. PBMCs were isolated from fresh blood, on a Ficoll gradient, followed by DNA extraction. The number of sjTREC copies per DNA content was determined by real-time Q-PCR. The absolute number of sjTRECs in a given sample was estimated using a standard curve of internal controls, and was normalized against RNaseP expression. A significant reduction ($t_{3,4} = -3.06$;

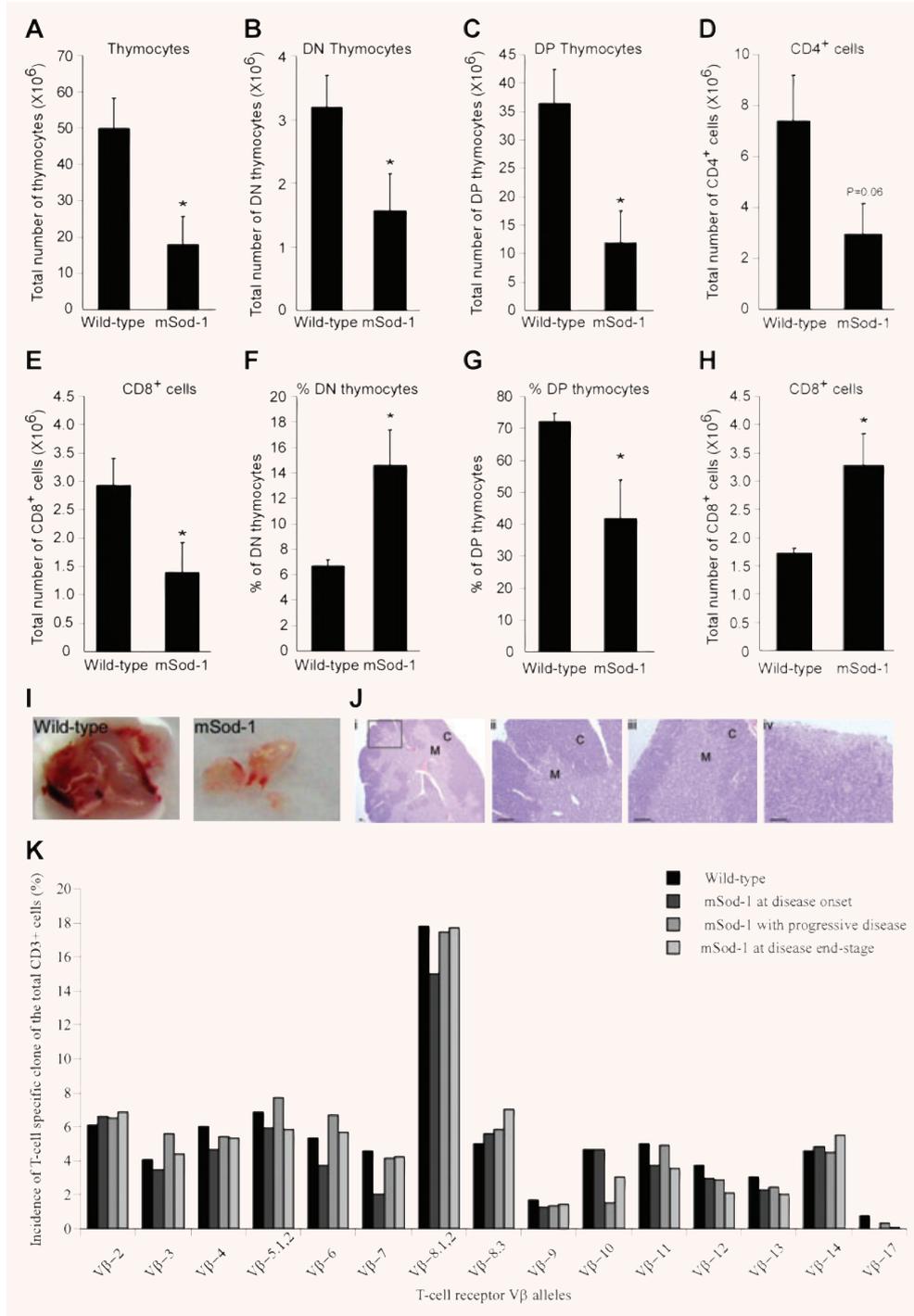


Fig. 1 Thymic cell content is reduced in mSOD-1 mice during the course of disease progression. Two-colour flow-cytometry was used to analyse the phenotypes and total cell number in the thymus of mSOD1 mice and their age-matched wild-type controls during the progressive stage of the disease (day 120; **A–G**) and prior to disease onset (day 60; **H**). (**A–E**) Total thymocyte number is significantly reduced at the progressive stage of the disease: (**A**) Total number of thymocytes ($t_{12} = -2.84$; $*P = 0.01$). (**B**) Total number of DN thymocytes ($t_{12} = -2.12$; $*P = 0.05$). (**C**) Total number of DP thymocytes ($t_{12} = -2.97$; $P = *0.01$). (**D**) Total number of CD4⁺ cells ($t_{12} = -2.07$; $P = 0.06$). (**E**) Total number of CD8⁺ cells ($t_{12} = -2.2$; $P = *0.04$). Reduction in thymic cellularity correlated with an increased proportion of DN cells (**F**, $t_{6,4} = 2.82$; $*P = 0.02$) and decreased proportion of DP cells (**G**, $t_{6,6} = -2.5$; $*P = 0.04$). (**H**) Significant elevation in the absolute number of CD8 cells was observed at day 60, prior to disease onset ($t_{4,23} = 2.7$; $*P = 0.05$). Values represent means \pm S.E.M. (**I**) Representative photomicrographs of thymus collected from mSOD-1 mouse at the end-stage of the disease and its age-matched wild-type control. (**J**) Thymic dysplasia was detected in mSOD1 mice upon disease progression. Sections

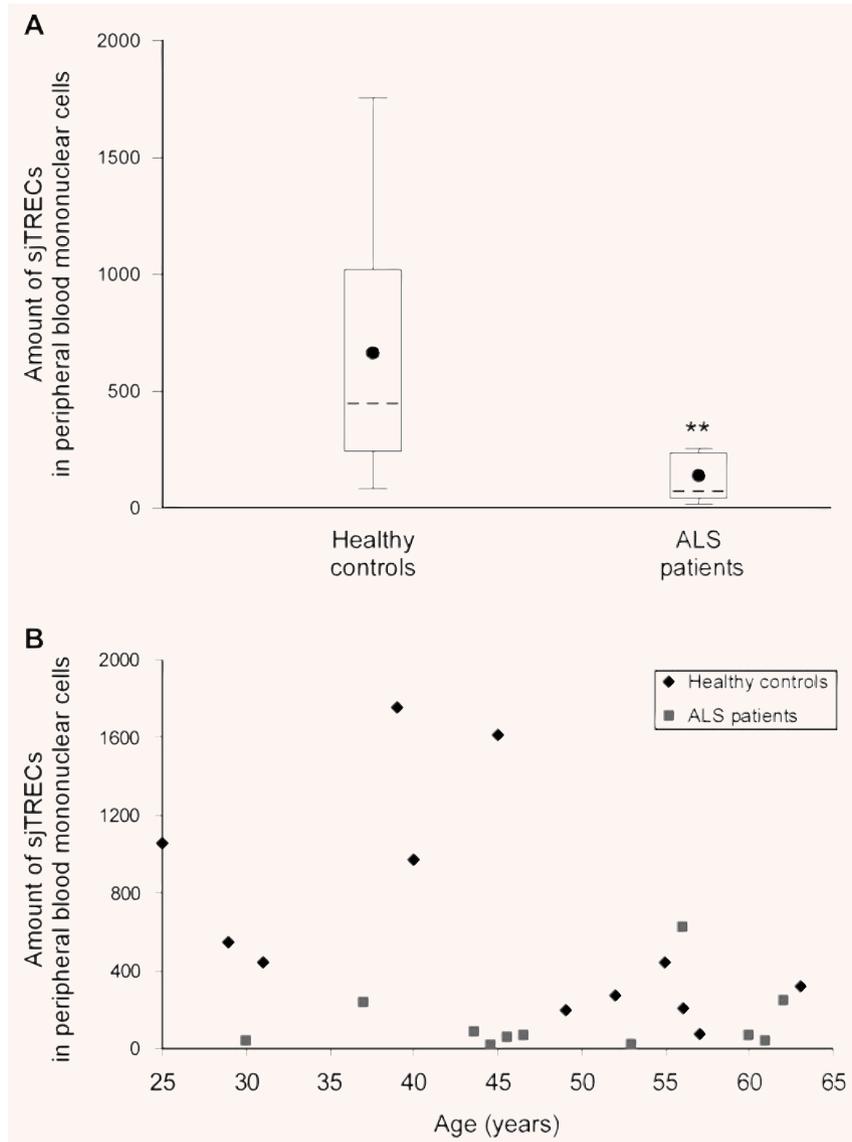


Fig. 2 Thymic output is severely reduced in ALS patients. Total DNA was purified from PBMCs that were collected from 11 ALS patients (30–62 years old; average age: 49.1 ± 10.3) and 12 age-matched (25–63 years old; average age: 45.1 ± 12.3 ; $P = 0.40$) healthy controls. **(A)** The amount of sjTREC was measured by Q-PCR, and was normalized against RNaseP. A significant reduction was found in the copies of sjTREC in the blood of ALS patients, compared to controls ($t_{3,4} = -3.06$; $**P = 0.008$). The graph shows the interquartile range of the measurements. The dashed line represents the median value; the circle indicates the average value. **(B)** Scatterplot of sjTREC levels by age.

$P = 0.008$) in the number of sjTRECs was found in the ALS patients relative to healthy controls (Fig. 2A). Moreover, plotting the sjTREC levels as a function of age (Fig. 2B), showed that although the numbers of sjTRECs in healthy donors decreased with age, as expected, the numbers of sjTRECs in the ALS patients was low throughout the entire range of ages tested. The age-inappropriate low sjTREC numbers observed in young ALS patients (ages 30–50; Fig. 2B), compared with age-matched controls, may indicate thymic malfunction. We compared the sjTREC values to disease severity, based on the most widely used clinical measure in ALS patients, the ALS Functional Rating Scale [40], which measures muscle strength and bulbar function through 12 parameters (speech, salivation, swallowing, handwriting, cutting food, dressing

and hygiene, turning in bed, walking, climbing stairs, dyspnoea, orthopnea, respiratory insufficiency – with five degrees of severity for each parameter). Based on this scale, despite the small number of patients, a rough correlation could be found between two groups of patients: the group with the lowest score (less severe disease) ($n = 4$) showed an average sjTREC levels of 333, whereas the other patients ($n = 11$) showed an average sjTREC levels of 75 ($P = 0.06$; one-tailed Student's *t*-test). For this analysis, we included all the 15 patients that we analysed, aged 30–73 years.

The dramatic reduction in sjTREC in the patients, which indicated thymic involution, prompted us to image the thymus of several young patients, to confirm that indeed the reduced TREC reflect anatomical changes. Three patients were examined, two by

MRI and one by X-ray. In all tested patients no traces of thymus tissue could be detected. An axial double inversion recovery image at the level of the ascending aorta in the examined ALS patients (males, 30 and 40 years old) showed clear retrosternal fat with no evidence of thymic remnants (Fig. S1).

The TCR is created through a series of sequential recombination events between the different TCR genes ($TCRD > TCRG > TCRB > TCRA$) as well as within each specific gene (e.g. $TCRD$: $D\delta 2$ - $D\delta 3$, $D\delta 2$ - $J\delta 1$, $V\delta$ - $J\delta 1$); different segments are excised during each stage of this sequential process [32, 41]. To further characterize thymic function in ALS patients, we measured the levels of two additional recombination events of $TCRD$, $D\delta 2$ - $D\delta 3$ (D-D) and $D\delta 2$ - $J\delta 1$ (D-J), which represent the early and late DN stage, respectively [32], and occur prior to the exclusion of the sjTRECs. QPCR results were normalized against $TCRA$ germline DNA (an indication of T-cell number). No difference was found between ALS patients and healthy donors in the two events tested, the D-D ($t_{21} = -0.15$; $P = 0.88$) and D-J ($t_{21} = -0.35$; $P = 0.73$) recombinations (Fig. 3A and B).

Because most T-cell recombination events occur at the transition stage between DN and DP progenitors [32], our results from patients are consistent with the increased proportion of DN thymocytes and decreased proportion of DP thymocytes in the thymus of $mSOD1$ mice (Fig. 1F and G), and the loss of thymic architecture in terms of a distinct cortex and medulla (Fig. 1J) [34, 42, 43]. These results also provide an explanation for the recent report showing that in ALS patients, levels of $CD45RA^+$ (naïve) T cells are diminished, whereas levels of $CD45RO^+$ (memory) T cells are increased, compared to age-matched controls [24].

An additional measure of the status of an individual's immune capabilities is the diversity of their TCR repertoire [44]. The TCR repertoire is comprised of naïve T cells that have recently migrated from the thymus, as well as a circulating pool of antigen-experienced peripheral T cells. Lymphoid clonality is often detected by PCR analysis of $TCR\gamma$ gene rearrangement, which represents the 'prototype' of restricted-repertoire targets [45]. The γ -chain gene, although typically not expressed on the cell surface, remains rearranged in the T-cell genome, and provides a conventional marker of clonality due to its expression in both $TCR\alpha\beta$ and $TCR\gamma\delta$ T-cells [46–48]. We therefore evaluated the peripheral $TCR\gamma$ repertoire in ALS patients. We found that the repertoire of the existing T-cell population in these patients was restricted (Fig. 4), although the repertoire did not constitute a monoclonal population, as found in cancer [48].

Changes in expression of immune-related genes in ALS patients relative to age-matched controls

In order to better understand the global immune deviation associated with ALS, the expression profile of 96 immune-related genes was studied using a predesigned array (TLDA) in PBMCs taken from two of the examined ALS patients compared to two healthy controls. The genes that showed the most significant differences (Table 3) were further confirmed in these patients and in an

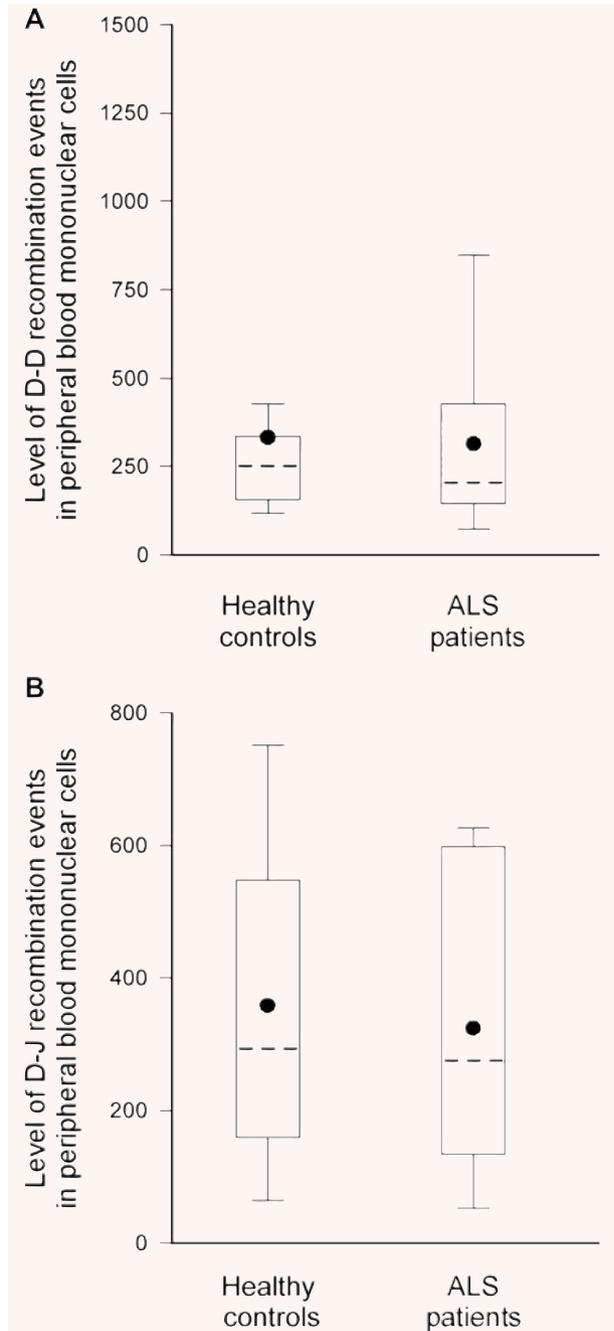


Fig. 3 Normal levels of early T-cell recombination events in ALS patients. Total DNA was purified from PBMCs that were collected from 11 ALS patients (30–62 years old; average age: 49.1 ± 10.3) and 12 age-matched (25–63 years old; average age: 45.1 ± 12.3 ; $P = 0.40$) control donors. The early recombination events were quantified using Q-PCR. No differences were found in the two tested events, D-D (**A**; $t_{21} = -0.15$; $P = 0.88$) and D-J (**B**; $t_{21} = -0.35$; $P = 0.73$). The graphs indicate the interquartile range of the measurements. The dashed line represents the median value; the circle indicates the average value.

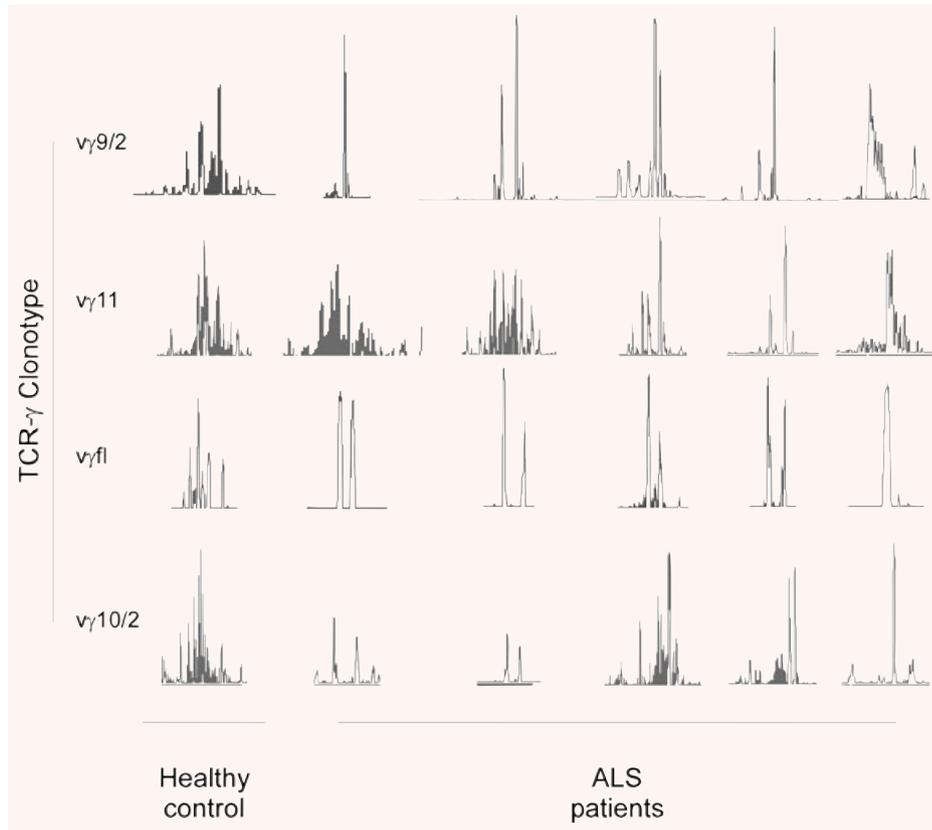


Fig. 4 Restricted TCR γ repertoire in ALS patients. Genescan display of four PCR-generated profiles of TCR γ rearrangements, from five representative patients. V γ 9/2 and V γ fl represent FAM labelled fluorescent variable region primers. V γ 11 and V γ 10/2 are HEX labelled fluorescent variable region primers. Nine additional patients were similarly analysed and demonstrated a similar profile, suggesting restricted polyclonality, though not monoclonality, relative to normal healthy individuals.

Table 3 Gene array expression analysis

Gene	Expression ratio (healthy/ALS) normalized to GUSB
<i>IFNG</i>	11.0
<i>IL18</i>	11.7
<i>CD80</i>	8.0
<i>CD86</i>	6.8
<i>IL8</i>	7.9

Differences in immune-related gene expression in PBMCs of ALS patients versus healthy controls. The table shows only selected genes that showed the most prominent differences. Values represent expression ratio (healthy controls/ALS patients), normalized to the housekeeping gene, GUSB.

additional six patients by quantitative real-time PCR. Altogether, eight ALS patients and six age-matched healthy controls were analysed for the expression of *CD80*, *CD86*, *IL8*, *IFNG* and *IL-18*. As can be seen (Fig. 5A–E), relative to the healthy controls, the patients showed a dramatic reduction in all of the above genes, all of which are key factors in the T-cell mediated immune response. Thus for example, IL-8 is a major chemoattractant that signals immune cells to arrive to the site of need, in the initiation of an

immune response [49]. CD80 and CD86 are the most important T-cell co-stimulatory molecules required for activation of the T cells [50]. Consistent with this trend is the observed reduction in the expression of *IL18* and *IFNG*; IL-18 is a pleiotropic cytokine involved in the polarization of the T-cell response and induces production of interferon (IFN)- γ , which is one of the major cytokines associated with CD4⁺ T cells [51].

To gain a molecular insight to the peripheral immune deficit in the ALS patients, we also compared in the mRNA of PBMCs from these patients the expression ratio of *BAX* to *BCL2*, which determines the susceptibility of a cell to apoptosis. We found a significant increase in the *BAX* to *BCL2* ratio in ALS patients (Fig. 5F), as a result of reduced *BCL2* expression (3.8 ± 3.5 versus 14.6 ± 10.6 in ALS patients and healthy controls, respectively; $Z = 2.26$, $P = 0.02$). Taken together with the reduction in sTREC, these results further demonstrate the peripheral immune cell morbidity in ALS patients and attribute the loss of T cells to their increased susceptibility to apoptosis.

Discussion

In this study, we present novel data demonstrating thymic malfunction in both a mouse model of ALS and in ALS patients.

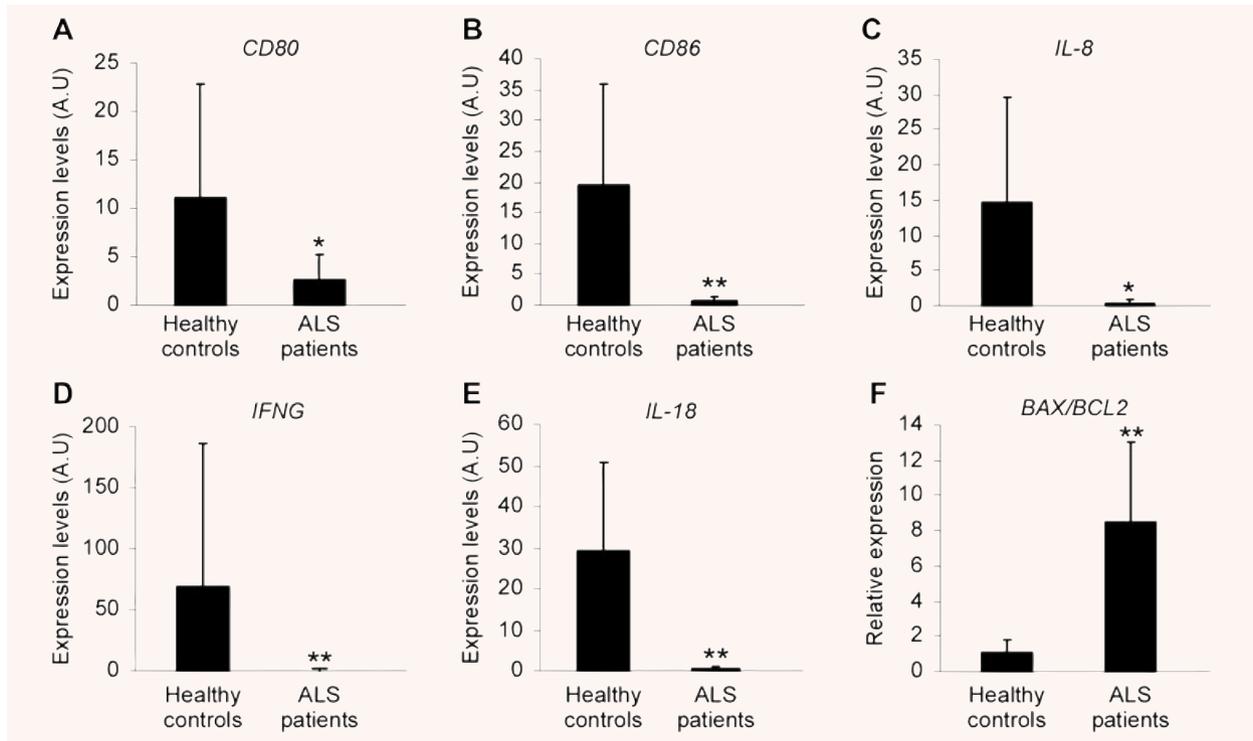


Fig. 5 Reduced immune potential and increased susceptibility to apoptosis in PBMCs from ALS patients. Expression levels of (A) *CD80* ($Z = 2.3$, $*P = 0.02$), (B) *CD86* ($Z = 3.0$, $**P = 0.002$), (C) *IL8* ($Z = -2.5$, $*P = 0.01$), (D) *IFNG* ($Z = 2.8$, $**P = 0.006$), (E) *IL18* ($Z = 3.0$, $**P = 0.002$) and (F) *BAX* to *BCL2* ratio ($Z = -3.0$, $**P = 0.002$). Expression was assessed by real-time Q-PCR. Values represent expression levels in arbitrary units, normalized against 18S in the same samples ($n = 8$ and $n = 6$ for ALS patients and healthy controls, respectively; each was tested in triplicate). Error bars represent S.D.

Specifically, we found in the mouse model, evidence of thymic dysplasia, characterized by progressive reduction in thymic cellularity and by distortion of thymic structure. In ALS patients, we found reduced sjTREC levels, a measure of thymic output potential in the blood, relative to their age-matched controls, with no significant reduction in two earlier events of TCR rearrangement (D-D and D-J). Moreover, in ALS patients, we demonstrate restricted T-cell clonality. Gene array revealed a significant reduction in expression of genes associated with T-cell activity, including *CD80*, *CD86*, *IL18* and *IFNG*. The loss of T cells in the patients was also associated with a reduction in expression of the anti-apoptotic gene, *BCL2*, and the complete disappearance of the thymus tested by imaging in three young patients.

The scarcity of blood-borne immune cells in healthy CNS parenchyma, in combination with the concept of the CNS being an 'immune privileged site', have contributed to the common view that, under normal conditions, the CNS functions most effectively in the absence of any immune cell activity. However, it recently became evident that the nervous and immune systems engage in an intense bidirectional dialogue. For example, immune cells (peripheral T cells and resident microglia) and immune-related

molecules (Toll-like receptors) were found to have a role in the different steps of neurogenesis, including progenitor cell proliferation and neuronal differentiation [12, 52, 53]. In the injured CNS, immune cells (infiltrating monocyte-derived macrophages and peripheral $CD4^+$ T cells) contribute to recovery and regulation of neurotoxicity by secretion of growth factors, removal of dying neurons and detoxification of the environment [52, 54–57]. In line with these findings, boosting of T-cell levels was shown to be beneficial in animal models of acute or chronic neurodegenerative disorders [8, 17, 24].

The present findings of thymic dysfunction that develops in ALS over the course of disease progression suggests that one way to halt disease progression might be through passive transfer of T cells. Indeed, an independent recent study demonstrated that passive transfer of activated polyclonal T cells increases the life expectancy of mSOD1 mice [24]. The main conclusion of our present study, demonstrating T-cell deficiency in ALS and attributing to this deficiency a role in the disease progression, is also supported by the observations that depletion of $CD4^+$ T cells exacerbates the disease. Thus, deficiency in $CD4^+$ T cells is associated with a local increase in the cytotoxicity of the microglia and

astrocytes [20, 24, 25]. In addition, it was shown that in mSOD1 mice bred onto a TCR β deficient background (mSOD1 (G93A)/TCR $\beta^{-/-}$ mice), disease progression is significantly accelerated, and insulin-like growth factor (IGF)-1 levels are reduced in spinal cords [25].

Thymic involution, which develops in mSOD1 mice, could be a reflection of increased T-cell death occurring in the periphery [24], followed by a compensatory attempt to overcome the deficit by elevation in the number of new thymic emigrants, resulting in accelerated thymic involution. In fact, in PBMCs collected from the patients, a significant reduction in expression of the anti-apoptotic gene *BCL2* was observed. In addition, complete thymic involution was observed in both the animal model and in the patients that were examined. Similar thymic abnormalities were reported in the context of Down's syndrome and were attributed to SOD1 hyper-expression in Ts65Dn cells, causing enhanced generation of reactive oxygen intermediates (specifically H₂O₂ production) in thymocytes [29]. It is also possible that elevated oxidative stress levels, associated with motor neuron death [58], lead to thymocyte apoptosis. This thymic behaviour in ALS patients is reminiscent of the demonstration in intracranial glioma models, attributed to alterations in the expression of WNT protein, an important factor in the DN to DP transition of thymocyte development (reviewed in [59]), and to the increased expression of Notch1 and its ligand, Jagged-1, which induce thymocyte apoptosis [28]. Accordingly, the death of T cells in ALS patients, regardless of the SOD1 mutation, could be a reflection of a response to stress that might be a primary or secondary event in the disease onset/progression.

Our present study, showing the rapid development of T-cell malfunction at a time when a protective immune response is most needed, could explain the rapid progression of the disease both in the mouse model and in human patients. Taken together with other published observations [20, 24, 25, 29], the current study suggests that spontaneous protective T-cell mediated immunity functions in mSOD1 mice only at an early disease stage, but becomes dysfunctional at later stages.

Importantly, as with other CNS degenerative conditions, our present findings of peripheral immune deficiency in ALS do not conflict with the observation of a local inflammatory response in the spinal cord of ALS mSOD1 mice and in patients [60, 61]. Rather, local inflammation is often cytotoxic and detrimental to the neural tissue; boosting peripheral immunity helps recruit immune cells (blood monocytes and CD4⁺ T cells) that can locally regulate this cytotoxicity and allows a shift in the local immune cells from cytotoxic towards protective [62, 63]. For example, 'alternatively activated' macrophages were shown to support CNS repair [64–66]. Cytotoxic microglia, which express high levels of tumour necrosis factor (TNF) α , fail to produce essential trophic factors such as IGF-1; suppressing microglial expression of TNF α was shown to be accompanied by production of IGF-1 [22, 52, 55, 67, 68]. Thus, thymic defects, resulting in reduced T-cell availability, might be a factor in the limited ability to recruit the peripheral blood-borne monocytes required for regulating the local microglial response and for restoration of homeostasis. This dis-

tingent contribution to the ALS pathology of the peripheral immune deficit and the local cytotoxic immune activation in the spinal cord, is in line with previous studies demonstrating increased levels of pro-inflammatory cytokines in the CSF of ALS patients [69], whereas the T-cell associated cytokine IFN- γ could not be detected [70]. The necessity for T-cell support for controlling the local inflammation in the diseased spinal cord is also consistent with the detrimental effect of treating ALS patients with the immunosuppressive drug, minocycline [71], or of frequent treatment of mSOD1 mice with adjuvant-free glatiramer acetate (Copaxone) [72], a regimen that suppresses peripheral immunity in multiple sclerosis [73].

Because a reduction in new thymic emigrants can occur transiently, as a result of acute diseases or treatment with immunosuppressive drugs, it is important to note that none of the patients, at the time that the blood samples were taken, exhibited any of these conditions or was being treated with immunosuppressive therapies. Although the ALS patients were found here to have a limited T-cell immunity, there are no reports describing an increased incidence of mortality in such patients as a result of infectious diseases. This could be due to the short life expectancy of these patients (3–5 years following diagnosis) and the long lifespan of naive T cells in the periphery (3–30 years) [74]. Yet, in agreement with our results, several reports indicated elevated susceptibility to infection in ALS patients, as compared to healthy controls: ALS patients show higher levels of plasma endotoxin/lipopolysaccharide [75], higher rates of retroviral enzymes detected in the blood [76, 77] and a higher frequency of systemic mycoplasmal infections [78]. Moreover, the AIDS virus was shown to cause a form of ALS-like disease [79].

We show in the present study that the malfunctioning of the thymus in ALS patients is manifested by a combination of several parameters: reduced levels of sjTRECs, (with no changes in levels of early T-cell recombination events), restricted T-cell polyclonality and disappearance of thymic tissue. In addition, a reduction in the potential activity of the remaining T cells was demonstrated by the reduced expression of the genes encoding two key co-stimulatory molecules, CD80 and CD86, and two cytokines, IFN- γ and IL-18. Reduction in sjTREC levels has also been observed in autoimmune diseases [80], cancer [81], aging and HIV infection [13]. Yet, the finding in the present study of a reduction in sjTRECs together with normal levels of the early T-cell recombination events differs from the profile observed in any other pathology investigated, such as cancer [41]. Importantly, reduced levels of naive T-cells also characterize thymic aging [82], autoimmune diseases [82] and early stages of HIV [83]. An overall reduction in all T-cell subsets, on the other hand, characterizes immunodeficiency states, such as the late stage of HIV [84].

In light of the existing literature and our present results, it is possible that the malfunction of the immune system in ALS patients represents a form of a sudden and rapid immunosenescence. We propose that the T-cell malfunction found in ALS be viewed as a co-morbidity factor. If this is demonstrated to be the

case, it suggests a therapeutic direction consisting of overcoming T-cell deficiency by means of thymic reconstitution, thymus transplantation, and induction of thymopoiesis.

Acknowledgements

We thank S. Schwarzbaum for editing the manuscript. M.S. is the incumbent of the Maurice and Ilse Katz Professorial Chair in Neuroimmunology. The study was supported by a grant from IsrALS, the Israeli ALS Research Association, by Israeli Academy of Science (given to M.S.) and by the Marciano Family Foundation. A.S. is a graduate student in the Cedars-Sinai Graduate Program in Biomedical Sciences and Translational Medicine.

References

1. Lee JR, Annegers JF, Appel SH. Prognosis of amyotrophic lateral sclerosis and the effect of referral selection. *J Neurol Sci.* 1995; 132: 207–15.
2. Rosen DR. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature.* 1993; 362: 59–62.
3. Boillee S, Yamanaka K, Lobsiger CS *et al.* Onset and progression in inherited ALS determined by motor neurons and microglia. *Science.* 2006; 312: 1389–92.
4. Clement AM, Nguyen MD, Roberts EA *et al.* Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science.* 2003; 302: 113–7.
5. Gowing G, Philips T, Van Wijmeersch B *et al.* Ablation of proliferating microglia does not affect motor neuron degeneration in amyotrophic lateral sclerosis caused by mutant superoxide dismutase. *J Neurosci.* 2008; 28: 10234–44.
6. Beers DR, Henkel JS, Xiao Q *et al.* Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA.* 2006; 103: 16021–6.
7. Hauben E, Butovsky O, Nevo U *et al.* Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J Neurosci.* 2000; 20: 6421–30.
8. Moalem G, Leibowitz-Amit R, Yoles E *et al.* Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med.* 1999; 5: 49–55.
9. Rollis A, Shechter R, London A *et al.* Two faces of chondroitin sulfate proteoglycan in spinal cord repair: a role in microglia/macrophage activation. *PLoS Med.* 2008; 5: e171.
10. Ron-Harel N, Segev Y, Lewitus GM *et al.* Age dependent spatial memory loss can be restored by immune activation. *Rejuvenation Research.* 2008; 11: 903–13.
11. Ziv Y, Avidan H, Pluchino S *et al.* Synergy between immune cells and adult neural stem/progenitor cells promotes functional recovery from spinal cord injury. *Proc Natl Acad Sci USA.* 2006; 103: 13174–9.
12. Ziv Y, Ron N, Butovsky O *et al.* Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci.* 2006; 9: 268–75.
13. Douek DC, McFarland RD, Keiser PH *et al.* Changes in thymic function with age and during the treatment of HIV infection. *Nature.* 1998; 396: 690–5.
14. Kipnis J, Cohen H, Cardon M *et al.* T cell deficiency leads to cognitive dysfunction: implications for therapeutic vaccination for schizophrenia and other psychiatric conditions. *Proc Natl Acad Sci USA.* 2004; 101: 8180–5.
15. Kipnis J, Yoles E, Schori H *et al.* Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response. *J Neurosci.* 2001; 21: 4564–71.
16. Yoles E, Hauben E, Palgi O *et al.* Protective autoimmunity is a physiological response to CNS trauma. *J Neurosci.* 2001; 21: 3740–8.
17. Hauben E, Agranov E, Gothilf A *et al.* Posttraumatic therapeutic vaccination with

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 An axial double inversion recovery MRI image at the level of the ascending aorta in a male with ALS, showing a clear retrosternal fat deposit (*arrow*) with no evidence of thymic remnants.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

18. Schori H, Kipnis J, Yoles E *et al.* Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. *Proc Natl Acad Sci USA.* 2001; 98: 3398–403.
19. Cohen H, Ziv Y, Cardon M *et al.* Maladaptation to mental stress mitigated by the adaptive immune system via depletion of naturally occurring regulatory CD4+CD25+ cells. *J Neurobiol.* 2006; 66: 552–63.
20. Beers DR, Henkel JS, Zhao W *et al.* CD4+ T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS. *Proc Natl Acad Sci USA.* 2008; 105: 15558–63.
21. Frenkel D, Maron R, Burt DS *et al.* Nasal vaccination with a proteasome-based adjuvant and glatiramer acetate clears beta-amyloid in a mouse model of Alzheimer disease. *J Clin Invest.* 2005; 115: 2423–33.
22. Butovsky O, Koronyo-Hamaoui M, Kunis G *et al.* Glatiramer acetate fights against Alzheimer's disease by inducing dendritic-like microglia expressing insulin-like growth factor 1. *Proc Natl Acad Sci USA.* 2006; 103: 11784–9.
23. Benner EJ, Mosley RL, Destache CJ *et al.* Therapeutic immunization protects dopaminergic neurons in a mouse model of Parkinson's disease. *Proc Natl Acad Sci USA.* 2004; 101: 9435–40.

24. **Banerjee R, Mosley RL, Reynolds AD et al.** Adaptive immune neuroprotection in G93A-SOD1 amyotrophic lateral sclerosis mice. *PLoS ONE*. 2008; 3: e2740.
25. **Chiu IM, Chen A, Zheng Y et al.** T lymphocytes potentiate endogenous neuroprotective inflammation in a mouse model of ALS. *Proc Natl Acad Sci USA*. 2008; 105: 17913–8.
26. **Angelov DN, Waibel S, Guntinas-Lichius O et al.** Therapeutic vaccine for acute and chronic motor neuron diseases: implications for amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA*. 2003; 100: 4790–5.
27. **Schwartz M, Ziv Y.** Immunity to self and self-maintenance: what can tumor immunology teach us about ALS and Alzheimer's disease? *Trends Pharmacol Sci*. 2008; 29: 287–93.
28. **Andaloussi AE, Han Y, Lesniak MS.** Progression of intracranial glioma disrupts thymic homeostasis and induces T-cell apoptosis *in vivo*. *Cancer Immunol Immunother*. 2008; 57: 1807–16.
29. **Paz-Miguel JE, Flores R, Sanchez-Velasco P et al.** Reactive oxygen intermediates during programmed cell death induced in the thymus of the Ts(1716)65Dn mouse, a murine model for human Down's syndrome. *J Immunol*. 1999; 163: 5399–410.
30. **Prass K, Meisel C, Hoflich C et al.** Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med*. 2003; 198: 725–36.
31. **Schwartz M, Ziv Y.** Immunity to self and self-maintenance: a unified theory of brain pathologies. *Trends Immunol*. 2008; 29: 211–9.
32. **Dik WA, Pike-Overzet K, Weerkamp F et al.** New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med*. 2005; 201: 1715–23.
33. **van Dongen JJ, Langerak AW, Bruggemann M et al.** Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98–3936. *Leukemia*. 2003; 17: 2257–317.
34. **Takahama Y.** Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol*. 2006; 6: 127–35.
35. **Pearse G.** Normal structure, function and histology of the thymus. *Toxicol Pathol*. 2006; 34: 504–14.
36. **Kim G, Kohyama K, Tanuma N et al.** Persistent expression of experimental autoimmune encephalomyelitis (EAE)-specific Vbeta8.2 TCR spectratype in the central nervous system of rats with chronic relapsing EAE. *J Immunol*. 1998; 161: 6993–8.
37. **Linton PJ, Dorshkind K.** Age-related changes in lymphocyte development and function. *Nat Immunol*. 2004; 5: 133–9.
38. **Taub DD, Longo DL.** Insights into thymic aging and regeneration. *Immunol Rev*. 2005; 205: 72–93.
39. **Hazenber MD, Verschuren MC, Hamann D et al.** T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med*. 2001; 79: 631–40.
40. **Voustianiouk A, Seidel G, Panchal J et al.** ALSFRS and appel ALS scores: discordance with disease progression. *Muscle Nerve*. 2008; 37: 668–72.
41. **Asnafi V, Beldjord K, Boulanger E et al.** Analysis of TCR, pT alpha, and RAG-1 in T-acute lymphoblastic leukemias improves understanding of early human T-lymphoid lineage commitment. *Blood*. 2003; 101: 2693–703.
42. **Hollander GA, Wang B, Nichogiannopoulou A et al.** Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature*. 1995; 373: 350–3.
43. **Shores EW, Van Ewijk W, Singer A.** Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur J Immunol*. 1991; 21: 1657–61.
44. **Nikolich-Zugich J, Slifka MK, Messaoudi I.** The many important facets of T-cell repertoire diversity. *Nat Rev Immunol*. 2004; 4: 123–32.
45. **van Krieken JH, Langerak AW, Macintyre EA et al.** Improved reliability of lymphoma diagnostics *via* PCR-based clonality testing: report of the BIOMED-2 Concerted Action BHM4-CT98–3936. *Leukemia*. 2007; 21: 201–6.
46. **Theodorou I, Raphael M, Bigorgne C et al.** Recombination pattern of the TCR gamma locus in human peripheral T-cell lymphomas. *J Pathol*. 1994; 174: 233–42.
47. **Ponti R, Quaglini P, Novelli M et al.** T-cell receptor gamma gene rearrangement by multiplex polymerase chain reaction/heteroduplex analysis in patients with cutaneous T-cell lymphoma (mycosis fungoides/Sezary syndrome) and benign inflammatory disease: correlation with clinical, histological and immunophenotypical findings. *Br J Dermatol*. 2005; 153: 565–73.
48. **Ponti R, Fierro MT, Quaglini P et al.** TCRgamma-chain gene rearrangement by PCR-based GeneScan: diagnostic accuracy improvement and clonal heterogeneity analysis in multiple cutaneous T-cell lymphoma samples. *J Invest Dermatol*. 2008; 128: 1030–8.
49. **Koch AE.** Review: angiogenesis: implications for rheumatoid arthritis. *Arthritis Rheum*. 1998; 41: 951–62.
50. **Greenfield EA, Nguyen KA, Kuchroo VK.** CD28/B7 costimulation: a review. *Crit Rev Immunol*. 1998; 18: 389–418.
51. **Nakanishi K, Yoshimoto T, Tsutsui H et al.** Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol*. 2001; 19: 423–74.
52. **Butovsky O, Ziv Y, Schwartz A et al.** Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci*. 2006; 31: 149–60.
53. **Rolls A, Shechter R, London A et al.** Toll-like receptors modulate adult hippocampal neurogenesis. *Nat Cell Biol*. 2007; 9: 1081–8.
54. **Shaked I, Tchoresh D, Gersner R et al.** Protective autoimmunity: interferon-gamma enables microglia to remove glutamate without evoking inflammatory mediators. *J Neurochem*. 2005; 92: 997–1009.
55. **Butovsky O, Talpalar AE, Ben-Yaakov K et al.** Activation of microglia by aggregated beta-amyloid or lipopolysaccharide impairs MHC-II expression and renders them cytotoxic whereas IFN-gamma and IL-4 render them protective. *Mol Cell Neurosci*. 2005; 29: 381–93.
56. **Kipnis J, Yoles E, Porat Z et al.** T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. *Proc Natl Acad Sci USA*. 2000; 97: 7446–51.
57. **Butovsky O, Bukshpan S, Kunis G et al.** Microglia can be induced by IFN-gamma or IL-4 to express neural or dendritic-like markers. *Mol Cell Neurosci*. 2007; 35: 490–500.

58. **Kanekura K, Suzuki H, Aiso S et al.** ER stress and unfolded protein response in amyotrophic lateral sclerosis. *Mol Neurobiol.* 2009; 39: 81–9.
59. **Staal FJ, Luis TC, Tiemessen MM.** WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol.* 2008; 8: 581–93.
60. **Graves MC, Fiala M, Dinglasan LA et al.** Inflammation in amyotrophic lateral sclerosis spinal cord and brain is mediated by activated macrophages, mast cells and T cells. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 2004; 5: 213–9.
61. **Weydt P, Weiss MD, Moller T et al.** Neuro-inflammation as a therapeutic target in amyotrophic lateral sclerosis. *Curr Opin Investig Drugs.* 2002; 3: 1720–4.
62. **Butovsky O, Kunis G, Koronyo-Hamaoui M et al.** Selective ablation of bone marrow-derived dendritic cells increases amyloid plaques in a mouse Alzheimer's disease model. *Eur J Neurosci.* 2007; 26: 413–6.
63. **Shechter R, London A, Varol C et al.** Essential role for myeloid derived cells in the recovery from spinal cord injury. Neuroscience 2008. Washington DC: The Society for Neuroscience's 38th Annual Meeting; *Plos Medicine.* 2009; 6: e1000113.
64. **Rossignol S, Schwab M, Schwartz M et al.** Spinal cord injury: time to move? *J Neurosci.* 2007; 27: 11782–92.
65. **Rapalino O, Lazarov-Spiegler O, Agranov E et al.** Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. *Nat Med.* 1998; 4: 814–21.
66. **Schwartz M, Yoles E.** Immune-based therapy for spinal cord repair: autologous macrophages and beyond. *J Neurotrauma.* 2006; 23: 360–70.
67. **Butovsky O, Landa G, Kunis G et al.** Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J Clin Invest.* 2006; 116: 905–15.
68. **Zhao W, Xie W, Xiao Q et al.** Protective effects of an anti-inflammatory cytokine, interleukin-4, on motoneuron toxicity induced by activated microglia. *J Neurochem.* 2006; 99: 1176–87.
69. **Mitchell RM, Freeman WM, Randazzo WT et al.** A CSF biomarker panel for identification of patients with amyotrophic lateral sclerosis. *Neurology.* 2009; 72: 14–9.
70. **Ford L, Rowe D.** Interleukin-12 and interferon-gamma are not detectable in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 2004; 5: 118–20.
71. **Gordon PH, Moore DH, Miller RG et al.** Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *Lancet Neurol.* 2007; 6: 1045–53.
72. **Bukshpan S, Kunis G, Schwartz M.** Daily injection of glatiramer acetate is risky in a mouse model of ALS: different requirements for multiple sclerosis versus other neurodegenerative diseases. *Biodrug.* 2008; 22: 293–9.
73. **Aharoni R, Teitelbaum D, Arnon R.** T suppressor hybridomas and interleukin-2-dependent lines induced by copolymer 1 or by spinal cord homogenate down-regulate experimental allergic encephalomyelitis. *Eur J Immunol.* 1993; 23: 17–25.
74. **Sprent J, Tough DF.** Lymphocyte life-span and memory. *Science.* 1994; 265: 1395–400.
75. **Zhang R, Miller RG, Gascon R et al.** Circulating endotoxin and systemic immune activation in sporadic amyotrophic lateral sclerosis (sALS). *J Neuroimmunol.* 2009; 206: 121–4.
76. **McCormick AL, Brown RH Jr, Cudkovic ME et al.** Quantification of reverse transcriptase in ALS and elimination of a novel retroviral candidate. *Neurology.* 2008; 70: 278–83.
77. **Steele AJ, Al-Chalabi A, Ferrante K et al.** Detection of serum reverse transcriptase activity in patients with ALS and unaffected blood relatives. *Neurology.* 2005; 64: 454–8.
78. **Nicolson GL, Nasralla MY, Haier J et al.** High frequency of systemic mycoplasma infections in Gulf War veterans and civilians with amyotrophic lateral sclerosis (ALS). *J Clin Neurosci.* 2002; 9: 525–9.
79. **Verma A, Berger JR.** ALS syndrome in patients with HIV-1 infection. *J Neurol Sci.* 2006; 240: 59–64.
80. **Prelog M, Schwarzenbrunner N, Sailer-Hock M et al.** Premature aging of the immune system in children with juvenile idiopathic arthritis. *Arthritis Rheum.* 2008; 58: 2153–62.
81. **Yamanaka K, Yawalkar N, Jones DA et al.** Decreased T-cell receptor excision circles in cutaneous T-cell lymphoma. *Clin Cancer Res.* 2005; 11: 5748–55.
82. **Stulnig T, Maczek C, Bock G et al.** Reference intervals for human peripheral blood lymphocyte subpopulations from 'healthy' young and aged subjects. *Int Arch Allergy Immunol.* 1995; 108: 205–10.
83. **Hazenber MD, Otto SA, Cohen Stuart JW et al.** Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med.* 2000; 6: 1036–42.
84. **Clark DR, de Boer RJ, Wolthers KC et al.** T cell dynamics in HIV-1 infection. *Adv Immunol.* 1999; 73: 301–27.