

Presence of Dendritic Cells, MCP-1, and Activated Microglia/Macrophages in Amyotrophic Lateral Sclerosis Spinal Cord Tissue

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Dendritic cells are potent antigen-presenting cells that initiate and amplify immune responses. To determine whether dendritic cells participate in inflammatory reactions in amyotrophic lateral sclerosis (ALS), we examined mRNA expression of dendritic cell surface markers in individual sporadic ALS (sALS), familial ALS (fALS), and nonneurological disease control (NNDC) spinal cord tissues using semiquantitative and real-time reverse transcription polymerase chain reaction (RT-PCR). Immature (DEC205, CD1a) and activated/mature (CD83, CD40) dendritic cell transcripts were significantly elevated in ALS tissues. The presence of immature and activated/mature dendritic cells (CD1a⁺ and CD83⁺) was confirmed immunohistochemically in ALS ventral horn and corticospinal tracts. Monocytic/macrophage/microglial transcripts (CD14, CD18, SR-A, CD68) were increased in ALS spinal cord, and activated CD68⁺ cells were demonstrated in close proximity to motor neurons. mRNA expressions of the chemokine MCP-1, which attracts monocytes and myeloid dendritic cells, and of the cytokine macrophage-colony stimulating factor (M-CSF) were increased in ALS tissues. The MCP-1 protein was expressed in glia in ALS but not in control tissues and was increased in the CSF of ALS patients. Those patients who progressed most rapidly expressed significantly more dendritic transcripts than patients who progressed more slowly. These results support the involvement of immune/inflammatory responses in amplifying motor neuron degeneration in ALS.

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Amyotrophic lateral sclerosis (ALS) is a progressive motoneuron disease characterized by the degeneration of upper and lower motoneurons, culminating in respiratory failure. Although the cause and pathogenesis of ALS are incompletely defined, increasing evidence indicates the presence of immune/inflammatory reactions that could contribute to motor neuron injury in ALS. In ALS spinal cord tissue, we and others have demonstrated the presence of T cells,^{1–4} IgG,⁵ activated microglia,^{2–4,7} macrophages,^{1–4,6,7} and reactive astrocytes,⁸ as well as other indications of inflammation.^{8–10} Analyses of blood, skin, and muscle from ALS patients indicate widespread inflammatory responses.^{11–15} In addition, in the mSOD1 transgenic mouse model of familial ALS (fALS), immune/in-

flammatory responses are present early in disease before any evidence of dysfunction.^{16–18} Such data suggest that the inflammatory responses could contribute to the pathogenesis of motor neuron injury.

Dendritic cells are the sentinels of the immune system, controlling both innate and adaptive immunity. Unlike other central nervous system (CNS) antigen-presenting cells, dendritic cells are able to prime naive T cells. A role for dendritic cells in the CNS has been demonstrated in multiple sclerosis (MS),^{19–20} experimental autoimmune encephalomyelitis (EAE),^{21,22} delayed-type hypersensitivity,²³ CNS infections,^{24,25} and after injury.²⁶ CNS dendritic cells can originate from infiltrating blood cells, from choroid plexus and/or meninges,^{24,25,27,28} or from microglia.^{21,26,29,30}

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Table 1. Patient Data

Patient No.	Sex	Age at Death (yr)	Location of Onset	Length of ALS Disease	SOD1 Mutation	Cause of Death	Analyses
sALS							
1	M	53	Bulbar	3.25	NA	Respiratory failure	R,I
2	M	70	Arm	0.9	NA	Respiratory failure	R
3	M	70	Arm	16.25	NA	Renal failure	R
4	F	54	Leg	3.0	NA	Respiratory failure	R
5	F	46	BP, Arm	4.5	NA	Respiratory failure	R
6	M	40	Bulbar	4.5	NA	Respiratory failure	R,I
7	M	77	Arm	3.3	NA	Respiratory failure	R,I
8	M	51	Leg	7.75	NA	Respiratory failure	R,I
9	F	71	Leg	0.9	NA	Respiratory failure	R
10	M	73	Bulbar	2.3	NA	Respiratory failure	I
11	M	47	Arm	7.0	NA	Respiratory failure	I
fALS							
12	M	60	Thighs	0.9	A4T	Respiratory failure	R,I
13	M	48	Bulbar	0.75	A4V	Respiratory failure	R
14	M	34	Hand	2.75	V148G	Respiratory failure	R
15	M	69	Abdomen	0.75	A4V	Respiratory failure	R
16	M	55	Leg	3.75	G85R	Respiratory failure	R
DC							
17	M	76	NA	NA	NA	MI	R
18	F	66	NA	NA	NA	Hypertension and sudden death	R
19	M	58	NA	NA	NA	Head trauma	R
20	M	33	NA	NA	NA	Asthma with sudden death	R
21	M	39	NA	NA	NA	CAD with pulmonary embolism	R
22	F	38	NA	NA	NA	Drug intoxication	R
23	M	60	NA	NA	NA	Cerebral infarct	I
24	F	75	NA	NA	NA	Alzheimer's disease	I
25	F	64	NA	NA	NA	Pneumonia	I
26	F	71	NA	NA	NA	Cerebral infarct	I
27	M	76	NA	NA	NA	Cerebral infarct	I
28	M	70	NA	NA	NA	MI	I
29	F	68	NA	NA	NA	Pulmonary embolism	I

DC = disease control; M = male; F = female; NA = not applicable; R = reverse transcription polymerase chain reaction; I = immunohistochemistry; MI = myocardial infarction; CAD = coronary artery disease; BP = brachial plexus.

Myeloid dendritic cells are most responsive to the chemokine MCP-1.^{24,27} Transgenic mice deficient in MCP-1 or its receptor, CCR2, are resistant to EAE and have a significant reduction in T-cell and monocyte infiltration.^{20,31,32} To determine whether immature and mature/activated dendritic cells and other immune/inflammatory cells were involved in the CNS inflammatory response in ALS and whether chemokine and cytokine expression levels were increased to recruit or activate such cells, we examined ALS spinal cord tissue and cerebrospinal fluid (CSF) using semiquantitative and real-time reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA).

Patients and Methods

Sporadic Amyotrophic Lateral Sclerosis, Familial Amyotrophic Lateral Sclerosis, and Nonneurological Disease Control Postmortem Tissues

Spinal cord autopsy tissues obtained from patients at our Muscular Dystrophy Association (MDA)/ALS Clinic at Bay-

lor College of Medicine (BCM; seven sporadic ALS [sALS], one fALS), the Department of Pathology, BCM (two non-neurological disease control [NNDC]), the Northwestern University Medical School (four fALS), and the University of Maryland Brain Bank (two sALS, four NNDC). The clinical diagnosis was confirmed pathologically for all sALS patients. No pathological changes were observed in the spinal cords of NNDC patients. Postmortem times were equivalent between ALS and NNDC patients (12.9 hours vs 13.4 hours, respectively). The relevant clinical data are summarized in Table 1.

Paraffin sections of the lumbar, thoracic, and cervical spinal cords were obtained from our MDA/ALS Clinic, BCM (six sALS, one fALS). Sections obtained from the Department of Pathology, BCM, from seven controls who died of Alzheimer's disease (one), ischemic cerebral infarct (three), and NNDC (four), as well as human tonsil, were examined.

In addition, CSF was obtained with informed consent from 20 sALS and 20 random control patients (neurodegenerative and nonneurodegenerative diseases) followed at BCM. The controls consisted of patients with Parkinson's disease, Alzheimer's disease, MS, neuropathy, Bell's Palsy, radiculopathy.

Table 2. Semiquantitative Reverse Transcription Polymerase Chain Reaction Primer Pairs and Conditions

Target	Primer Pairs	Product Length	Annealing Temperature (°C)	No. of Cycles	Reference or Accession No.
β-actin	F, 5'-GTGGGGCGCCCCAGGCACCA-3' R, 5'-CTCCTTAATGTACAGCAGGATTTTC-3'	539	60	28	Draude and Lorenz ⁴⁴
CD1a	F, 5'-TTCCGTATACGCACCATTCCG-3' R, 5'-GTTGCGCGGAGATACCATGTC-3'	535	58	40	XM 048792
CD123	F, 5'-ATGGTCCCTCTTTGGCTCAC-3' R, 5'-CAAGTTTTCTGCACGACCTG-3'	1135	68	45	Leveque and colleagues ⁴⁵
CD11b	F, 5'-CAGAGCGTGGTCCAGCTTCAG-3' R, 5'-CCTTCATCCGCCGAAAGTCAT-3'	406	63	35	Noguera and colleagues ⁴⁶
CD11c	F, 5'-ATGACCAGGACCAGGGCAGCAGTC-3' R, 5'-AAAACCTGGGTGCTGGGTCTCTGGA-3'	564	63	40	Uwai and colleagues ⁴⁷
CD14	F, 5'-GGTGCCGCTGTGTAGGAAAGA-3' R, 5'-GGTCCCTCGAGCGTCAGTTCCCT-3'	453	65	35	Cario and colleagues ⁴⁸
CD18	F, 5'-TCGTGACAAGACCGTGCTGC-3' R, 5'-CTACTGGTCACCGCGAAGGTCG-3'	462	60	37	Luk and colleagues ⁴⁹
CD40	F, 5'-CAGAGTCACTGAAACGGAATGCC-3' R, 5'-TGCCCTGCCTGTTGCACAACC-3'	380	61	40	Lienenlueke and colleagues ⁵⁰
CD68	F, 5'-CATTCCCCTATGGACACCTCA-3' R, 5'-GTCTCCGGATGATGCAGAAAG-3'	391	67	39	XM 008237
CD83	F, 5'-AAGACCACCTCAGGGGACAGC-3' R, 5'-TGGGATCCCCTTCCAGATGTT-3'	573	58	38	XM 052592
DEC-205	F, 5'-TGGGCAACTCGAAGACTG-TA-3' R, 5'-AACATGACCCATGAAAGCC-3'	350	58	40	Kato and colleagues ⁵¹
GM-CSF	F, 5'-TGCAGAGCCTGCTGCTCTTG-3' R, 5'-CAAGCAGAAAGTCCTTCAGG-3'	390	71	40	Liu and colleagues ⁵²
HLA-DRα	F, 5'-TGGGACCATCTTCATCATCAAGG-3' R, 5'-GGGCATTCATAGCAGAGACAGAC-3'	380	61	37	Lichtenberger and colleagues ⁵³
IL-1β	F, 5'-CGATCACTGAACTGCACGCTCCG-3' R, 5'-GCCGGTCCATATTTGACTGAAGTGG-3'	431	71	45	Lesnikov and colleagues ⁵⁴
IL-6	F, 5'-CAGCTATGAACTCCTTCTCCACAAG-3' R, 5'-TGCCCATGCTACATTTGCCGAAGAG-3'	652	71	40	McGuinness and colleagues ⁵⁵
INF	F, 5'-CAGCTCTGCATCGTTTTGGGT-3' R, 5'-AGTGATGGCTGAACTGTCCGCC-3'	404	71	45	E 00692
M-CSF	F, 5'-CAGTTGTCAAGGACAGCAC-3' R, 5'-GCTGGAGGATCCCCTCGGACTG-3'	670	60	38	Atkins and colleagues ⁵⁶
MCP-1	F, 5'-ACTGAAGCTCGCACTCTC-3' R, 5'-CTTGGGTTGTGGAGTGAG-3'	359	58	37	De Rossi and colleagues ⁵⁷
MIP-1α	F, 5'-TCACCTGCTCAGAATCATGC-3' R, 5'-TCCATAGAAGAGGTAGCTGTGG-3'	404	60	45	Hatano and colleagues ⁵⁸
RANTES	F, 5'-ATGAAGTCTCCGCGGCACGCC-3' R, 5'-CTAGCTCATCTCCAAAGAGTTG-3'	275	63	40	Hatano and colleagues ⁵⁸
SR-A	F, 5'-CCAGGACATGGGAATGCAA-3' R, 5'-CCAGTGGGACCTCGATCTCC-3'	365	60	36	Draude and Lorenz ⁴⁴
TGF-β	F, 5'-CAAGTGGACATCAACGGGTT-3' R, 5'-GCTCCAAATGTAGGGGCAGG-3'	297	62	39	Koller and colleagues ⁵⁹
TNF-α	F, 5'-TGCTGCACTTTGGAGTGATC-3' R, 5'-GTTGACCTTGGTCTGGTAGGAG-3'	356	62	39	Lesnikov and colleagues ⁵⁴

RNA Isolation, Semiquantitative Reverse Transcription Polymerase Chain Reaction, and Analyses

RNA was extracted from homogenized frozen cervical spinal cord specimens according to the manufacturer's recommendations (RNeasy; Qiagen, Chatsworth, CA) and ethanol precipitated. The concentrations were determined spectrophotometrically (Beckman Coulter, DU-64, Miami, FL). RT-PCR was performed on 10ng of RNA according to the manufac-

turer's recommendations (OneStep RT-PCR; Qiagen). The primers, hybridization temperature, and cycles used, selected to allow amplification within the linear range, are summarized in Table 2. The RT-PCR products were resolved by ethidium bromide-stained gel electrophoresis and photographed under ultraviolet illumination. The negatives were scanned (HP ScanJet 6300C) and densitometric analyses performed (Image J program, National Institutes of Health). All expression levels were normalized to β-actin.

Table 3. Real-time Reverse Transcription Polymerase Chain Reaction Primer Pairs, Probes, and Conditions

Target	Primer Pairs and Probes	Annealing Temperature (°C)	No. of Cycles
β-actin	F, 5'-GGCATCCACGAAACTACCTTCA-3' R, 5'-GCAGTGATCTCCTTCTGCATC-3' P, 5'-TexR-CTGTACGCCAACACAGTGCT-BHQ-2-3'	62	45
DEC205	F, 5'-ACTGGGAAGAAGCTGAACGA-3' R, 5'-GATCACTCCATTGCCAGGAT-3' P, 5'-FAM-TAGCTTCAGCCATGTGGATG-BHQ-1-3'	62	45
CD83	F, 5'-CACTATCATCAGAAGGGGCAA-3' R, 5'-TGCAGAGTGCACCTGTATGTC-3' P, 5'-FAM-AAGGCCCTATTCCCTGAAGA-BHQ-1-3'	62	45
CD68	F, 5'-GGGAATGACTGTCTCACAAA-3' R, 5'-GTGGTTTTGTGGCTCTTGTA-3' P, 5'-FAM-ACTTTGCTGCCATCCTTCAC-BHQ-1-3'	62	45
MCP-1	F, 5'-CCCCAGTCACCTGCTGTTAT-3' R, 5'-TGGAATCCTGAACCCACTTC-3' P, 5'-FAM-ATTGTGGCCAAGGAGATCTG-BHQ-1-3'	62	45

Real-time Reverse Transcription Polymerase Chain Reaction

RT reactions were performed on 2 μg of RNA according to the manufacturer's recommendations (Omniscript Reverse Transcriptase; Qiagen). Real-time RT-PCR was performed on 1 μl of the reverse transcription reaction using IQ Supermix (Bio-Rad, Richmond, CA) and the iCycler iQ Real-time PCR Detection System (Bio-Rad) according to the manufacturer's instructions. The primers and probes listed in Table 3 were designed using Primer3 software.

Enzyme-Linked Immunosorbent Assay

The CSF was examined for the following cytokines and chemokines: tumor necrosis factor (TNF)-α (Quantikine; R&D Systems, Minneapolis, MN), transforming growth factor (TGF)-β (Duo SET, R&D Systems), interleukin (IL)-6 (Quantikine; R&D Systems), M-CSF (Quantikine; R&D Systems), granulocyte macrophage (GM)-CSF (Quantikine; R&D Systems), MIP-1α (Duo SET, R&D Systems), and MCP-1α (OpiEI; PharMingen, San Diego, CA) according to the manufacturers' instructions. Numerical results were obtained using the Microplate autoreader EL311 (Bio-Tek Instruments, Winooski, VT).

Statistical Analyses

RT-PCR data were analyzed using the two-tailed Student's *t* test (Excel software) and analyses of variance (Sigma Stat software, Jandel Scientific). Group means were plotted ± standard error of the mean; *p* value up to 0.05 was considered statistically significant. ELISA data were analyzed with the Wilcoxon-Mann-Whitney test (NCSS software); *p* value up to 0.05 was considered statistically significant.

Immunohistochemistry

For CD68, CD1a, and CD83 staining, spinal cord samples were fixed in 10% neutral formaldehyde and embedded in paraffin. Five-micrometer sections were cut, deparaffinized, rehydrated, and rinsed (10mM phosphate-buffered saline [PBS]; pH 7.4). The sections were blocked for endogenous peroxidase activity (0.3% H₂O₂ in distilled water, 30 min-

utes). Antigen retrieval techniques were used according to the primary antibody data sheets. The sections were blocked with 5% normal horse serum (Vector Laboratories, Burlingame, CA; 1 hour, room temperature). The primary antibodies (CD68, CD1a, CD83; Serotec, Bicester, UK), diluted in PBS containing 3% normal horse serum (1:40, undiluted, 1:50, respectively) were incubated (overnight, 4°C). As a negative control, the primary antibodies were omitted. After rinsing in PBS, the sections were incubated with biotin labeled anti-mouse IgG (1:200 dilution in PBS containing 3% normal horse serum; 2 hours, room temperature). After washing in PBS, the sections were incubated further with biotin-avidin complex containing peroxidase (Vector Elite kit; Vector Laboratories; 1 hour, room temperature). After washing in PBS, the peroxidase reaction was visualized by exposing the sections to Immunopure Metal enhanced DAB substrate kit (Pierce, Rockford, IL; 15 minutes) and then washed. The sections were dehydrated and cleared in xylene. The immunostained sections were examined using a Zeiss (Thornwood, NY) Axioskop microscope equipped with a DXC-970-MD CCD camera (Sony Corp, Japan) and digital image analysis system (Optimas 6.2; Optimas Corp, Bothell, WA).

For MCP-1 staining, ALS and control spinal cord samples, snap-frozen in liquid nitrogen and stored at -70°C, were sectioned at 15 μm, dried, and fixed in ice-cold acetone (2 minutes). The sections were pretreated as above and incubated overnight with 1 to 50 dilution of goat anti-human MCP-1 (R&D Systems). The sections were processed and photographed as above.

Results

Dendritic Cell Transcript Levels in Amyotrophic Lateral Sclerosis Tissues

To determine levels of dendritic cell surface marker mRNA expression in sALS, fALS, and NNDC tissues, we examined expression of the mRNAs encoding CD1a and DEC-205, immature dendritic cell markers; CD83 and CD40, mature dendritic cell markers;

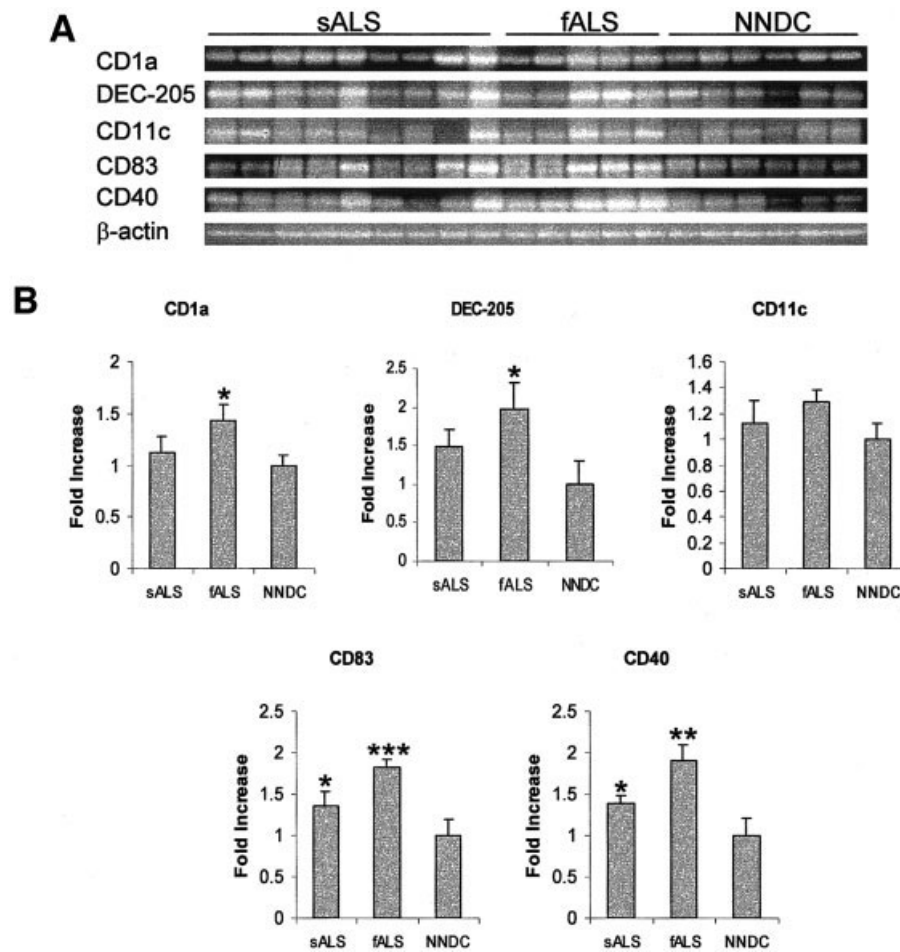


Fig 1. Dendritic cell marker transcripts increased in amyotrophic lateral sclerosis (ALS) tissues. (A) The products of reverse transcription polymerase chain reactions (RT-PCRs), performed with mRNA from nine sporadic ALS (sALS), five familial ALS (fALS), and six non-neurological disease control (NNDC) spinal cords and with the CD1a, DEC-205, CD11c, CD83, or CD40 primers pairs listed in Table 2, were separated using ethidium bromide-stained gel electrophoresis and photographed under ultraviolet illumination. (B) The bands were scanned and densitometric analyses performed. Expression levels were normalized to β -actin. Group means were plotted \pm SEM. * $p < 0.05$, ** $p = 0.007$, *** $p = 0.0007$.

CD11c, a myeloid dendritic cell marker; and CD123, a lymphoid dendritic cell marker; normalized to β -actin. Figure 1 demonstrates that CD1a ($p = 0.03$) and DEC-205 ($p = 0.04$) mRNA expression was significantly increased in the fALS tissue compared with controls. CD83 and CD40 mRNA expression was significantly increased in both the sALS ($p = 0.05$ and $p = 0.05$, respectively) and fALS ($p = 0.0009$ and $p = 0.007$, respectively) tissues compared with controls. Although not significant, there was a trend toward increased DEC-205 mRNA expression in sALS tissues ($p = 0.19$) and increased CD11c mRNA expression in the fALS tissue ($p = 0.07$) compared with controls. CD123 mRNA was not detected in sALS, fALS, or NNDC tissues, suggesting the absence of lymphoid dendritic cells within ALS spinal cord tissue.

Dendritic Cell Immunohistochemistry in Sporadic Amyotrophic Lateral Sclerosis Spinal Cords

To confirm the presence of dendritic cells, we stained ALS and control spinal cord sections for CD1a and CD83 dendritic antigens. In ALS spinal cords, CD1a-immunopositive cells with the morphology of dendritic cells were present both in ventral horns (Fig 2A,C) and

in degenerating corticospinal tracts (see Fig 2B,D). The number of CD1a⁺ cells was less than 5% of the number of CD68⁺ cells (see Fig 5 below). Most of the cells in the white matter were located proximal or adjacent to vessels (see Fig 2B,D), whereas in the ventral horn, parenchymal CD1a-expressing cells were common. Only a few macrophages and perivascular monocytoïd cells were immunostained (see Fig 2). Several CD1a-immunopositive perivascular cells decorated the vessels in the ventral roots (see Fig 2E). There were clearly no cells that stained positive for CD1a present in the NNDC spinal cords (see Fig 2F). As a positive control, subepithelial dendritic (see Fig 2G) and interfollicular cells showed ample staining in tonsil tissue.

The immunostaining pattern of CD83 antigen was found to be similar to CD1a immunostaining. However, the intensity of staining was weaker over cells with dendritic morphology than seen with the CD1a staining (Fig 3A) but remained strong on perivascular cells with monocytoïd morphology (see Fig 3B). There were no cells stained in control spinal cords (see Fig 3D). As a positive control, interfollicular cells with dendritic shape showed strong immunoreactivity in tonsil tissue (see Fig 3C).

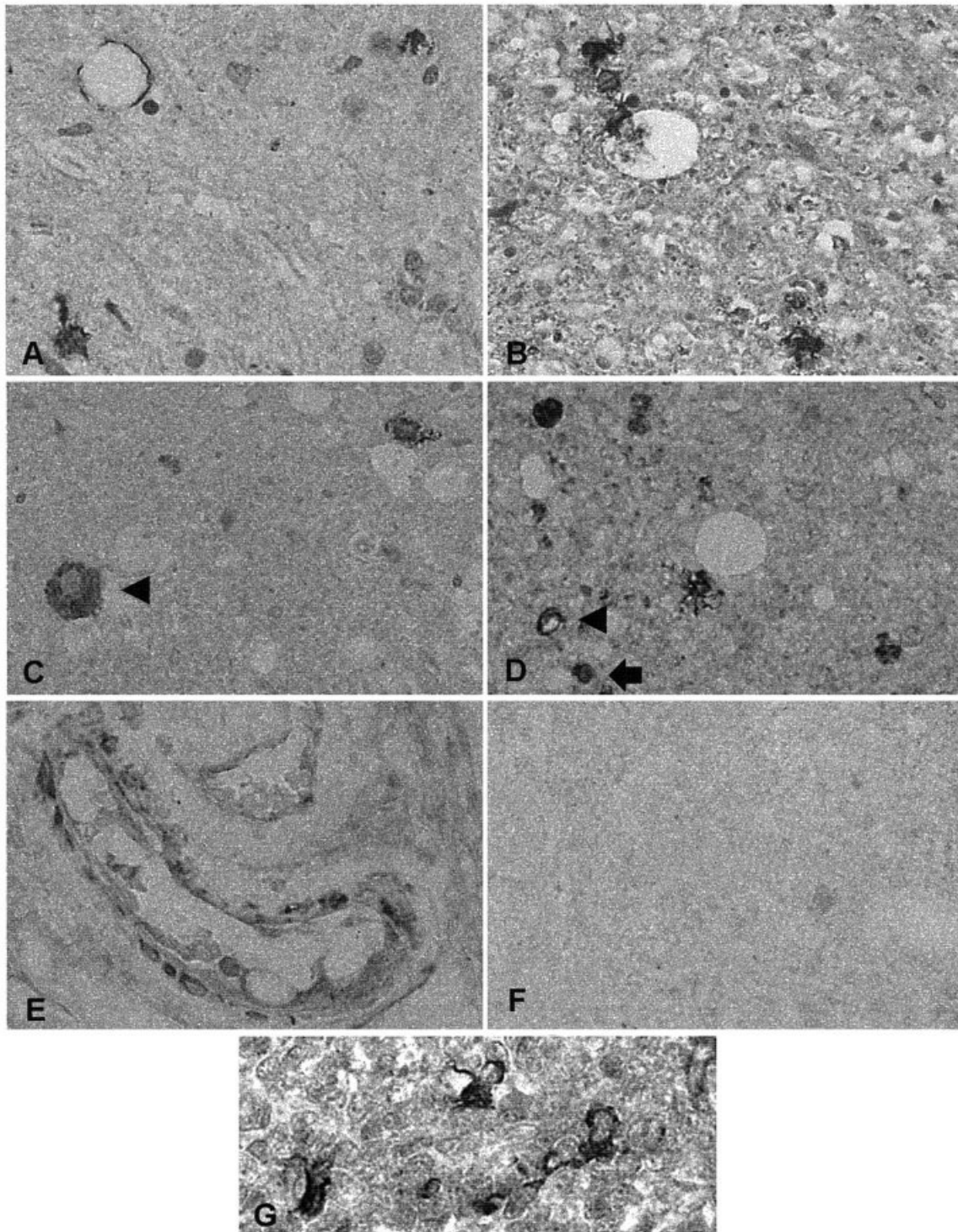


Fig 2. CD1a-immunopositive cells in amyotrophic lateral sclerosis (ALS) sections, not in control sections. (A) Dendritic cells immunostained for CD1a (bottom left and top right corner) in the ventral horn of an ALS spinal cord. (B) CD1a-immunostained dendritic cells in the region of the lateral corticospinal tract. One of them is in close contact with a small vessel. (C) Large CD1a⁺ macrophage (arrowhead) and a CD1a⁺ dendritic cell in the ventral horn of an ALS spinal cord. (D) CD1a-immunopositive monocyte (arrow) and macrophage cells (arrowhead), and a dendritic cell (connected to a small vessel) in the lateral corticospinal tract of an ALS spinal cord. (E) A vessel surrounded by CD1a⁺ cells in the ventral root of an ALS spinal cord. (F) Control spinal cord showing no cells immunostained for CD1a. (G) CD1a-positive dendritic cells in the subepithelial layer of the tonsil. (A–G) Peroxidase reaction; (A–D, F) magnification $\times 280$; (E) $\times 200$.

Monocytic/macrophage/microglial cell transcript levels in ALS tissue

To ascertain the expression levels of activated microglia and monocyte/macrophage transcripts in ALS tissues, we examined the expression of mRNA encoding the following microglia/monocyte/macrophage cell surface markers in sALS, fALS, and NNDC tissues normalized to β -actin: CD14, CD18, CD11b, CD68, SR-A, and HLA-DR. As shown in Figure 4, the expression of CD14, CD18, CD68, and SR-A mRNAs were significantly increased in sALS spinal cord tissue compared with controls ($p = 0.00017$, $p = 0.030$, $p = 0.00010$, $p = 0.046$, respectively). CD18 and CD68 mRNAs were also significantly increased in the fALS spinal cord tissue compared with controls ($p = 0.030$, $p =$

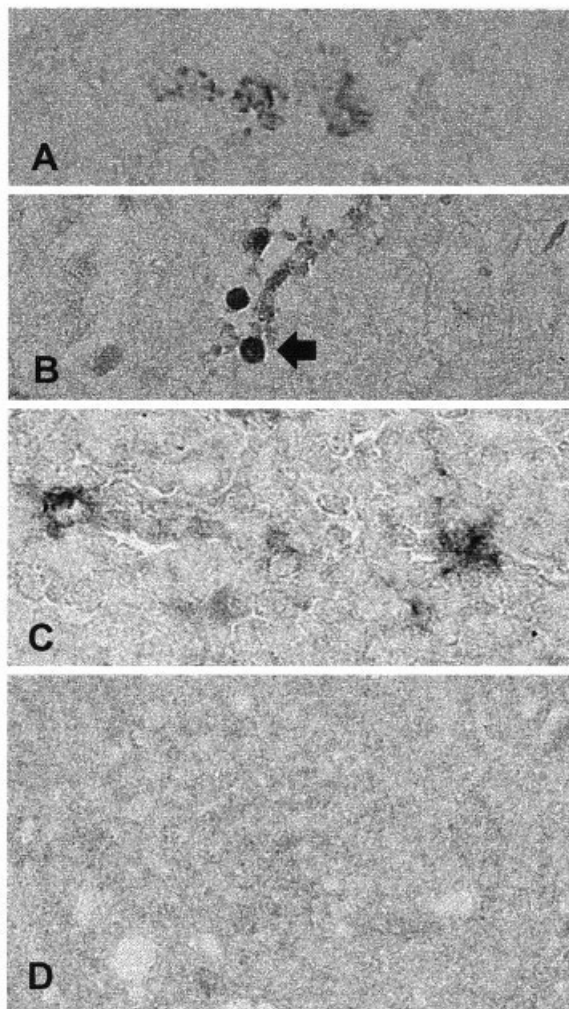


Fig 3. Immunostaining for CD83. (A) CD83-immunopositive dendritic cells in the lateral corticospinal tract. (B) CD83-positive monocytic cells (arrow) close to a vessel in the lateral corticospinal tract. (C) CD83-positive dendritic cells in the interfollicular region of the tonsil. (D) Control spinal cord showing no cells immunostained for CD83. (A–D) Peroxidase reaction, magnification $\times 280$.

0.000053 , respectively). Although not significant, there was a trend toward increased CD14 and SR-A mRNA expression in the fALS tissue ($p = 0.10$ and $p = 0.19$, respectively) and increased HLA-DR mRNA expression in the sALS tissue ($p = 0.066$) compared with controls. Fc γ R γ subunit mRNA was not detected in sALS, fALS, or control tissues.

Immunostaining for CD68 Antigen

CD68 immunostained microglia were present in the spinal cords of ALS and control sections. In control spinal cords, CD68 immunostaining was present in ramified cells in the ventral horn (Fig 5B) and white matter (see Fig 5D), but not in proximity to motor neurons, the vasculature, or the corticospinal tracts. However, in the ventral horns of ALS spinal cord sections, there was an increased density of CD68-positive cells with enlarged cell processes, often in close proximity to normal appearing motor neurons (see Fig 5A). Several CD68-positive cells laden with lipofuscin also were present in proximity to destroyed neurons. The density of ramified cells decreased in regions of the degenerating corticospinal tracts; instead, the cells transitioned to rounded macrophages of different sizes mostly with cleared cytoplasm (phagocytized lipids dissolved during paraffin embedding; see Fig 5C). Cells with monocyte morphology were also present (see Fig 5E). Approximately 5% of the immunostained cells had dendritic cell morphology, typically in a perivascular location.

Cytokine mRNA Levels

To determine if cytokines were increased, the expression levels of mRNA encoding the following cytokines were examined normalized to β -actin: TNF- α , TGF- β , IL-1 β , IL-6, interferon INF, GM-CSF, and M-CSF. As indicated in Figure 6, M-CSF mRNA expression was significantly increased in the fALS tissue as compared with controls ($p = 0.017$). Although not significant, there was a trend toward decreased TGF- β and IL-1 β expression in sALS tissue ($p = 0.18$ and $p = 0.11$, respectively) compared with controls. IL-6 mRNA was detected in only two sALS tissues, but not in fALS or control tissues. INF- γ and GM-CSF mRNAs were not detected, although they were detected in lipopolysaccharide-activated monocytes (positive control). The others were not significantly different from control levels.

Chemokine mRNA Levels

To ascertain if there was an increase in chemokines known to attract inflammatory cells, we examined the relative expression of MCP-1, MIP-1, and RANTES mRNA normalized to β -actin. Figure 7 demonstrates that MCP-1 mRNA was drastically increased in the sALS tissue compared with controls ($p = 0.000044$).

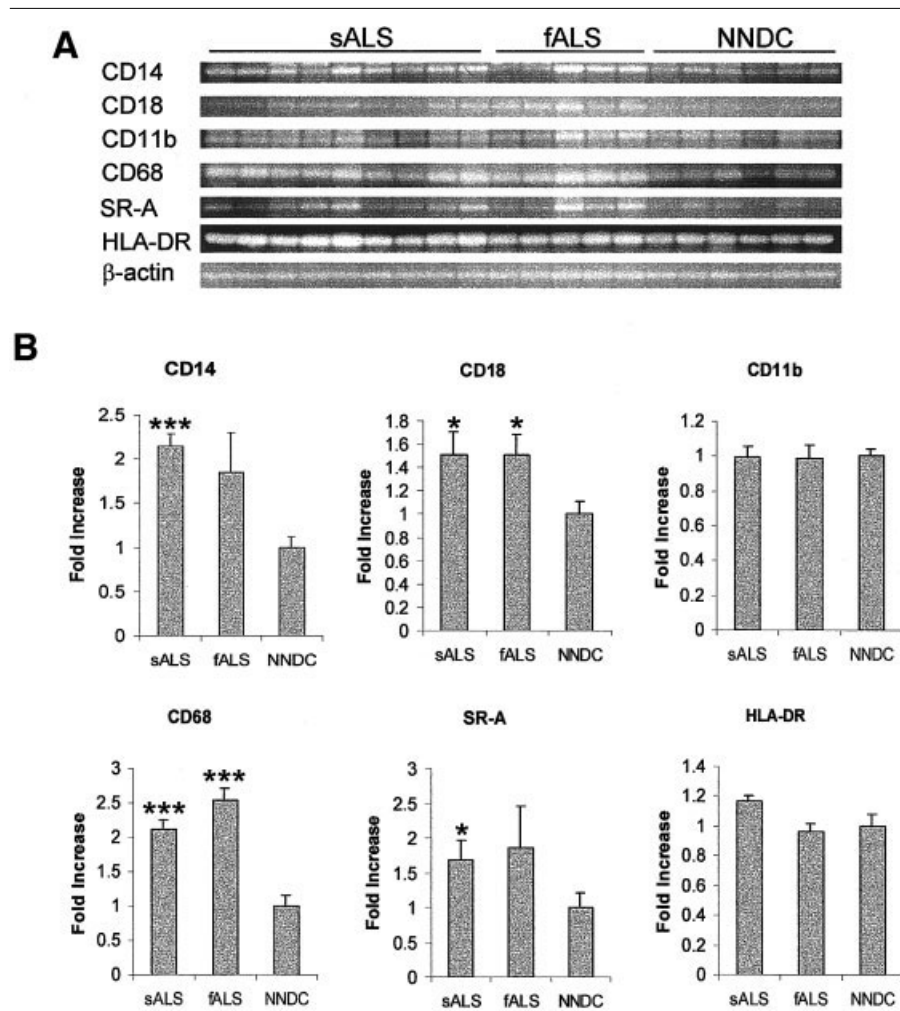


Fig 4. Monocyte/macrophage cell surface marker transcripts are increased in amyotrophic lateral sclerosis (ALS) tissue. (A) The products of reverse transcription polymerase chain reactions performed with mRNA from nine sporadic ALS (sALS), five familial ALS (fALS), and six nonneurological disease control (NNDC) spinal cords and with the CD14, CD18, CD11b, CD68, SR-A, or HLA-DR primers pairs listed in Table 2, were separated using ethidium bromide-stained gel electrophoresis and photographed under ultraviolet illumination. (B) The bands were scanned and densitometric analyses were performed. Expression levels were normalized to β -actin. Group means were plotted \pm SEM. * $p < 0.05$, *** $p \leq 0.0001$.

Although not significant, there was a trend toward increased MCP-1 mRNA expression in the fALS tissue compared with controls ($p = 0.063$). In addition, there was a trend toward decreased RANTES expression in the sALS tissue compared with controls ($p = 0.083$). MIP-1 mRNA was not significantly different between ALS and control tissues.

MCP-1 Expression in Glia

To determine the source of the MCP-1 chemokine seen in the tissues and CSF, ALS spinal cord tissue was examined immunohistologically for the expression of MCP-1. MCP-1 was readily detected in ALS spinal cord tissues in the lateral (Fig 8A) and posterior (see Fig 8C) columns in the cytoplasm of glia (probably astrocytes) and some macrophages. MCP-1 was not detected in control tissues (see Fig 8 B,D).

Cytokines and Chemokines in Cerebrospinal Fluid

We used ELISA to examine expression levels of the cytokines TNF- α , TGF- β 1, M-CSF, GM-CSF, and IL-6 and the chemokines MCP-1 and MIP-1 α in CSF (Fig

9). Levels of the cytokines TNF- α , GM-CSF, IL-6, and the chemokine MIP-1 α were low and variably detectable in ALS and control patients but were not significantly different in the two populations. Levels of the cytokines TGF- β 1 and M-CSF were readily detectable but were not significantly different in ALS versus controls. MCP-1 was also readily detectable and was the only cytokine or chemokine that was significantly elevated in ALS CSF compared with disease control ($p = 0.032$).

Real-time Reverse Transcription Polymerase Chain Reaction

To obtain more quantitative measurements of the alterations we observed using semiquantitative RT-PCR and immunohistochemistry, we used real-time RT-PCR to examine DEC205, CD83, CD68, and MCP-1 relative to β -actin (Fig 10). The immature dendritic transcript DEC205 was significantly increased in both the sALS and fALS samples compared with controls ($p = 0.038$ and $p = 0.032$, respectively). The mature dendritic transcript CD83 was significantly increased in the fALS samples compared with controls ($p = 0.0076$), and, al-

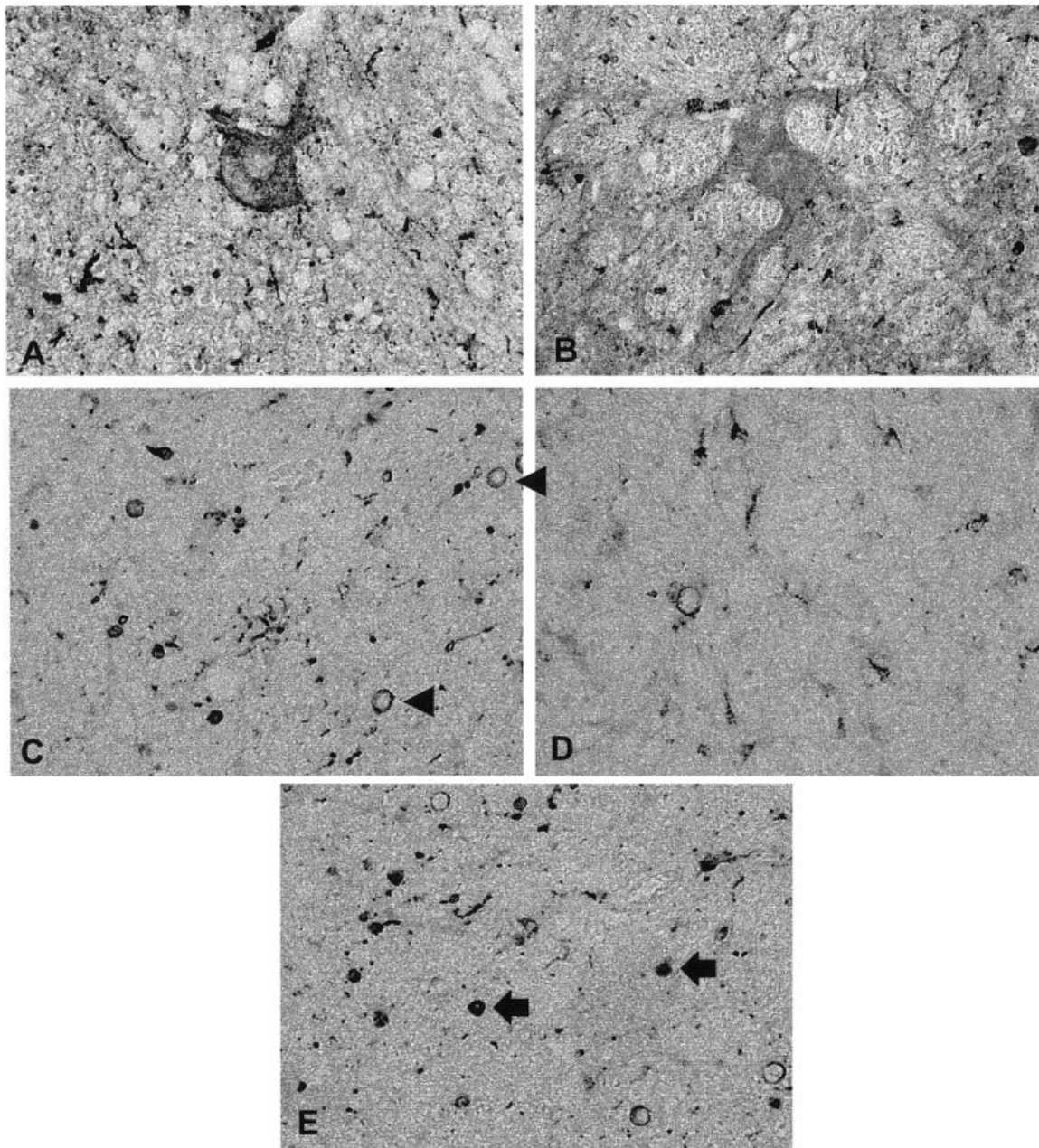


Fig 5. Immunostaining for CD68. (A) The external membrane (partially tangentially sectioned) of a motoneuron in the ventral horn of an amyotrophic lateral sclerosis (ALS) spinal cord is decorated by microglial processes immunostained for CD68. (B) There is no CD68⁺ reactivity of microglia in proximity of the motoneuron in a control spinal cord. (C) Many of the CD68⁺ microglial cells transitioned to rounded macrophages in the lateral corticospinal tract of an ALS spinal cord (arrowheads). The overall density of the cells immunostained for CD68 is increased. (D) The density and shape of microglial cells in the lateral corticospinal tract of a normal spinal cord. (E) The CD68⁺ macrophages and also monocytes tend to gather in the vicinity of a vessel (arrows). (A–E) Peroxidase reaction, A,B, $\times 140$; C–E, $\times 180$.

though not significant, there was a clear trend toward increased expression in the sALS samples ($p = 0.066$). The microglial transcript CD68 was significantly increased in both the sALS and fALS samples compared with controls ($p = 0.0023$ and $p = 0.0094$). In addition MCP-1 transcripts were increased in the both the sALS ($p = 0.00028$) and fALS ($p = 0.0031$) samples

compared with controls. Thus, the real-time RT-PCR verified the results seen with semiquantitative RT-PCR.

Correlation of survival with presence of dendritic cell markers

Two sALS patients and three fALS patients died within 1 year from first symptom. To determine whether

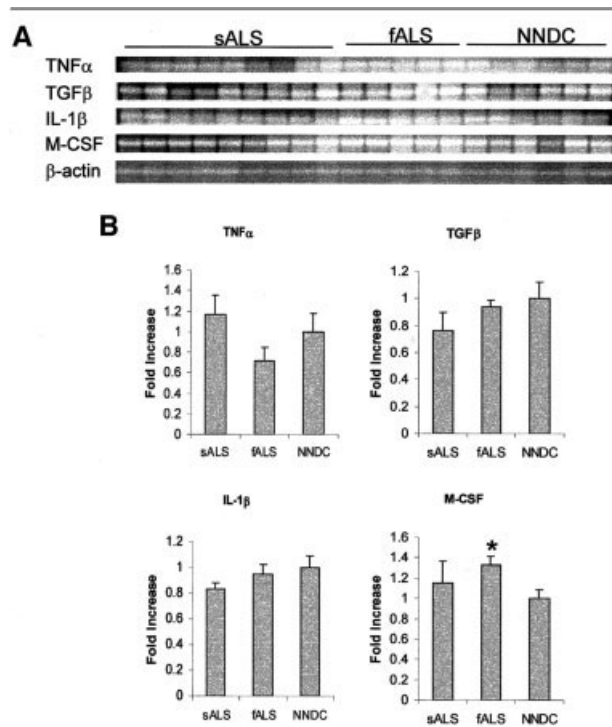


Fig 6. Cytokine transcription levels in amyotrophic lateral sclerosis (ALS) tissues. (A) The products of reverse transcription polymerase chain reactions, performed with mRNA from nine sporadic ALS (sALS), five familial ALS (fALS), and six non-neurological disease control (NNDC) spinal cords and with the TNF- α , TGF β , IL-1 β , IL-6, or M-CSF primers pairs listed in Table 2, were separated using ethidium bromide-stained gel electrophoresis and photographed under ultraviolet illumination. (B) The bands were scanned and densitometric analyses were normalized to β -actin. Group means were plotted \pm SEM. * $p = 0.017$.

there was a pattern of expression in these rapidly progressing patients, we ranked the ALS patients from 1 to 14 based on their level of expression for each transcript. The patients who progressed the most rapidly ranked higher in dendritic transcripts, but not in the monocytic/macrophage/microglia transcripts. To assess if this correlation was significant, we grouped the rapidly progressing patients and the slower progressing patients and compared the expression of dendritic cell, monocytic/macrophage/microglial, cytokine, or chemokine transcripts (Fig 11). We performed analyses of variances to determine if the rapidly progressing patients expressed an increase in transcripts compared with the slower progressing patients. Both the fast and slow progressing ALS groups were significantly different compared with the controls for dendritic cell ($p = 0.0000067$ and $p = 0.0020$, respectively), monocytic/macrophage/microglial ($p = 0.028$ and $p = 0.0012$, respectively), and chemokine ($p = 0.017$ and $p = 0.0058$, respectively) transcripts. However, only the dendritic cell transcripts ($p = 0.050$) were significantly

increased in the rapidly progressing patients compared with the slower progressing patients (monocytic/macrophage/microglial transcripts: $p = 0.355$, cytokine transcripts: $p = 0.499$, chemokine transcripts: $p = 0.853$). Thus, the more rapidly progressing ALS correlated with increased dendritic cell transcripts.

Discussion

Dendritic cells are a diverse set of cells that function as members of the innate as well as adaptive immune systems and in the latter capacity possess a highly efficient antigen-presenting capacity to stimulate naive T cells.³³ Dendritic cells have been demonstrated in the CNS in autoimmune diseases such as MS and the animal model, EAE.^{19–21} A role for dendritic cells has been established in both causing EAE and inducing tolerance.^{22,34} To determine whether dendritic cells are similarly present in ALS, we assayed for expression of dendritic cell transcripts in sALS and fALS tissues compared with controls. We demonstrated a significant increase of CD83 and CD40 (mature/activated dendritic cells markers) mRNA in spinal cord tissue of the sALS patients and a significant increase of CD83, CD40, CD1a, and DEC-205 (the latter two expressed on dendritic cells before maturation) mRNA in spinal cord tissue of fALS patients compared with controls. Immunohistochemical staining of sALS spinal cord sections showed that both parenchymal and perivascular CD1a and CD83-positive cells were present in the ventral horn, whereas mostly perivascular-positive cells were present in the degenerating corticospinal tract. Both CD1a- and CD83-positive cells were conspicuously ab-

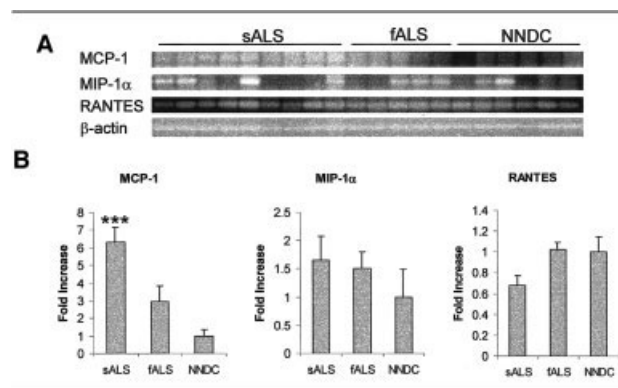


Fig 7. Chemokine mRNA levels in amyotrophic lateral sclerosis (ALS) tissues. (A) The products of reverse transcription polymerase chain reactions performed with mRNA from nine sporadic ALS (sALS), five familial ALS (fALS), and six non-neurological disease control (NNDC) spinal cords and with the MCP-1, MIP-1 α , or RANTES primers pairs listed in Table 2 were separated using ethidium bromide-stained gel electrophoresis and photographed under ultraviolet illumination. (B) The bands were scanned and densitometric analyses were performed. Expression levels were normalized to β -actin. Group means were plotted \pm SEM. *** $p = 0.000044$.

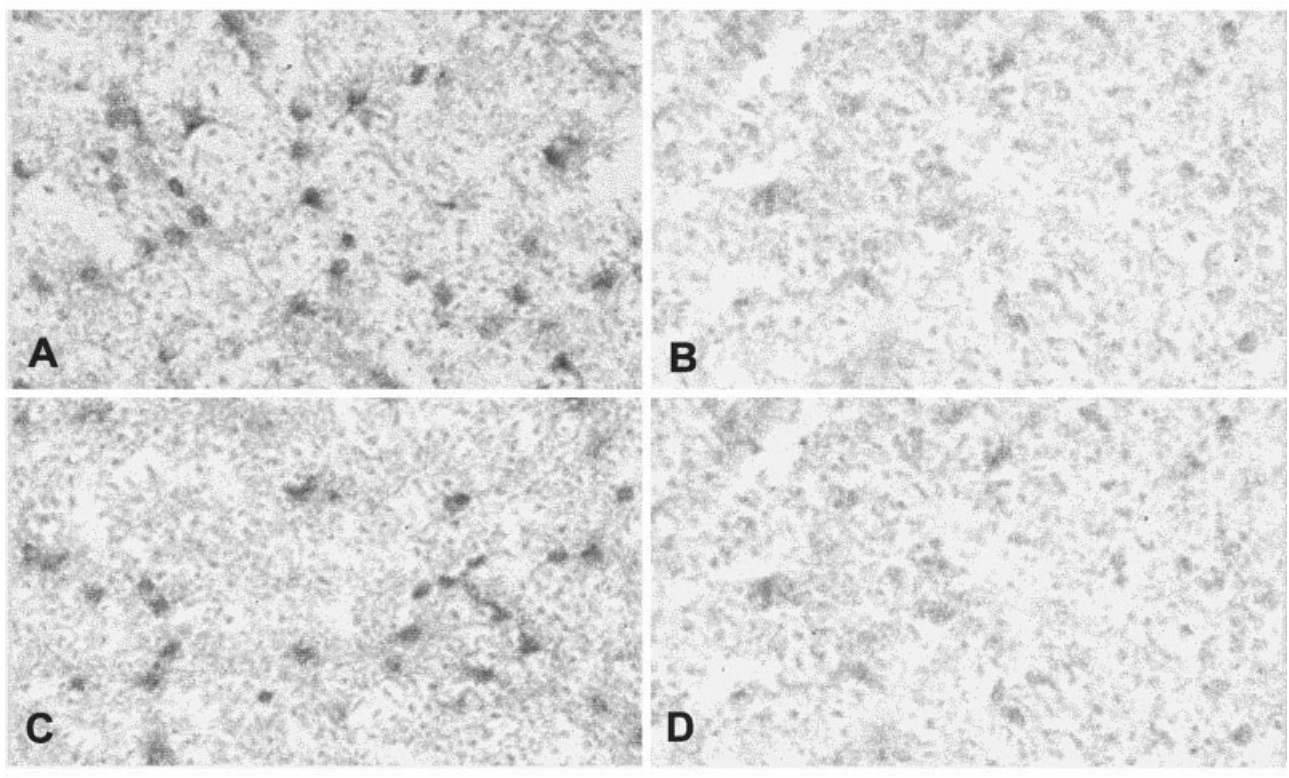


Fig 8. MCP-1 immunohistochemistry. Immunohistochemistry was used to detect MCP-1 expression in amyotrophic lateral sclerosis (ALS) and control tissues. (A) MCP-1-immunopositive glia and macrophages in the lateral column of an ALS spinal cord. (C) MCP-1-immunopositive glia in the posterior column of an ALS spinal cord. (B, D) There are no immunolabeled cells for MCP-1 in the lateral column or in the posterior column of the control spinal cord. (A–D) Peroxidase reaction, $\times 100$.

sent in control tissue. The tissues used for the RT-PCR analyses were frozen and not perfused; thus, mRNA from any blood cells present in the spinal cord vessels would be in the RNA pool. Therefore, the dendritic cell transcripts detected in controls, when no dendritic cells were present in control tissues, could be transcripts from the blood cells in the vessels.

The presence of immature dendritic cells in ALS tissue, which can take up and process antigen as well as stimulate other cellular constituents of the innate immune system (such as microglia or natural killer cells), suggests an involvement in the ongoing immune/inflammatory reaction. It is also relevant that among the factors acting on immature dendritic cells are reactive oxygen intermediates, aggregated IgG, and cytokines.³³ All of these are well-known changes present in ALS and could contribute to the enlistment of immature dendritic cells in CNS degeneration. As dendritic cells mature, they lose their phagocytic properties and their ability to capture antigen, but express MHC class II as well as costimulatory molecules and become potent antigen-presenting cells.³⁵ The presence of both immature and mature dendritic cells in ALS, and their absence in control specimens, argues for their involvement in accelerating the pathogenesis of motor neuron injury in ALS.

Our previous results and the results of others have established the presence of monocyte/macrophage cells and activated microglia in spinal cord tissue of most ALS patients.^{1,4,6} The increased expression of mRNAs encoding proteins expressed on monocytic/macrophage/microglial cells (CD14, CD18, and SR-A) in spinal cord clearly confirms the presence of such constituents in ALS tissues. In addition, CD68⁺ monocyte/macrophage and activated microglia cells were seen dispersed in degenerating white matter, less extensively in the ventral horns, and packed around blood vessels of ALS spinal cord tissues. Although the specific role of these monocyte/macrophage cells is unclear, their frequent presence in proximity to normal appearing motor neurons in ALS ventral horns would suggest an active role, and not merely a phagocytic role, in motor neuron injury. The presence of activated microglia and the therapeutic benefit of minocycline, an inhibitor of microglial p38 MAPK, in the presymptomatic mSOD1 transgenic mouse models of fALS provide additional evidence for the potential role of microglia in motor neuron injury in ALS.^{16,17,36–38}

To determine whether inflammatory cells are recruited to the CNS and/or differentiate from endogenous precursor cells, we examined the expression levels of various cytokine and chemokine mRNAs. The most

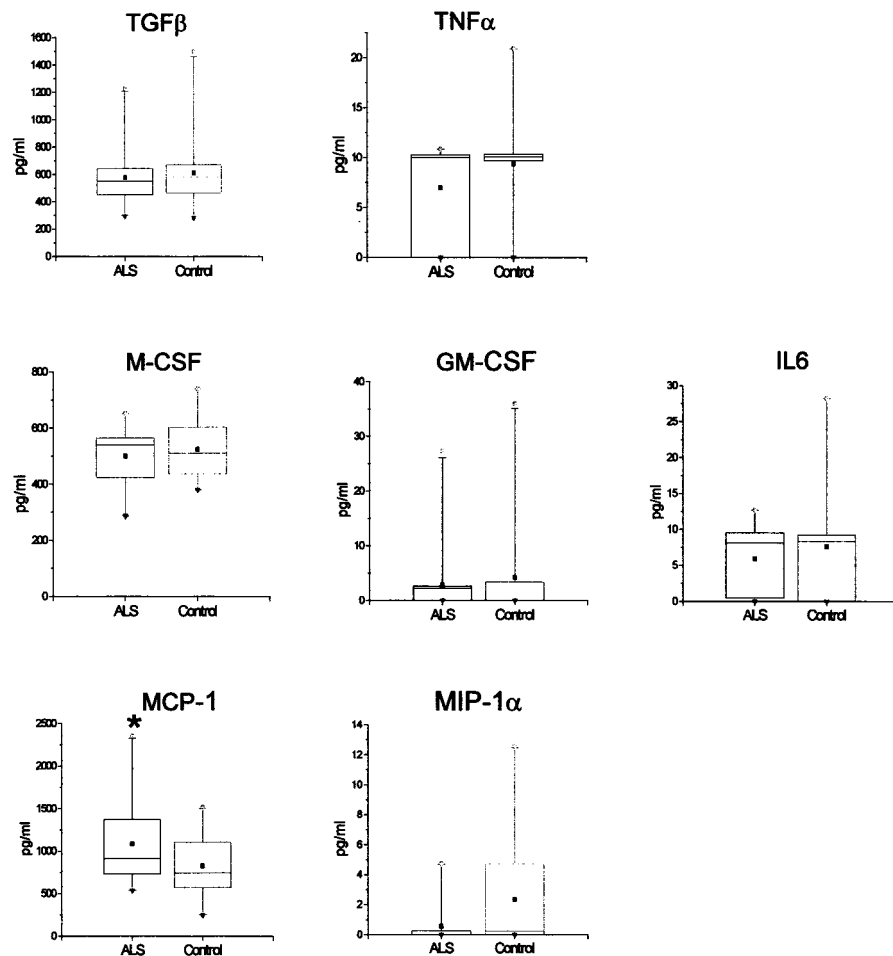


Fig 9. MCP-1 increased in cerebrospinal fluid (CSF). ELISA analyses were performed on CSF samples from 20 amyotrophic lateral sclerosis (ALS) and 20 control patients. MCP-1 levels from ALS patients were significantly increased over the diseased control group (* $p = 0.05$); none of the other cytokines or chemokines were significantly different. The boxes indicate the scores from 25 to 75%. The lines in the boxes indicate the median; the small black squares indicate the mean. The vertical bars indicate the scores from 5 to 95%. All of the TGF β , M-CSF, and MCP-1 levels were within the linear range; 25% of the ALS and 15% of the control TNF- α levels were below the linear range; 40% of the ALS and 30% of the control IL-6 levels were below the linear range; 95% of the ALS and 80% of the control GM-CSF levels were below the linear range; and 100% of the ALS and 90% of the control MIP-1 α levels were below the linear range.

significant increase in ALS tissue was the 23-fold increase in MCP-1 mRNA compared with controls. No other mRNA tested was increased as much. We determined that MCP-1 expression was mostly in glia and in some macrophages in the CNS. In addition, MCP-1 in the CSF of sALS patients was increased compared with controls.

MCP-1 is a potent chemoattractant and activating peptide that is expressed mostly in astrocytes but also in neurons, microglia, and macrophages after ischemia, hypoxia, or excitotoxicity. Excessive MCP-1 can exacerbate an injury. It attracts CCR2-expressing myeloid dendritic cells, microglia/monocytes, and activated T cells but may also regulate Th1 and Th2 responses. Mice deficient in MCP-1 or its receptor, CCR2, are resistant to EAE and have a significant reduction in T cell and monocyte infiltration.^{20,31,32} Thus, MCP-1 is important in the recruitment of immune/inflammatory cells into the CNS, and more specifically the recruitment of dendritic cells and monocytes. Furthermore, the immunohistochemical demonstration of such perivascular dendritic cells in both ventral horn and

white matter are in accord with recruitment from the circulation.

A third key finding, in addition to the presence of immature and mature/activated dendritic cells in ALS tissue and to the increased MCP-1 expression in CSF and tissue in ALS, is that increased expression of dendritic cell transcripts correlated with the more rapidly progressing ALS. Two sALS patients and three fALS patients died within 1 year from first symptom; the remainder of the patients died between 2.75 and 16.25 years after first symptom. The five rapidly progressing patients expressed significantly more dendritic transcripts, compared with the slower progressing patients, but not more monocytic/macrophage/microglial, cytokine, or chemokine transcripts. Thus, the correlation of rapidly progressing ALS and increased dendritic cell transcripts support the involvement of the immune/inflammatory system in amplifying the disease process.

Profiling mRNAs expressed in sALS and mSOD1 transgenic mouse spinal cord tissues has recently been used to investigate ALS.³⁹⁻⁴² As each array examined different sets of genes, the genes identified from each

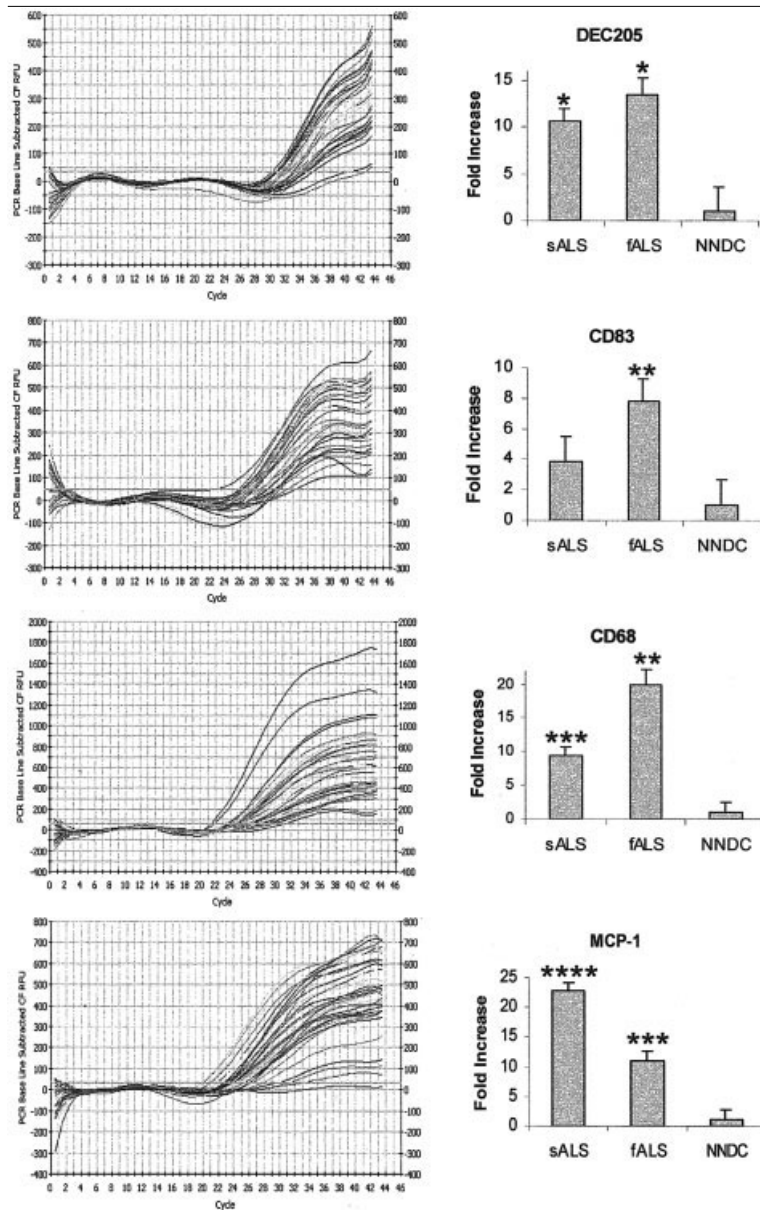


Fig 10. Real-time reverse transcription polymerase chain reaction (RT-PCR). Real-time RT-PCR was used to examine DEC205, CD83, CD68, and MCP-1 relative to β -actin. The immature dendritic transcript DEC205 was significantly increased in both the sporadic amyotrophic lateral sclerosis (sALS) and familial ALS (fALS) samples compared with the nonneurological disease control (NNDC) samples. The mature dendritic transcript CD83 was significantly increased in the fALS samples compared with the NNDC samples. The microglial transcript CD68 was significantly increased in both the sALS and fALS samples compared with the NNDC samples. In addition, MCP-1 transcripts were increased in both the sALS and fALS samples compared with the NNDC samples. Thus, the real-time RT-PCR verified the results seen with semiquantitative RT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0005$.

screen were different. Despite this, some categories of genes identified were the same. All four of the sALS and mSOD1 transgenic mouse screens showed increased expression of genes involved in inflammation. Although the two sALS screens did not identify genes encoding dendritic cell surface proteins, the list of genes in the array we were able to obtain did not include genes for dendritic cell markers (list provided by Ishigaki). In addition, one of the mSOD1 transgenic mouse screens detected an increased expression of CD63, a microglial activation marker that can be expressed at high levels on a subset of dendritic cells.^{41,43}

In summary, we have demonstrated the presence of immature and mature dendritic cells, increased numbers of activated microglia/macrophages in ALS spinal

cord tissue, and increased levels of the chemokine MCP-1 that can recruit these cells from the systemic circulation. Although something other than the immune system may well have initiated the motor neuron injury and although macrophages could be playing a phagocytic role, we propose that the presence of immature dendritic cells, of the chemokine that attracts them and their maturation to activated dendritic cells, indicate that dendritic cells may well be exacerbating the motoneuron injury in ALS. This is especially apparent in those patients who had the most rapid rate of decline and expressed higher levels of dendritic cell marker mRNA. Thus, therapies specifically targeted to dendritic cells and to the chemokine MCP-1 and its receptors may well offer meaningful pharmacological approaches to ALS.

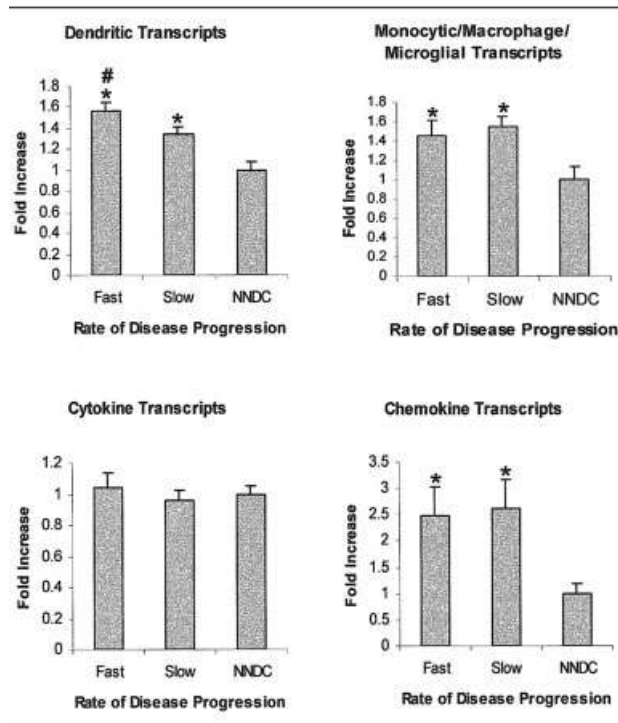


Fig 11. Patients with rapidly progressing amyotrophic lateral sclerosis (ALS) expressed significantly more dendritic transcripts than those patients who progressed more slowly. Patients who died within 1 year from first symptom were grouped and compared with those patients who progressed more slowly. Their dendritic transcript expression levels were compared. Patients with rapidly progressing ALS expressed significantly more dendritic transcripts than those patients who progressed more slowly ($p = 0.050$). Patients with rapidly progressing ALS expressed equivalent monocytic/macrophage/microglial transcripts, cytokine transcripts, and chemokine transcripts as those patients who progressed more slowly. Both the rapidly progressing and slower progressing patients expressed more dendritic transcripts, monocytic/macrophage/microglial transcripts, and chemokine transcripts than the nonneurological disease control (NNDC) patients (*).

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