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Evidence for systemic immune system alterations in sporadic amyotrophic lateral sclerosis (sALS)

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Abstract

Sporadic amyotrophic lateral sclerosis (sALS) is a progressive neuroinflammatory disease of spinal cord motor neurons of unclear etiology. Blood from 38 patients with sALS, 28 aged-match controls, and 25 Alzheimer's disease (AD) patients were evaluated and activated monocyte/macrophages were observed in all patients with sALS and AD; the degree of activation was directly related to the rate of sALS disease progression. Other parameters of T-cell activation and immune globulin levels showed similar disease associated changes. These data are consistent with a disease model previously suggested for AD, wherein systemic immunologic activation plays an active role in sALS.

Keywords: Amyotrophic lateral sclerosis (ALS); Alzheimer's disease (AD); Motor neuron disease; Immune activation; Monocyte/macrophage

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurological disease characterized by gradual degeneration of spinal cord motor neuron cells leading to progressive weakness, paralysis of muscle and death. Almost 90% of ALS patients are characterized as having sporadic ALS (sALS) with 10% having a familial form, a subset of whom have point mutations in their superoxide dismutase (SOD) gene. The pathogenesis of ALS is incompletely understood, although different hypotheses have been suggested, including mitochondria dysfunction (Richter et al., 1988; Wong et al., 1995; Curti et al., 1996), mutation in the superoxide dismutase gene (Deng et al., 1993; Durham et al., 1997), defects in neuronal glutamate transports (Rothstein et al., 1995; Fray et al., 1998), and autoimmunity (Appel et al., 1993). In addition, several recent studies have suggested that the immune system may be actively involved in the disease process of ALS, with observations of activated microglia, IgG deposits, and dysregulation of cytokine expression in the spinal cord of ALS patients (Troost et al., 1989; Engelhardt and Appel, 1990; Schiffer et al., 1996; Hayashi et al., 2001). However, despite intensive investigation ALS has no known cause or effective therapy.

Inflammatory mechanisms and immune reactivity have been hypothesized to play a role in the pathogeneses of central nervous system (CNS) disease (Akiyama et al., 2000; Minagar et al., 2002; Bar-Or et al., 2003; Filion et al., 2003, Hirsch et al., 2003; Floris et al., 2004; Ringheim and Conant, 2004), including ALS (Alexianu et al., 2001; McGeer and McGeer, 2002; Henkel et al., 2004; Simpson et al., 2004). Furthermore, various studies have suggested that monocyte/macrophage activation may play a significant role in the pathogenesis of many neurological diseases, such as Alzheimer's disease (AD) (Smits et al., 2000; Fiala et al.,

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2002), multiple sclerosis (MS) (Minagar et al., 2002; Filion et al., 2003), Parkinson's disease (McGeer et al., 1988), and HIV-associated dementia (HAD) (Pulliam et al., 1997; Diesing et al., 2002). Indeed, the best pathological correlate for HIV-associated neurologic disorders, especially HAD, is the number of activated mononuclear phagocytes present in the brain (perivascular and parenchymal blood-derived macrophages and microglia), and not the absolute level of HIV in the brain (Glass et al., 1995; Adamson et al., 1999). Similar findings have been reported for simian AIDS related encephalopathy (SIVE) (Williams et al., 2002). Studies on blood from patients with HAD (Liu et al., 2000) and monkeys with SIVE (Williams et al., 2002) have shown a direct relationship between the presence of activated blood macrophages and CNS disease. These activated macrophages are thought to mediate blood–brain barrier (BBB) breakdown and directly contribute to CNS pathogenesis. Given evidence of macrophage activation in spinal cords of patients with ALS disease (Engelhardt and Appel, 1990; Appel et al., 1993; Obal et al., 2001; McGeer and McGeer, 2002; Wilms et al., 2003; Henkel et al., 2004), a pathogenic process similar to these may occur in ALS.

In addition to local evidence for tissue associated macrophage activation, increasing numbers of studies on peripheral immune activation levels in CNS disease patients such as AD (Shalit et al., 1995; Scali et al., 2002; Casal et al., 2003), HIV-associated neurologic disorder (Pulliam et al., 1997; Sanchez-Ramon et al., 2003) and MS (Bar-Or et al., 2003; Filion et al., 2003) support the hypothesis of a peripheral immune reaction which might be correlated with the clinical stages of disease. In order to examine whether macrophage activation observed in spinal cords of ALS patients would be reflected in the systemic immune system, the current study was designed: (1) to detect differences, if any, in the expression of monocyte/macrophage and T-cell activation markers in peripheral blood mononuclear cells (PBMC) between sALS patients and controls and (2) to test whether the peripheral immune activation state in sALS patient blood would correlate with clinical stage of disease. This study shows for the first time that the pattern of immunologic activation in blood cells from patients with sALS correlates with ALS disease state and that rate of disease progression is directly related to the degree of systemic monocyte/macrophage activation. These data are consistent with a model of sALS that includes systemic immunologic dysfunction as a cofactor in disease pathogenesis, not dissimilar to pathogenic models for AD, MS and HAD as described above.

2. Methods

2.1. Subjects

Thirty-eight patients diagnosed with sALS (12 females and 26 males, mean age 59.3±13.4 years) by El Escorial criteria (Brooks, 1994) at the Forbes Norris MDA/ALS Research Center (San Francisco, CA, USA) had blood drawn in accordance with the CPMC (California Pacific Medical Center) and UCSF committees on human research guidelines, coordinated by the UCSF AIDS and Cancer Specimen Resource (ACSR) program. The Revised ALS Functional Rating Scale (ALSFRS-R), scored 0–48, was used to evaluate overall patient functional status (Cedarbaum et al., 1999). All scores were updated within a month of blood testing. Patients had sALS for 4–93 months with a range of ALSFRS-R scores of 8–43. Demographic information on sALS patients whose specimens were studied is shown in Table 1, in which 12 patients were using various anti-inflammatory medications with standard dose (Celebrex, Vioxx, Naproxyn, Excedrin), and 30 patients were taking riluzole (50 mg twice daily); 10 patients received both medications.

Two control groups were used in the study. The first control group consisted of 28 age-matched normal blood samples (10 females and 18 males, mean age 56.0±15.1 years), which were obtained from blood draws at Stanford University Blood Center and processed in a similar manner to the sALS patient blood specimens. Normal control samples for IgG and IgM studies consisted of plasma from 80 blood donors and were also obtained from the Stanford University Blood Center. The second control group obtained from the same source as sALS, had Alzheimer's disease (AD). This group consisted of 25 AD patients (11 females and 14 males, mean age 77.5±7.9), and was used as neurological disease controls.

2.2. Flow cytometry

About 10 ml of peripheral blood was drawn from each patient and normal controls into heparinized tubes and transferred to the laboratory at room temperature for same day immunologic studies. Cellular immunologic activation was evaluated by quantitating levels of CD38 on T-cell subsets and HLA-DR on CD14 cells. CD16 (Fc gamma III receptor) expression on CD14 cells was used as another marker for monocyte differentiation and has been an antigen associated with cytokine expression patterns characteristic of tissue macrophages (Ziegler-Heitbrock et al., 1993; Frankenberger et al., 1996). The monocyte granularity associated with its differentiation was measured by CD14-associated "backgating" on side light-scatter characteristics (SSC). Whole blood was stained with CD14-fluorescein isothiocyanate (FITC), CD16-phycoerythrin (PE) (DAKO, Carpinteria, CA, USA), CD8-FITC, CD38-PE, HLA-DR-PE, and CD4-peridinin chlorophyll protein (PerCP) (Becton-Dickinson, San Jose, CA, USA) for 15 min at room temperature. Negative controls consisted of aliquots stained with isotype IgG-FITC, IgG-PE, and IgG-PerCP; all staining was performed as per manufacturers specifications. Samples were then lysed with FACS Lysing Solution (Becton-Dickinson) for 10 min at room temperature

Table 1
Clinical summary of sALS patients

Patient ID no.	Patient age (years)	Patient gender	Therapy		Duration of illness (months)	ALSFRS-R score
			riluzole[a]	NSAID[b]		
Patient 1	76	F	No	No	46	19
Patient 2	59	M	Yes	No	15	30
Patient 3	77	F	Yes	No	37	34
Patient 4	57	M	Yes	Celebrex	19	34
Patient 5	63	F	Yes	Vioxx	10	N/A
Patient 6	75	F	No	No	78	33
Patient 7	64	M	Yes	No	43	13
Patient 8	58	F	Yes	No	42	28
Patient 9	72	M	Yes	No	18	26
Patient 10	40	F	Yes	No	12	28
Patient 11	58	M	Yes	Celebrex	21	18
Patient 12	55	M	Yes	No	85	N/A
Patient 13	82	M	No	Celebrex	4	N/A
Patient 14	67	M	Yes	Celebrex	45	20
Patient 15	79	M	Yes	No	14	15
Patient 16	49	M	No	No	88	16
Patient 17	60	M	No	No	18	32
Patient 18	49	M	Yes	N/A	26	29
Patient 19	37	M	Yes	Celebrex	82	8
Patient 20	70	M	Yes	No	29	N/A
Patient 21	49	M	Yes	No	14	39
Patient 22	41	F	Yes	No	33	37
Patient 23	58	M	Yes	No	20	32
Patient 24	30	M	No	No	24	35
Patient 25	65	M	Yes	No	18	42
Patient 26	41	M	Yes	No	43	43
Patient 27	58	M	Yes	Celebrex	25	26
Patient 28	66	F	Yes	No	6	39
Patient 29	63	F	Yes	Excedrin	18	34
Patient 30	65	M	Yes	Celebrex	33	34
Patient 31	34	M	Yes	Celebrex	41	15
Patient 32	47	M	Yes	No	17	38
Patient 33	62	M	Yes	No	57	43
Patient 34	87	M	No	No	93	25
Patient 35	64	F	Yes	Celebrex	45	35
Patient 36	60	F	Yes	No	27	23
Patient 37	65	F	Yes	No	27	30
Patient 38	53	M	No	Naproxyn	45	37

[a] Fifty milligrams twice daily.
[b] Standard dose.

followed by 0.1% sodium azide+PBS $Ca^{+}Mg^{+}$ free wash. The stained cells were then resuspended in 1 ml of fixing solution (1% paraformaldehyde in PBS, with 0.1% sodium azide). Analysis was accomplished by acquisition of data on a FACScan flow cytometer (Becton-Dickinson) with Cellquest software where at least 20,000 cells were counted per analysis.

2.3. Detection of Serum IgG and IgM

Plasma from sALS patient blood was obtained by Percoll gradient centrifugation, and was frozen at −70 °C until use. Standard ELISA for determination of serum antibody: Anti-Human IgG Fab or anti-Human IgM (Sigma, St. Louis, MO, USA) were coated (100 μl /well) into 96-well ELISA plates (Nunc, Roskilde, Denmark) by incubation for at least one hour at 37 °C. The plates were washed one time with TBS (150 mM NaCl, 20 mM Tris–HCl, pH7.4), then blocked for 30 min by addition of 150 μl/well of BLOTTO (TBS plus 0.1% Tween-20, 2.5% normal goat serum, 2.5% non fat dry milk) at room temperature, with gentle rocking. ELISA plates were subsequently washed once (1×) with TBS. Serial dilutions of serum were added to coated plates (duplicate wells each dilution, 100 μl/well) and allowed to react for 90 min, room temperature. A standard calibration series (0–5 μg/ml) for IgG and IgM (Sigma) was prepared, added to ELISA wells, and incubated in parallel. BLOTTO was used in all dilutions. Following the 90-min incubation, all fluids were removed by aspiration, then all plates were washed 3× with TBS. Bound IgG antibodies were detected by adding 100 μl/well of anti-Human IgG alkaline phosphatase-conjugate (Promega, Madison, WI, USA) diluted 1:10000 in BLOTTO. Bound IgM antibodies were

detected by adding 100 μl/well of anti-Human IgM alkaline phosphatase-conjugate (Kirkegaard and Perry, Gaithersburg, MD, USA) diluted 1:5000 in BLOTTO. Antibody conjugates were incubated for 1 h at room temperature with gentle agitation. Conjugates were removed by aspiration and plates washed 4× with TBS. Development of color reaction was effected by addition of 100 μl of PNPP substrate (Sigma) to each well, followed by incubation for 20 min at room temperature. The optical density (O.D.) in each well was read at 405 nm. Any sera with exceptionally low or high values were re-tested. Raw IgG and IgM values from ALS samples were multiplied by a conversion factor to account for the different means of preparation from normal plasma.

2.4. Statistical analysis

Cut-off values for defining cell activation as "positive" and "negative" for sALS patients and disease controls were determined by comparison with values from normal ALS-negative, healthy donors. Results are expressed as the mean±SD. Statistical analysis of group differences, linear regressions and Pearson correlations were performed by GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA, USA). Distribution of groups was analyzed by Kolmogorov-Smirnov test. Between-group comparisons were then made using one-way ANOVA with Newman–Keuls test. For all analysis, a value of $p<0.05$ was considered significant.

3. Results

A cross-sectional study of immune activation was performed on blood from 38 patients diagnosed with sALS as compared to control groups with initial statistical analyses performed independent of drug treatment status. In the present investigation two control groups were chosen to compare with sALS patients: 28 age-matched normal controls and 25 AD patients as neurological disease controls. Blood cells from patients with sALS, similar to disease control AD patients, showed abnormal levels of activation. Table 2 summarizes the results of this study. Patients with sALS and AD had significantly higher proportional levels of the CD4 T lymphocyte subset as compared to normal controls ($p<0.05$). By contrast, the CD8 T-cell level and the ratio of CD4/CD8 were similar in all three groups. No evidence of lymphocytic activation above normal in T-cell subsets was observed in patients with sALS and disease controls.

Analysis of monocyte/macrophage markers showed that CD14+monocytes from patients with sALS and AD expressed significantly higher than normal levels of major histocompatibility (MHC) antigen class II (HLA-DR) ($p<0.001$) but no difference was found in the absolute percent of CD14 cells within the total white blood cell count in either of the sALS and AD patient blood specimens as compared to normal controls (Table 2). Almost half of the CD14 cells in sALS and AD blood had characteristics of tissue macrophages, expressing significantly higher than normal levels of the CD16 antigen ($p<0.001$). The aberrant monocytic phenotype defined by higher expression of HLA-DR and CD16 was associated with significant differences in CD14-associated SSC (measure of granularity and differentiation) between patients with sALS and normal controls. Compared with normal controls, monocytes from sALS patients had statistically increased granularity (higher SSC values) ($p<0.01$). Finally, the overall status of humoral immunity was evaluated by quantitating levels of serum-IgG and -IgM in patients with sALS and normal controls (Table

Table 2
Comparative analysis of serum antibodies and differentiation antigen expression in blood of sALS patients, normal controls and AD

Parameter	sALS (n=38)	Normal Controls (n=28)	AD (n=25)	p Value (sALS vs. Normal Controls)	p Value (AD vs. Normal Controls)	p Value (sALS vs. AD)
CD4/CD8	2.87±1.56	2.33±1.59	3.43±2.72	NS	NS	NS
%CD4	47.43±8.04	39.81±11.30	47.37±11.22	<0.01	<0.05	NS
%CD4CD38	27.21±11.76	32.24±10.51	25.67±12.02	NS	NS	NS
Med CD4CD38[a]	13.02±14.54	19.15±16.71	14.08±16.12	NS	NS	NS
%CD8	20.38±8.22	21.19±8.53	19.93±12.43	NS	NS	NS
%CD8CD38	13.67±8.20	12.41±6.85	15.86±11.46	NS	NS	NS
Med CD8CD38[a]	3.43±6.46	1.93±2.40	5.85±16.19	NS	NS	NS
%CD14	2.34±1.01	2.51±0.93	2.49±1.00	NS	NS	NS
Mean CD14DR[b]	825.60±206.62	582.56±144.35	911.93±341.80	<0.001	<0.001	NS
CD14 SSC[c]	466.3±159.6	346.5±42.3	434.7±226.5	<0.01	NS	NS
%CD14CD16	42.44±11.22	23.90±10.60	41.77±18.97	<0.001	<0.001	NS
Serum-IgG (mg/ml)[d]	7.80±5.76	11.26±5.57	ND[e]	<0.003	ND	ND
Serum-IgM (mg/ml)[d]	2.28±2.30	1.37±1.14	ND	<0.03	ND	ND

[a] Median CD38 fluorescence expressed on CD4 and CD8 T-Cell.
[b] Mean DR fluorescence expressed on CD14 monocyte.
[c] CD14-associated side light-scatter characteristics.
[d] n=80 for control samples for serum-IgG and -IgM.
[e] ND, not data.

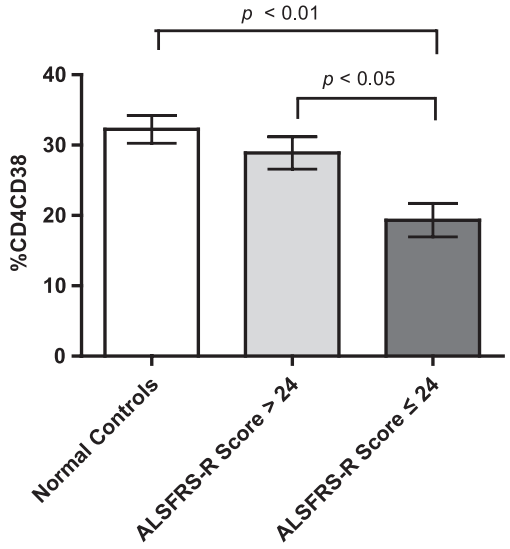

Fig. 1. Relationship of the revised ALS Functional Rating Score (ALSFRS-R) to CD4 T-cell co-expression of the activation antigen CD38 in sALS patients. Patients with sALS were divided into two groups based on a score of 24, the midpoint of the ALSFRS-R scale. The CD4 activation marker CD38 was significantly lower in patients with severe impairment (ALSFRS-R score of 0–24, n=9) compared to normal controls (p<0.01) and patients with milder impairment (ALSFRS-R score>24, n=25) (p<0.05), but no difference was found between normal controls and patients with milder impairment.

2); serum-IgG levels in patients with sALS were significantly lower than normal controls (p<0.003), whereas, serum-IgM concentrations were significantly higher (p<0.03) (sera from the AD patients were not available for study).

3.1. CD4 T-cell activation is decreased in advanced ALS disease

To test whether T lymphocytic activation would be related to duration or severity of disease, the T-cell activation results of patients with sALS from Table 2 were compared with the clinical ALS values shown in Table 1. To simplify clinical correlative analyses, sALS patients were divided into two groups based on disease severity as documented by their ALSFRS-R score (0–48, no disease=48). Those with severe impairment (an ALSFRS-R score of 0–24, n=9) were compared to those with milder impairment (ALSFRS-R score>24, n=25). As shown in Fig. 1, T-cell activation levels as quantitated by detection of CD38 antigens on the surface of CD4 T cells were significantly different between the two groups (p<0.05). Compared with normal controls, CD4/CD38 reactivity was significantly lower in patients with ALSFRS-R scores of 24 or lower (p<0.01) whereas no difference of CD4/CD38 reactivity was found in sALS patients with less severe disease (ALSFRS-R score>24). No significant disease associated changes were observed in any of the other T-cell (CD4 or CD8) parameters measured.

3.2. Macrophage activation and ALS disease progression

To evaluate whether systemic monocyte/macrophage activation would be related to duration or severity of disease, sALS macrophage activation parameters from Table 2 were subjected to the same analysis shown in Fig. 1 to test whether any disease specific changes would be present. Fig. 2a shows that the degree of CD14 co-expression of HLA-DR was independent of severity of disease as defined by ALSFRS-R score with both categories significantly higher than normal controls (p<0.001). Further analysis was performed based on rate of disease progression. When the rate of ALS disease progression (ALSFRS-R score change per month) was compared to CD14 cell HLA-DR expression, a direct and significant relationship was observed. Fig. 2b shows that higher CD14-DR levels were associated with a more rapid progression of ALS disease (Pearson r=0.3519, p<0.05). Finally, the elevated level of macro-

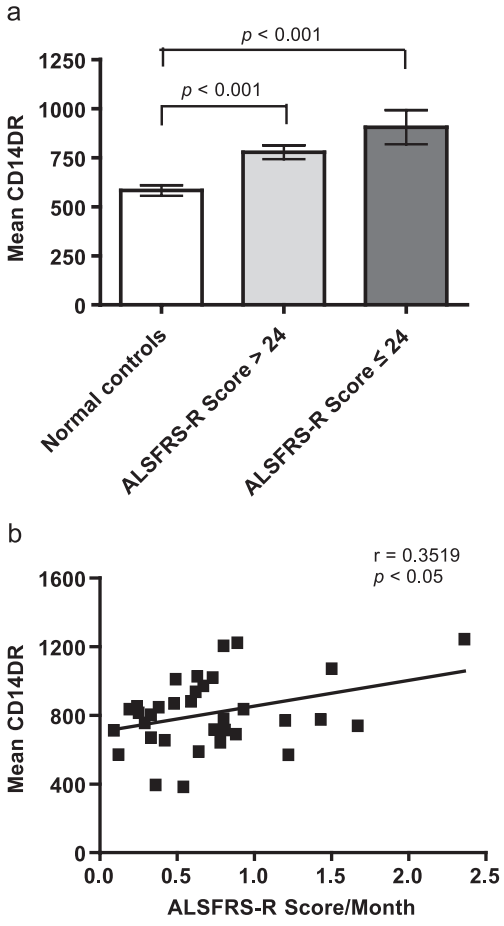

Fig. 2. Analyses of macrophage activation defined by CD14 co-expression of HLA-DR in patients with sALS. (a) Relationship of ALSFRS-R to CD14 co-expression of HLA-DR in sALS patients. Expression of HLA-DR on sALS blood monocytes was significantly higher than normal control (p<0.001) in patients with severe impairment and with milder disease, but CD14-DR levels was independent of disease severity defined by ALSFRS-R score. (b) Positive correlation of levels of HLA-DR on sALS CD14 cells with the rate of disease progression (ALSFRS-R score change per month) (Pearson r=0.3519, p<0.05).

phage differentiation antigen CD16 co-expression on the CD14 expressing monocytes in patients with sALS was independent of severity of disease (data not shown).

3.3. Changes of serum-IgG and -IgM in patients with sALS

Table 2 shows that the concentration of IgG and IgM in serum was significantly different in patients with sALS as compared to normal controls. Levels of serum-IgG and -IgM also varied with disease severity. sALS patients with ALSFRS-R scores of 0–24 had significantly lower levels of serum-IgG than individuals with milder disease ($p<0.05$) and normal controls ($p<0.01$); serum-IgG levels were similar in individuals with milder disease and normal controls (Fig. 3a). However, serum-IgM levels were significantly higher in individuals with earlier stage disease ($p<0.01$) than normal controls and not significantly different between normal controls and in individuals with severe disease (Fig. 3b).

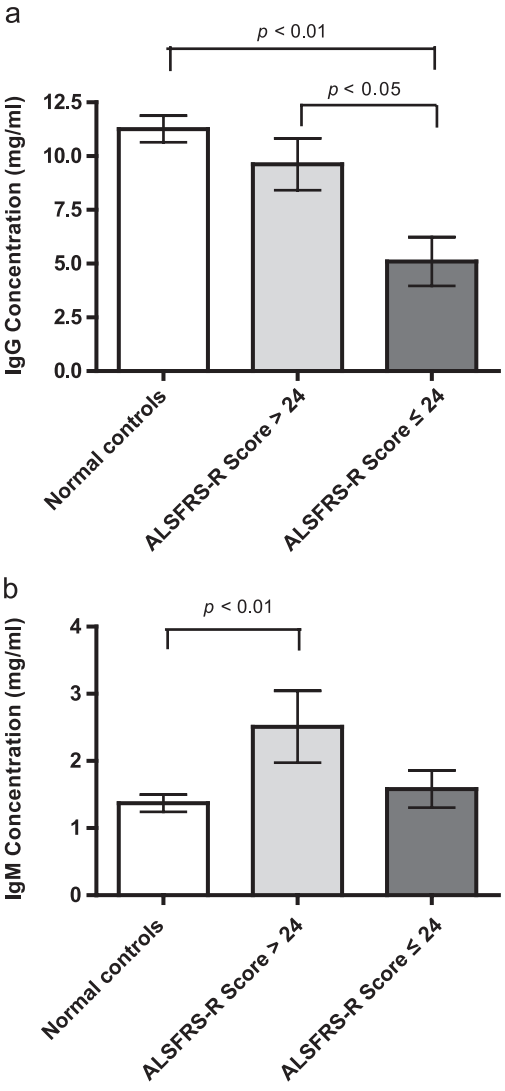

Fig. 3. Comparison of serum -IgG and -IgM levels between normal controls and sALS patient groups by ALSFRS-R categories. (a) Significantly lower levels of serum-IgG were found in sALS patients with severe impairment compared to patients with milder disease ($p<0.05$) and normal controls ($p<0.01$), but with no difference between patients with milder impairment and normal controls. (b) Levels of serum-IgM in patients with milder impairment were significantly higher than normal controls ($p<0.01$), but with no difference between patients with severe impairment and normal controls.

3.4. Therapy related changes in ALS specific immune activation status

Table 1 shows the medications that patients with sALS were taking at the time of assessment in the current study. The drugs fell into two different categories; riluzole approved for slowing ALS disease progression and non-steroidal anti-inflammatory drugs (NSAID). Table 3 summarizes the effects of medication treatments on immune activation measurements in patients with sALS. Levels of macrophage activation and differentiation as measured by HLA-DR and CD16 did not change with therapy. Even the inclusion of NSAID was not associated with lower levels of macrophage activation (Table 3). Similarly, there were no significant differences between patients in the three treatment categories regarding the levels of CD4/CD38 co-expression and serum-IgG. However dual therapy (riluzole+NSAID) was associated with normalization of serum-IgM levels, whereas, the riluzole alone group was no different from untreated patients.

4. Discussion

Recent clinical and pathological studies have shown that involvement of microglia/macrophage activation and immune reactivity is relatively common in spinal cord tissues of patients with ALS (Provinciali et al., 1988; Engelhardt and Appel, 1990; Appel et al., 1993; Alexianu et al., 2001; Obal et al., 2001; McGeer and McGeer, 2002; Wilms et al., 2003; Henkel et al., 2004). Although microglia/macrophage activation and immune-inflammatory response in ALS disease progression has been suggested, few studies to date have explored the status of the systemic immune response in patients with ALS.

In the current study, we performed immunophenotypic analyses and humoral immunity assessment of blood from patients with sALS to determine whether systemic immune alteration might be present in sALS. Two control groups, age-matched normal control and AD neurological disease control, were chosen to compare with sALS patients. In concordance with previous findings of activated microglia/macrophages in the spinal cords of ALS patients, persistently activated macrophages were observed in the blood of patients with sALS, similar to the neurological disease control AD group. The high levels of macrophage activation and differentiation were persistent throughout the course of sALS. In addition, macrophage activation defined by CD14 co-expression of HLA-DR was directly related to the rate of

Table 3
Comparative analyses of serum antibodies and differentiation antigen expression in blood of normal controls and sALS patients with or without medications

Parameter	Normal Controls (n=28)	sALS patients		
		Untreated (n=6)	riluzole (n=19)	riluzole+NSAID (n=10)
CD4/CD8	2.33±1.59	2.59±1.46	2.81±1.57	2.74±1.44
%CD4	39.81±11.30	44.62±8.09	46.45±7.48	49.02±9.20
%CD4CD38	32.24±10.51	20.52±7.92	28.21±10.95	30.81±14.87
Med CD4CD38[a]	19.15±16.71	5.91±5.47	13.13±12.85	19.75±20.12
%CD8	21.19±8.53	21.25±9.59	20.20±7.90	21.43±8.27
%CD8CD38	12.41±6.85	15.05±6.70	13.54±9.75	12.47±5.49
Med CD8CD38[a]	1.93±2.40	2.18±1.99	5.11±8.73	1.70±2.16
%CD14	2.51±0.93	2.39±1.10	2.32±1.24	2.33±0.66
Mean CD14DR[b]	582.56±144.35	779.95±336.69	805.62±167.06	829.43±181.55
CD14 SSC[c]	346.5±42.3	509.6±220.3	457.6±157.4	467.2±162.3
%CD14CD16	23.90±10.60	41.20±8.19	43.69±13.21	37.83±8.62
Serum-IgG (mg/ml)[d]	11.26±5.57	8.82±5.77	7.64±4.55	8.59±8.35
Serum-IgM (mg/ml)[d]	1.37±1.14	2.48±1.08	2.69±3.09	1.53±0.87

[a] Median CD38 fluorescence expressed on CD4 and CD8 T-Cell.
[b] Mean DR fluorescence expressed on CD14 monocyte.
[c] CD14-associated side light-scatter characteristics.
[d] n=80 for control samples for serum-IgG and -IgM.

sALS disease progression. Moreover, the macrophage activation status was not improved in sALS patients treated by riluzole (the only currently approved treatment for ALS) or NSAID. The direct relationship between degree of blood macrophage activation and rate of ALS disease progression suggests a link between the blood and pathogenic processes ongoing in the CNS.

Modulation of HLA-DR on blood monocytes has been associated with a variety of pathogenic states and blood measurements have been shown to have clinical significance (Pulliam et al., 1997; Gascon et al., 2002; Gu and van Oeveren, 2003; Melichar et al., 2003). The significantly higher levels of HLA-DR on the circulating monocytes in patients with sALS and AD observed in the current study therefore might be the reaction of peripheral immune system to motor neuron injury, extending from the reaction of microglia/macrophages in the spinal cord. By this model, the blood macrophage activation level might reflect degree of spinal cord injury/ongoing pathogenesis and have no relationship directly with spinal cord pathology. In this respect, sALS appears similar to other neurodegenerative diseases such as MS (Bar-Or et al., 2003; Filion et al., 2003), AD (Kusdra et al., 2000; Casal et al., 2003) and HAD (Pulliam et al., 1997; Liu et al., 2000), where activated macrophages correlate with the clinical stages of disease and/or the change of disease pathophysiology. The current study on blood from sALS patients and neurological disease controls added to previous reports on blood from patients with HAD (Pulliam et al., 1997) and AD (Kusdra et al., 2000) suggest certain shared pathogenic features in three neurodegenerative diseases that involve persistent macrophage activation.

The high levels of HLA-DR on sALS and AD CD14 cells was coupled with an elevation in the proportion of CD14 cells co-expressing the tissue macrophage marker, CD16. Characterized as tissue macrophages (Ziegler-Heitbrock et al., 1993), the circulating activated CD14+/CD16+monocytes in patients with neurodegenerative diseases enter the CNS (Fischer-Smith et al., 2001), and expose neural cells to neuro-toxic factors similar to those released by activated macrophage reported to cause neural-cell damage in vitro (Pulliam et al., 1997). Severity of neurological disorders such MS, AD, HAD and now, sALS may be due in part to neuro-toxic factors released by these activated monocyte/macrophages when migrating into the CNS and crossing the BBB (Pulliam et al., 1997; Fischer-Smith et al., 2001; Minagar et al., 2002; Filion et al., 2003).

Although blood macrophage abnormalities persisted throughout the sALS process in this cross sectional study, T-cell measurements showed changes related to disease severity. In the study of T-cell activation markers, CD38 levels decreased on CD4 T cells with sALS disease progression. However, the CD8/CD38 reactivity remained within the normal range. These data suggest that the adaptive component of the (T-cell) immune system did not become active during sALS pathogenesis even in the face of persistent systemic macrophage activation. Compared with normal controls, sALS patients had a significant increase in the percentage of T cells expressing CD4, and the percentage of CD8+T cells was found to be in the normal range. The absence of T-cell activation response in our study agrees with studies on an animal model of familial ALS (Alexianu et al., 2001; Hensley et al., 2002), which suggest that lymphocytes, unlike microglia/macrophages, play a minor role in the active ALS spinal cord associated neuro-inflammatory reaction. Therefore, the neuro-inflammatory process in ALS may be minimally dependent upon lymphocyte infiltration but rather is driven by macrophage activity.

In the current study, concentrations of serum-IgG and -IgM antibodies were significantly different compared to normal controls, and levels changed with disease progression. Patients with sALS had a normal IgG concentration and higher levels of IgM in early stages of disease. Lower levels of serum-IgG with a concomitant normalization in serum-IgM secretion were observed with disease progression in patients with sALS. Normalization of serum-IgM in sALS patients was associated with combined riluzole and NSAID therapy. Alteration of serum antibodies was observed in several studies in different subtypes of ALS patients (Appel et al., 1986; Provinciali et al., 1987, 1988; Apostolski et al., 1991; Duarte et al., 1991). Our study confirmed the abnormality of serum antibodies in ALS patients, and showed the correlation between levels of serum-IgG and -IgM and ALS disease progression. Specificities of serum antibodies remain unclear, but some functional tests have showed the relationship between serum antibodies and neuron injury related glutamate release in ALS (Andjus et al., 1997; La Bella et al., 1997). Serum-IgM changes with disease progression and combined drug therapy in the current study suggest some form of autoimmune process in sALS Pathogenesis.

Evidence accumulating over the past decade indicates that inflammatory processes may be involved in the progressive neuronal death in many neurodegenerative diseases, including AD (McGeer and McGeer, 1998; Akiyama et al., 2000), MS (Bar-Or et al., 2003; Floris et al., 2004), Parkinson's disease (Hirsch et al., 2003), and ALS (Alexianu et al., 2001; McGeer and McGeer, 2002; Henkel et al., 2004). Whether anti-inflammatory drugs would be useful for treatment of neurodegenerative diseases is still an open question. In the current study, blood macrophages from patients with sALS remained activated throughout ALS disease even in patients treated with anti-inflammatory medication and riluzole, although combined drug treatment was associated with normalization of serum-IgM levels. Other macrophage targeted therapeutic approaches under development for ALS may hold promise, as drug studies in transgenic ALS mice found a delay in disease onset and mortality in association with the drug minocycline, an inhibitor of microglia/macrophage activation and proliferation (Tikka et al., 2001; Van Den Bosch et al., 2002). This type of result further supports the active role of macrophages in ALS disease.

This investigation, for the first time, demonstrates a systemic alteration of blood cell activation in patients with sALS. Persistent macrophage activation was observed in sALS blood and levels of HLA-DR on CD14 cells was directly associated with the rate of sALS disease progression. Previous studies of macrophage activation in spinal cord (Engelhardt and Appel, 1990; Appel et al., 1993; Obal et al., 2001; Wilms et al., 2003; Henkel et al., 2004) and the current study confirm systemic macrophage activation in sALS, suggesting a potentially active role of macrophages in sALS disease. Abnormally activated macrophages without evidence of concomitant T-cell activation was observed in sALS blood. These observations along with extensive spinal cord microglial activation before the onset of clinical symptoms (Alexianu et al., 2001) and prior to evidence of significant motor neuron loss in an ALS mouse model, suggest that systemic immune dysregulation plays a role in ALS disease. Therefore, it may be hypothesized that measuring the activation-and inflammation-related markers of circulating monocytes, such as HLA-DR and CD16, as well as the status of T-cell activation in patients with ALS might provide valuable assistance in monitoring the treatment of ALS as an immune dysfunction disease. Moreover, these findings suggest that therapeutic intervention aimed at reducing inflammation/inflammatory macrophage levels in patients with sALS appears worthy of further investigation.

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