Increased ratio of circulating neutrophils to monocytes in amyotrophic lateral sclerosis

ABSTRACT

Objective: To elucidate amyotrophic lateral sclerosis (ALS) biomarkers and potential mechanisms of disease, we measured immune cell populations in whole blood from a large cohort of patients with ALS.

Methods: Leukocytes were isolated from the blood of 44 control patients and 90 patients with ALS. The percentages and total numbers of each cell population were analyzed using flow cytometry and matched with patient ALS Functional Rating Scale–Revised (ALSFRS-R) score to correlate leukocyte metrics with disease progression.

Results: We show a significant increase in the percentage of neutrophils and a significant decrease in the percentage of CD4 T cells and CD16\(^{+}\) monocytes in the blood of patients with ALS compared to controls; however, only CD16\(^{+}\) monocyte levels correlated with disease progression. We also examined the monocyte surface expression of CCRL2 and CCR3; CD16\(^{+}\) monocytes displayed decreased percentages and total numbers expressing CCR3, but these numbers did not correlate with ALSFRS-R score. We found that combining multiple disease metrics yielded the most accurate predictor of disease progression: the ratio of neutrophils to CD16\(^{+}\) monocytes (N:M ratio) is significantly increased in patients with ALS and better correlates with disease progression than any other single metric.

Conclusions: These observations implicate neutrophils and monocytes as important factors in late disease progression. *Neurol Neuroimmunol Neuroinflamm* 2016;3:e242; doi: 10.1212/NXI.0000000000000242

GLOSSARY

ALS = amyotrophic lateral sclerosis; ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised; CCR = chemokine receptor; N:M ratio = neutrophil to CD16\(^{+}\) monocyte ratio; SSC = side scatter.

Sporadic amyotrophic lateral sclerosis (ALS) may arise from a myriad of environmental and genetic sources. Neuroinflammation is universally observed in ALS, suggesting all ALS forms ultimately funneled through the immune system, thereby making it an incredibly attractive source of biomarkers and potential therapies.\(^1\) The immune response in ALS, however, is incredibly complex. This is attributable to the phased nature of the immune system during ALS pathology: the early immune response is dominated by protective cytokines and immune polarization, while the later response is destructive and correlates with rapid disease progression.\(^2\) Different immune cell types may have a dominant role during each phase, or the same cell type may have opposite roles based on the immune environment.\(^1\)–\(^3\) Thus, there is a critical need to elucidate immune mechanisms of ALS and identify potential biomarkers.

In the present study, we phenotyped immune cells in the peripheral blood of patients with ALS and healthy controls to identify immune cell populations linked with ALS progression. We show that several cell populations in patients with ALS are altered relative to healthy controls...
and that these alterations correlate with disease progression. In particular, we found that the percentage and number of CD16– monocytes, as well as the rate of chemokine receptor 3 (CCR3) and CCRL2 surface marker expression, are reduced in ALS. In addition, we found that the neutrophil to CD16– monocyte ratio (N:M ratio) correlates best with disease progression.

METHODS Study population. The study population included patients with ALS and healthy controls recruited under an approved protocol (University of Michigan Medical School Institutional Review Board [HUM00028826]). All patients provided written informed consent. Patients with ALS were diagnosed with "probable" or "definite" ALS using the El Escorial World Federation of Neurology criteria at the University of Michigan ALS Clinic between March 2011 and May 2014. Disease progression was monitored in patients with ALS using the ALS Functional Rating Scale–Revised (ALSFRS-R) in which lower scores correlate with increasing functional impairment. Healthy control patients were recruited via UMClinicalStudies.org; patients with a family history of ALS, previous diagnosis of a neurodegenerative disorder, chronic inflammatory disease, collagen vascular disease, use of immunomodulatory medication, or any fever or sickness at the time of blood draw were excluded.

Standard protocol approvals, registrations, and patient consents. Patients with ALS and healthy controls were consented by the principal investigator or designee using a comprehensive written consent. In brief, the study was reviewed in detail to each patient, patient questions were answered to their satisfaction, consent was reviewed, and the consent form was signed by the patient and principal investigator (or designee).

Blood sample collection and flow cytometry analyses. Blood samples were obtained from patients with ALS and healthy controls by peripheral venipuncture into a BD Vacutainer sodium heparin tube (BD Biosciences, San Jose, CA), placed at 4°C, and processed within 6 hours of collection. A 200-μL aliquot of whole blood was lysed with a red blood cell lysing buffer (0.8% NH₄Cl, 0.098% KHCO₃, 0.1 mM EDTA, and 13.8 mM HEPES), washed with FACS buffer (phosphate-buffered saline, 0.8% fetal bovine serum, and 0.1% sodium azide), and kept on ice throughout the processing and in darkness throughout immunostaining. Cells were counted and incubated in human TruStain FcX block (BioLegend, San Diego, CA) before staining. Following isolation from peripheral blood, leukocytes were identified using CD16. T cell gates were drawn from SSC vs CD14; subpopulations were then drawn as follows: the neutrophil gate was drawn to eliminate eosinophils. Monocyte gates were drawn from the SSC-low population followed by SSC vs CD14; subpopulations were identified using CD16. T cell gates were drawn from SSC vs CD3 followed by CD4 vs CD8. Total numbers of each cell population were determined by multiplying the percentage of each cell type with the total number of leukocytes.

RESULTS Peripheral blood leukocyte levels are altered in patients with ALS. Forty-four healthy control patients (23 women and 21 men) ranging in age from 46 to 76 years (mean 59.20 years) and 90 patients with ALS (37 women and 53 men) ranging in age from 38 to 92 years (mean 61.99 years) were enrolled in the study (table 1). Following isolation from peripheral blood, leukocytes from each patient were counted, stained with fluorescently labeled antibodies, and analyzed using flow cytometry. Based on cell surface marker expression, we identified neutrophils, CD16+ monocytes, CD16– monocytes, CD4+ T cells, and CD8+ T cells in the peripheral blood. The total number of blood leukocytes was not significantly different between patients with ALS and healthy human controls (figure 1A). We found that the percentage of neutrophils within the leukocyte population, but not the total number, was increased in patients

Table 1. Study population demographics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy controls</th>
<th>Patients with ALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>44</td>
<td>90</td>
</tr>
<tr>
<td>Age range, y</td>
<td>46–76</td>
<td>38–92</td>
</tr>
<tr>
<td>Age, y, mean ± SD</td>
<td>59.20 ± 7.64</td>
<td>61.99 ± 11.67</td>
</tr>
<tr>
<td>Males, n</td>
<td>21</td>
<td>53</td>
</tr>
<tr>
<td>Females, n</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>ALSFRS-R score, mean ± SD</td>
<td>NA</td>
<td>30.96 ± 7.48</td>
</tr>
<tr>
<td>ALSFRS-R range</td>
<td>NA</td>
<td>11–44</td>
</tr>
</tbody>
</table>

Abbreviations: ALS = amyotrophic lateral sclerosis; ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised; NA = not applicable.
with ALS relative to healthy controls (figure 1B). Levels of CD16⁺ monocytes were unaffected in patients with ALS, and we observed a trend toward a lower percentage of blood-borne CD16⁺ monocytes and fewer total CD16⁺ monocytes, although numbers observed were not significantly different from healthy control patients (figure 1B). Similarly, we observed a significant decrease in the percentage and total number of CD8 T cells, although the levels of CD8 T cells were not altered in patients with ALS.

Percentages and total number of each cell population were then compared with patient ALSFRS-R score to determine positive or negative correlations between cell types and severity of disease. Total number of leukocytes showed no relationship to the ALSFRS-R score (data not shown). We observed that the percentage and total number of neutrophils both increased as disease progressed, but the relationship did not reach statistical significance, indicating a lack of correlation between the 2 metrics of disease (figure 2). Similarly, we saw no correlation between CD16⁺ monocytes or CD8 T cells and patient ALSFRS-R scores. However, we observed a significant reduction in the percentage of CD16⁺ monocytes within the total leukocyte population, although total numbers of CD16⁺ monocytes did not correlate with disease progression. Of note, despite the reduced percentage and number of CD4 T cells observed in the blood of patients with ALS (figure 1B), there was no correlation between ALSFRS-R score and either of these metrics, suggesting that CD4 T cell levels in the blood are reduced at all stages of disease.

CCR3 and CCRL2 monocyte surface expression is altered during ALS. Based on these findings, we wished to examine whether surface markers on CD16⁺ monocytes were altered during the course of ALS. We have previously shown that markers that are expressed by monocytes are upregulated in the CNS of patients with ALS, particularly CCRL2 and CCR3.8 We therefore determined whether the frequency of CCRL2 and CCR3 surface marker expression was different in patients with ALS and healthy control patients in CD16⁺ monocytes populations. We observed that in CD16⁺ monocytes, there was a significant increase in the percentage of cells expressing CCRL2, but there was no difference in the total number of expressing cells (figure 3A). Similarly, we observed no changes in the expression pattern of CCRL2 in CD16⁺ monocytes.

In contrast, CCR3 expression was altered in CD16⁺ monocytes rather than CD16⁺ monocytes: CD16⁺ monocytes displayed a significantly reduced percentage of cells expressing CCR3 as well as a significantly reduced number of CCR3-positive CD16⁺ monocytes (figure 3B). However, when data for both CD16⁺ and
CD16^- monocytes were correlated with parameters of disease, we observed no significant correlation between CCR3 or CCRL2 surface expression and ALSFRS-R, duration of disease, progression rate, or site of onset (data not shown). These data indicate that changes in CD16^- monocyte CCR3 expression are not significantly affected by the disease stage.

N:M ratio correlates with disease progression. To better correlate immune cell populations with ALS progression, we examined 2 separate cell populations simultaneously. Having observed an increase in neutrophils during disease while observing a decrease in CD16^- monocytes, we calculated the N:M ratio for each individual patient. We observed that the N:M ratio
was significantly elevated in patients with ALS compared to healthy control patients; the N:M ratio increased nearly 50% (figure 4A). Even more striking, when correlated with ALFSRS-R score, there was a highly significant relationship between disease score and the N:M ratio: as the ALFSRS-R score dropped (and disease became more severe), the N:M ratio significantly increased (figure 4B). Finally, the N:M ratio was significantly linked only to ALFSRS-R score, as other metrics of disease such as progression rate and disease duration were not significantly correlated (data not shown). This indicates that the N:M ratio is a remarkable predictor of ALS disease state that is not significantly altered by outside variables.

DISCUSSION

The present study examined the role of blood-borne leukocytes and their role in driving ALS. More specifically, we attempted to correlate leukocyte numbers and their surface marker expression with clinical metrics of disease in order to identify potential biomarkers and mechanisms of disease that will ultimately lead to new and effective therapies. We obtained peripheral blood from patients with ALS and healthy controls and used flow cytometry to assess the total number of leukocytes as well as surface marker expression in specific populations of cells. The analysis of ALS using peripheral blood leukocytes is important for several reasons. First, it is now well established that ALS progression features a key immunologic component. Second, ALS requires multiple cell types to drive disease, as neuronal death alone does not trigger disease pathology; immune polarization has a critical role in determining whether the CNS environment is protective or destructive. Third, previous in vivo mouse studies have managed to alter ALS progression by removing specific cell populations in the periphery via antibody depletion or genetic knockout; manipulation of the immune system outside the CNS can therefore have an effect on disease pathology. Fourth, analysis and treatment focusing on blood-borne leukocytes is minimally invasive and does not require potentially damaging ingress into the fragile CNS.

Consistent with previous reports, we observed an increase in the percentage of neutrophils within the total leukocyte populations. This is important, as neutrophils may have several roles in disease (table e-1 at Neurology.org/nn). Most obviously, they may have a proinflammatory role in driving neurodegeneration and may aid disease pathology by contributing to the breakdown of the brain–spinal cord barrier, which has been shown to drive ALS. However, treatment of humans and mouse ALS models with granulocyte-colony stimulating factor, which induces granulocytosis, does not exacerbate disease symptoms. An alternate possibility is that neutrophils are playing a role in neuronal repair; neutrophils are some of the first cells to respond and are responsible for initiating the repair response in the CNS. Thus, the observed increase in blood-borne neutrophils may result from the exclusion of these cells following the initiation of the repair response.

We also observed a decrease in the percentage of CD16+ monocytes in the peripheral blood of patients with ALS, with reduction correlating with disease progression. A previous study profiling CD16+ monocytes in humans and mice showed decreased numbers in the peripheral blood, but accumulation in the CNS of mice. Therefore, reduced CD16+ monocytes in the blood of human patients may reflect CNS infiltration: monocytes are found in the CNS...
The N:M ratio is significantly increased in patients with ALS and correlates with disease progression.

(A) The N:M ratio in healthy control patients (filled black circles) and patients with ALS (empty circles) is shown. Average and SEM are displayed. Asterisks above bars indicate significance (**p < 0.001). (B) The N:M ratio in the blood of patients with ALS is correlated with the ALSFRS-R score at the time of blood draw. Data are displayed with best-fit line and 95% confidence intervals. \( R^2 \) value and \( p \) value are also shown. ALSFRS-R scores are displayed from high (left) to low (right) to illustrate disease progression. ALS = amyotrophic lateral sclerosis; ALSFRS-R = ALS Functional Rating Scale-Revised; N:M ratio = neutrophil to CD16\(^+\) monocyte ratio.

Near dying neurons in patients with ALS,\(^{26,27}\) T cells or memory T cells,\(^{13}\) as CD4 T cells have been shown to have a protective role in ALS.\(^{14}\)

Based on the significant correlation between reduced CD16\(^+\) monocyte levels and disease progression, we wished to determine whether surface marker expression on the monocytes of patients with ALS was significantly altered compared to healthy human patients. We focused on expression levels of CCRL2 and CCR3, as we had previously detected that these 2 markers were upregulated in the spinal cord during ALS.\(^8\) CCRL2 is a chemokine receptor expressed at high levels in activated T cells, neutrophils, and monocytes that binds the chemokine ligand CCL11/eotaxin, which is increased in ALS serum and CSF.\(^{34}\) We observed that the percentage of CD16\(^+\) monocytes expressing CCRL2 was significantly increased in patients with ALS but that the total number of these cells expressing CCRL2 was not significantly altered. We also observed that the CD16\(^+\) monocyte population displayed a reduced percentage expressing CCR3 as well as a reduced total number expressing the surface marker. Therefore, decreased expression of CCR3 on CD16\(^+\) inflammatory monocytes may indicate recruitment of cells expressing high levels of this receptor. Further studies will be required to determine the role of CCR3 in ALS pathology.

Despite the changes observed in monocyte surface marker expression, we did not observe a significant correlation between CCR3 or CCRL2 expression in ALS and duration of disease, progression rate, or site of onset. Based on the increased percentage of neutrophils and decreased percentage of CD16\(^+\) monocytes observed in patients with ALS, we attempted to establish a correlation between more than 2 cell types and disease progression: for each patient we established a ratio of neutrophils to CD16\(^+\) monocytes—the N:M ratio—and compared this ratio to the ALSFRS-R score to assess the correlation between neutrophil percentages, monocyte percentages, and disease progression. We found that the N:M ratio was significantly elevated in patients with ALS compared to healthy controls, and that this ratio significantly increased as the ALSFRS-R score dropped and disease worsened. Moreover, other metrics of disease—including duration of disease—did not significantly alter the N:M ratio. The observed increase is likely attributable to a higher rate of production in the short-lived neutrophil population coupled with monocyte depletion resulting from recruitment (figure e-1). In addition, the
increased level of neutrophils late in disease suggests that neutrophils are playing a pathogenic role during disease rather than participating in the repair mechanism. The N:M ratio therefore represents a potential biomarker of ALS. Further studies will be required to assess the potential utility of this new metric.

Overall, our results show that changes in the number and surface marker expression of peripheral leukocytes are associated with ALS disease progression. These data support previous studies suggesting that immune cells in the blood affect the immune response in the CNS as well as subsequent neurodegeneration, although differences in the sex composition of the control and ALS patient groups may have an effect as well. However, the role of immune cells in ALS may be similar in all patients, as we observed no significant difference in cellular percentage, numbers, or ratio based on patient age or site of disease onset (data not shown). The identified CCR3+ subset of CD16+ monocytes that is increased in ALS is worthy of further investigation as a potential disease biomarker. Similarly, the increased neutrophil and decreased monocyte frequencies represented as an N:M ratio in patients with ALS suggest that progressive alterations in peripheral myeloid populations may contribute to functional disease-related changes.

Further evaluation and consideration of the N:M ratio as a potential prognostic indicator may prove useful in diagnosis and prognosis of disease. In particular, long-term longitudinal studies of individual patients over time will allow us to further assess the role of the immune system in ALS.

AUTHOR CONTRIBUTIONS
B.J.M. and D.E.B. designed the study, acquired and analyzed the data, and wrote the manuscript. S.R.K. acquired and analyzed the data. C.F.-R. and C.B. helped design the study, acquired data, and critically reviewed the manuscript. B.C.C. and S.A.G. collected clinical outcome data, supervised the statistical analyses, and revised the manuscript. E.L.F. designed and funded the study and revised the manuscript.

ACKNOWLEDGMENT
The authors thank Andrea Smith, Blake Swihart, Crystal Paez, and Laura Wyant for technical support, Dr. Stacey Sakowski for editing assistance, and Drs. Benjamin Segal and David Irani for their scientific advising and equipment support (all at the University of Michigan).

STUDY FUNDING
Supported by the University of Michigan Program for Neurology Research & Discovery, the A. Alfred Taubman Medical Research Institute, and the NIH (NS007222 postdoctoral fellowship to D.E.B.).

DISCLOSURE
B.J. Murdock served as a temporary grant writer for the American Neurological Association, received research support from CReATe Consortium. D.E. Bender and S.R. Kashlan report no disclosures. C. Figueroa-Romero received research support from Katherine Rayner Fund, A. Alfred Taubman Medical Research Institute. C. Beckus holds a patent for TεK C antibody, produced at UCSF. B.C. Callaghan consulted and was on the advisory board for PCORI grant, received travel funding from the American Academy of Neurology, World Federation of Neurology, consulted for Advance Medical, ALS Association, certify Centers, received honoraria from British Medical Journal, received research support from Impeno Medical Inc., NIH. S.A. Goutman received research support from Neuralexem, CDC/ATSDR. E.L. Feldman received research support from Centers for Disease Control, NIH, National Institute of Neurological Disorders and Stroke, NIDDK, Novo Nordisk Foundation, Juvenile Diabetes Research Foundation, US-Israel Binational Science Foundation, American Diabetes Association. Go to Neurology.org/jn for full disclosure forms.

Received February 1, 2016. Accepted in final form March 24, 2016.

REFERENCES


Increased ratio of circulating neutrophils to monocytes in amyotrophic lateral sclerosis
Benjamin J. Murdock, Diane E. Bender, Samy R. Kashlan, et al.

*Neurol Neuroimmunol Neuroinflamm* 2016;3;
DOI 10.1212/NXI.0000000000000242

This information is current as of June 1, 2016

<table>
<thead>
<tr>
<th>Updated Information &amp; Services</th>
<th>including high resolution figures, can be found at: <a href="http://nn.neurology.org/content/3/4/e242.full.html">http://nn.neurology.org/content/3/4/e242.full.html</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Supplementary material can be found at: <a href="http://nn.neurology.org/content/suppl/2016/06/01/3.4.e242.DC1">http://nn.neurology.org/content/suppl/2016/06/01/3.4.e242.DC1</a></td>
</tr>
<tr>
<td>References</td>
<td>This article cites 35 articles, 10 of which you can access for free at: <a href="http://nn.neurology.org/content/3/4/e242.full.html##ref-list-1">http://nn.neurology.org/content/3/4/e242.full.html##ref-list-1</a></td>
</tr>
<tr>
<td>Citations</td>
<td>This article has been cited by 1 HighWire-hosted articles: <a href="http://nn.neurology.org/content/3/4/e242.full.html##otherarticles">http://nn.neurology.org/content/3/4/e242.full.html##otherarticles</a></td>
</tr>
<tr>
<td>Subspecialty Collections</td>
<td>This article, along with others on similar topics, appears in the following collection(s): All Immunology <a href="http://nn.neurology.org/cgi/collection/all_immunology">http://nn.neurology.org/cgi/collection/all_immunology</a> Amyotrophic lateral sclerosis <a href="http://nn.neurology.org/cgi/collection/amyotrophic_lateral_sclerosis">http://nn.neurology.org/cgi/collection/amyotrophic_lateral_sclerosis</a>_</td>
</tr>
<tr>
<td>Permissions &amp; Licensing</td>
<td>Information about reproducing this article in parts (figures, tables) or in its entirety can be found online at: <a href="http://nn.neurology.org/misc/about.xhtml#permissions">http://nn.neurology.org/misc/about.xhtml#permissions</a></td>
</tr>
<tr>
<td>Reprints</td>
<td>Information about ordering reprints can be found online: <a href="http://nn.neurology.org/misc/addir.xhtml#reprintsus">http://nn.neurology.org/misc/addir.xhtml#reprintsus</a></td>
</tr>
</tbody>
</table>