

Increased ratio of circulating neutrophils to monocytes in amyotrophic lateral sclerosis

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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 funnel 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

Supplemental data
at Neurology.org/nn

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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)⁴ and CCRL2^{5,6} 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 criteria⁷ 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 (ALSFERS-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, 4% 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.

For flow cytometry, a predefined amount of specific monoclonal fluorochrome–labeled antibodies was added to the appropriate tubes and incubated. Flow cytometry was then performed immediately or the cells were fixed with BD Cytofix/Cytoperm for analysis within 48 hours. Phenotyping was performed on a BD FACSCanto II cytometer (BD Biosciences), with voltages set in FACSDiva software 6.1.1. using BD calibration beads (BD CaliBRITE 3; BD Biosciences), while compensations were determined using single color stained and unstained cells. Isotype control gates were set at ≤1.0%.

Fluorochrome-conjugated antibodies, including mouse anti-human CD3-FITC (UCHT1), CD4-PerCP (SK3), CD8-APC (SK1), CD14-APC Cy7 (MφP9), CD15-PE Cy5 (H198), CD16-PE (3G8), HLA-DR, DP, DQ-FITC (Tu39), and immunoglobulin isotype-matched control antibodies were purchased from BD. Fluorochrome-conjugated mouse anti-human CCR3-PE (5E8), CCRL2-PE (K097F7), CD16-AlexaFluor 647 (3G8), CD8-APC-Cy7 (HIT8a), and immunoglobulin isotype-matched control antibodies were purchased from BioLegend. Initial gating was based on forward vs side scatter (SSC). Subsets for each population were then drawn as follows: the neutrophil gate was drawn from SSC-high population vs CD15 followed by CD16 expression 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.

Statistical analyses. Power analysis indicated a sample size of 60 with an α value of 0.05, and $p = 0.5$ provides an estimated power of 0.87958. Statistical analyses and graphing were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Comparisons between groups were performed using a Student 2-tailed *t* test utilizing a Bonferroni correction for multiple comparisons; all data are presented as mean \pm SEM. Correlations between immune variables and ALSFRS-R score were expressed using regression analysis in Prism with R^2 and p values displayed. A value of $p < 0.05$ was considered statistically significant. Further statistical analyses of the CCR subsets CCRL2 and CCR3 on CD16⁺ and CD16⁻ monocytes were performed on a subgroup of the patients in the study; for these analyses concerning CCR3 and CCRL2, extreme outliers were removed using robust regression and outlier removal with a *Q* coefficient of 1.

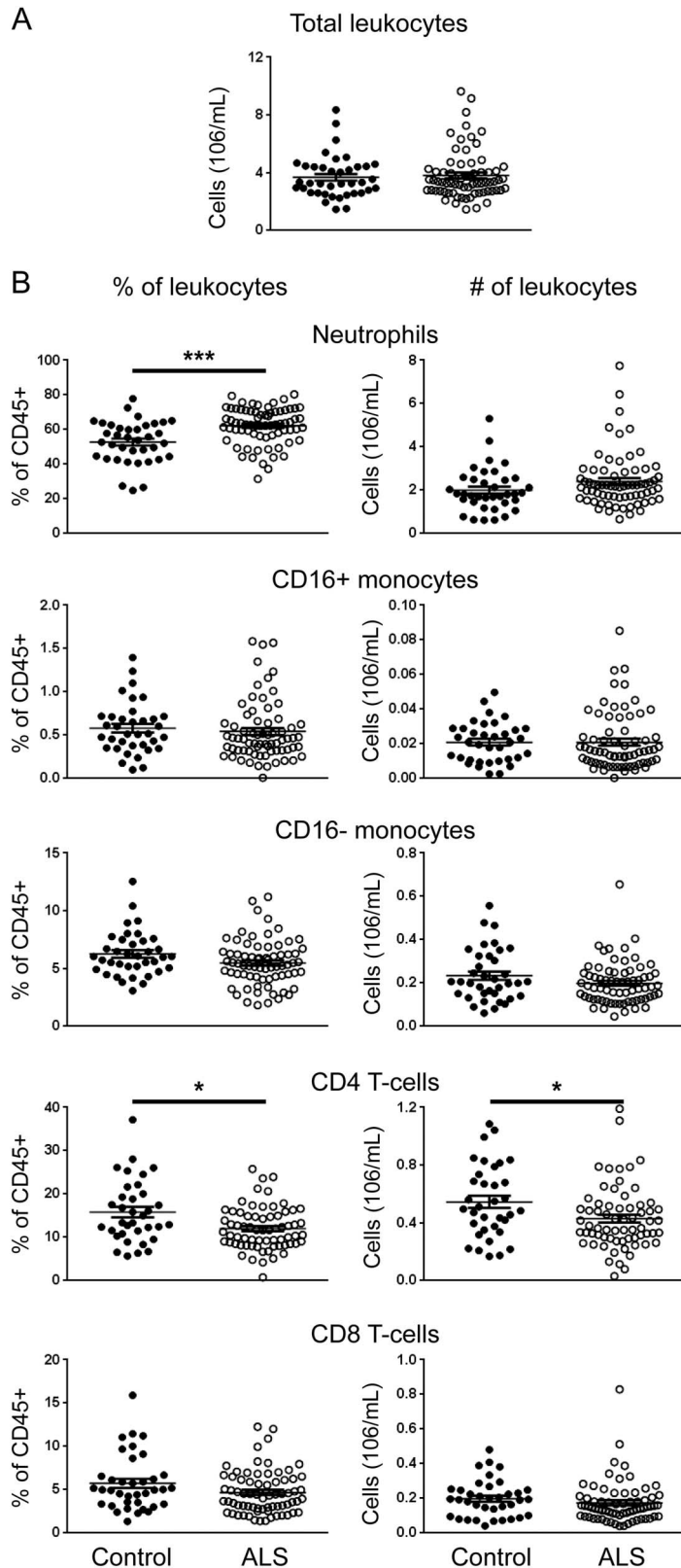
RESULTS Peripheral blood leukocyte levels are altered in patients with ALS. Forty-four healthy control patients (23 women and 21 men) ranging in age from 46 to 76 years (mean 59.20 years) and 90 patients with ALS (37 women and 53 men) ranging in age from 38 to 92 years (mean 61.99 years) were enrolled 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

Characteristics	Healthy controls	Patients with ALS
No. of patients	44	90
Age range, y	46–76	38–92
Age, y, mean \pm SD	59.20 \pm 7.64	61.99 \pm 11.67
Males, n	21	53
Females, n	23	37
ALSFERS-R score, mean \pm SD	NA	30.96 \pm 7.48
ALSFERS-R range	NA	11–44

Abbreviations: ALS = amyotrophic lateral sclerosis; ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised; NA = not applicable.

Figure 1 Immune cell populations are altered in the peripheral blood of patients with ALS



(A) The total number of leukocytes and (B) the percent and total number of specific leukocyte populations are shown. Filled black circles represent individual healthy human control patients, and empty circles represent patients with ALS. Average and SEM are displayed. Asterisks above bars indicate significance (% of CD45⁺ cells: **p* < 0.01, ****p* < 0.0001; # of leukocytes: **p* < 0.008). ALS = amyotrophic lateral sclerosis.

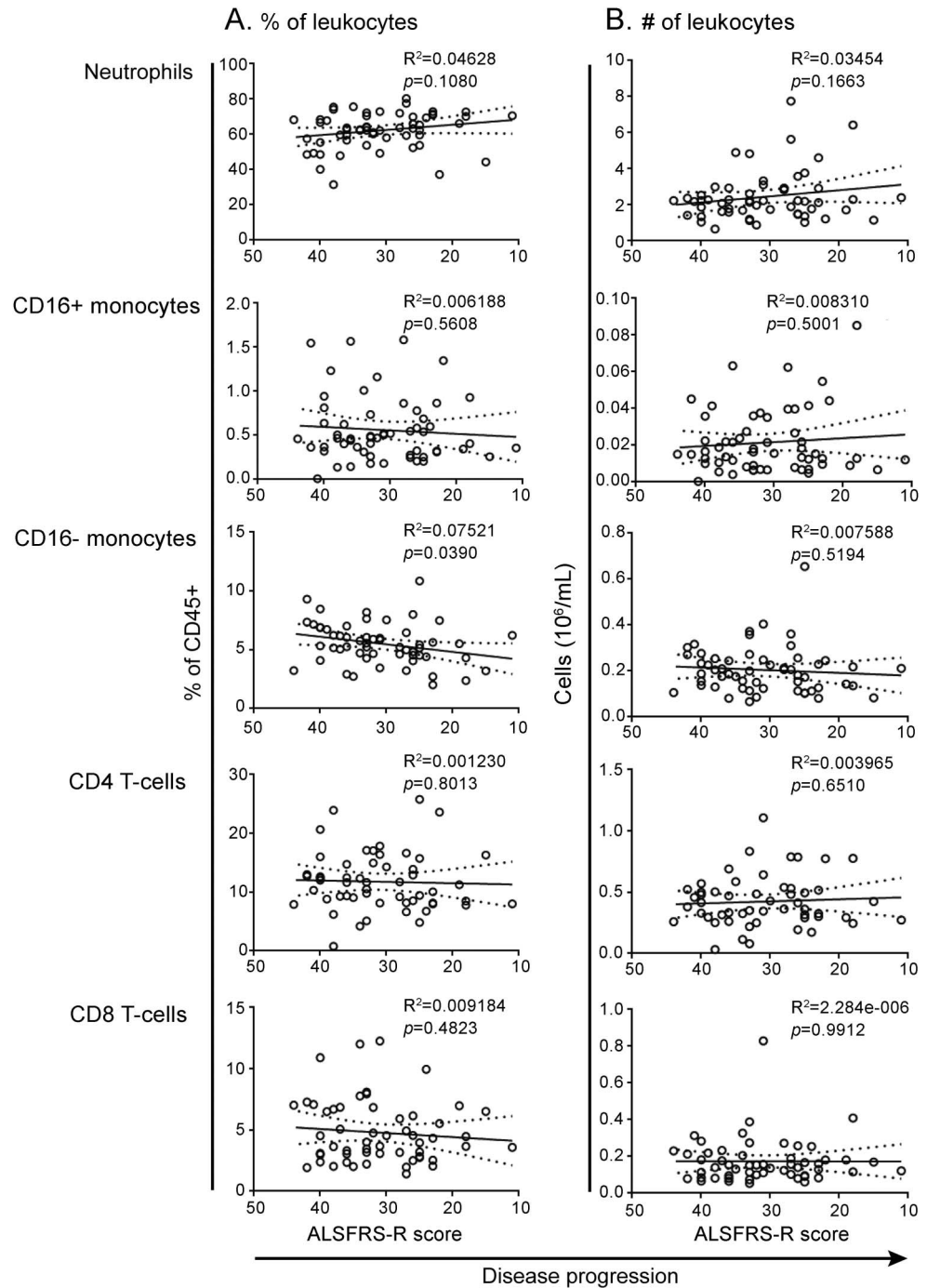
with ALS relative to healthy controls (figure 1B). Levels of CD16⁺ monocytes were unaffected in patients with ALS, and we observed a trend toward a lower percentage of blood-borne CD16⁻ monocytes and fewer total CD16⁻ monocytes, although numbers observed were not significantly different from healthy control patients (figure 1B). Similarly, we observed a significant decrease in the percentage and total number of CD4 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⁺ and CD16⁻ monocyte 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

Figure 2 Percentage of CD16⁻ monocytes in the blood is reduced during disease progression

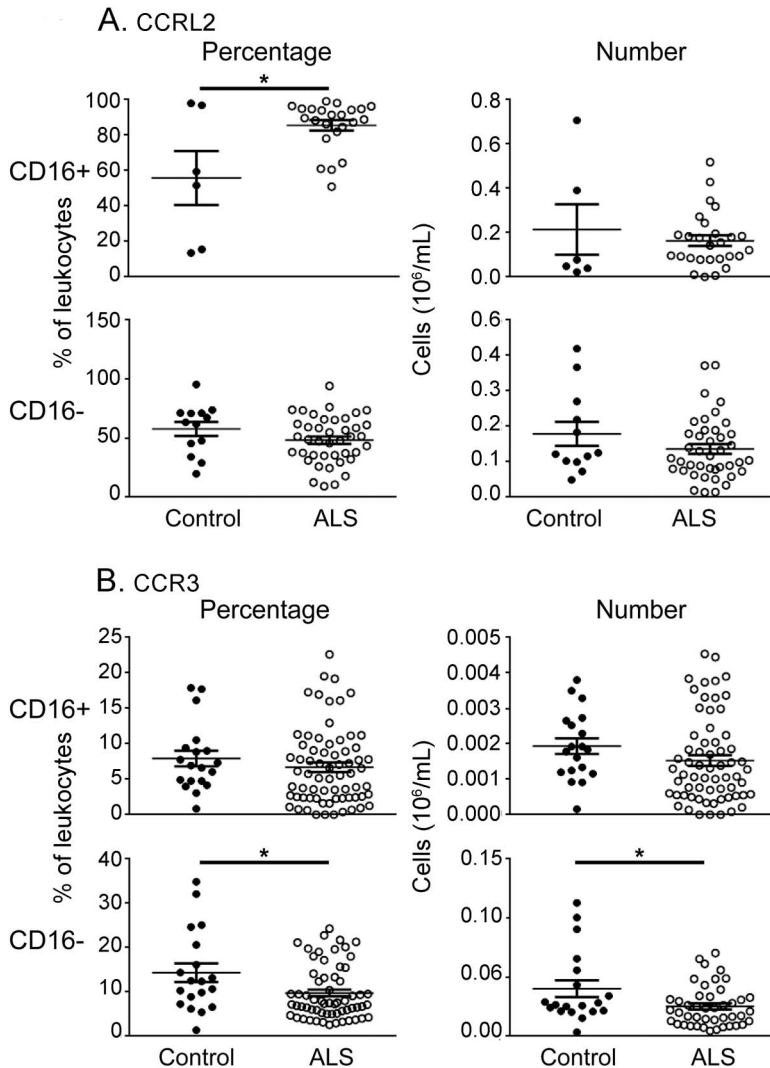


The percentage and total number of leukocytes in the blood of patients with amyotrophic lateral sclerosis are correlated with their ALSFRS-R score at the time of blood draw. Data are displayed with best-fit line and 95% confidence intervals. R² value and p value are also shown. ALSFRS-R scores are displayed from high (A) to low (B) to illustrate disease progression. ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised.

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

Figure 3 Percentage and total number of CD16⁺ monocytes expressing CCR3 is reduced in patients with ALS



The percentage and total number of CD16⁺ and CD16⁻ monocytes expressing CCRL2 (A) and CCR3 (B) in the peripheral blood are shown. Filled black circles represent individual healthy human control patients, and empty circles represent patients with ALS. Average and SEM are displayed. Asterisks above bars indicate significance (* $p < 0.025$). ALS = amyotrophic lateral sclerosis.

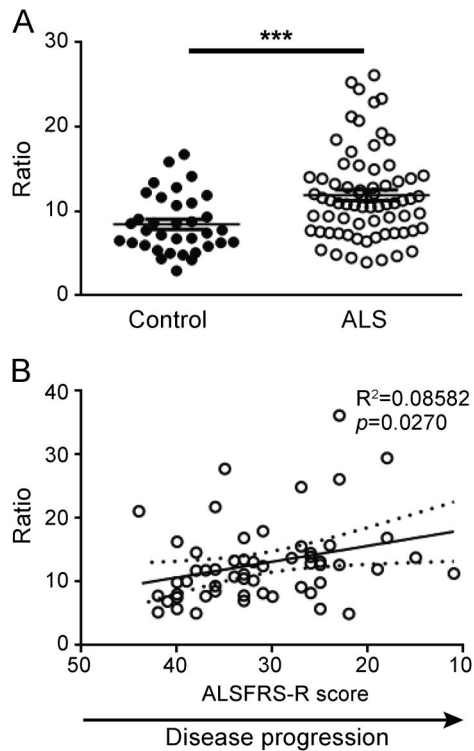
was significantly elevated in patients with ALS compared to healthy control patients; the N:M ratio increased nearly 50% (figure 4A). Even more striking, when correlated with ALSFRS-R score, there was a highly significant relationship between disease score and the N:M ratio: as the ALSFRS-R score dropped (and disease became more severe), the N:M ratio significantly increased (figure 4B). Finally, the N:M ratio was significantly linked only to ALSFRS-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.^{2,3} Second, ALS requires multiple cell types to drive disease, as neuronal death alone does not trigger disease pathology^{9,10}; immune polarization has a critical role in determining whether the CNS environment is protective or destructive.^{3,11} 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^{12–15}; 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,^{13,16–18} 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,¹⁹ which has been shown to drive ALS.²⁰ However, treatment of humans and mouse ALS models with granulocyte-colony stimulating factor, which induces granulocytosis, does not exacerbate disease symptoms.^{21,22} 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.^{23–25} 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.¹² Therefore, reduced CD16⁻ monocytes in the blood of human patients may reflect CNS infiltration: monocytes are found in the CNS

Figure 4 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} and may promote the activation of microglia.^{12,28} Alternatively, they may suppress the inflammatory response.²⁹ Whether these cells are capable of crossing the brain–spinal cord barrier in ALS is still a matter of debate,^{12,30} but it has been shown that depleting inflammatory monocytes in a mouse model of ALS delays disease onset and extends survival.¹² Of note, whereas another group has observed increased numbers of CD4⁺ T cells in the peripheral blood,³¹ we observed a significant reduction in the percentage and total number of peripheral CD4⁺ T cells. This difference is likely attributable to methodology: previous studies examined CD4⁺ T cells within the mononuclear subfraction of leukocytes, while we examined CD4⁺ T cells within the entire population of leukocytes. Despite the observed differences, CD4⁺ T cell levels did not correlate with ALSFRS-R score, indicating that cellular levels were reduced during the entirety of disease, not just an early or late phase. These changes could reflect the recruitment of specific subpopulations of CD4⁺ T cells, such as regulatory

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 CCL2 is a chemokine receptor expressed at high levels in activated T cells, neutrophils, and monocytes that binds the chemoattractant plasma protein chemerin,³² which is a potent chemoattractant for immature macrophages and dendritic cells.³³ It has also been established that CCRL2 expression is upregulated in activated macrophages following both M1- and M2-polarizing forms of stimulation.^{5,6} Similarly, monocytes expressing the CCR3 receptor can bind 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.³⁵ 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,^{12–15} 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.

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