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Early Immune System Changes in Amyotrophic Lateral Sclerosis Correlate with Later Disease Progression

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Summary

Background: Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease with no cure and limited treatment options. The immune system is implicated in disease pathology, unlocking a potential therapeutic avenue. However, it is unclear whether immune changes are a cause or consequence of disease progression.

Methods: Peripheral immune cells were longitudinally measured at monthly intervals in 55 ALS and 50 control participants. 22 peripheral immune markers in the blood were assessed using flow cytometry, and clinical progression was assessed using the revised ALS functional rating scale (ALSFRS-R). Individual immune markers, their trajectories, and overall variability were compared in ALS versus control participants; ALS participants were also stratified by clinical progression

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Author Contributions:

BJM designed the study and IRB protocol, collected data, validated data, designed the statistical approaches, and drafted the manuscript. BZ designed the study, validated data, designed the statistical approaches, and drafted the manuscript. IFW-D collected data, validated data, and helped draft the manuscript. SJT collected data, validated data, and helped draft the manuscript. KDP designed the study, collected data, validated data, and helped draft the manuscript. JPF designed the study, collected data, validated data, and helped draft the manuscript. CEP designed the study, collected data, validated data, and helped the manuscript. DGJ validated data, designed statistical approaches, and drafted the resubmission of the manuscript. ELF designed the study and IRB protocol and drafted the manuscript. LZ designed the study, validated data, designed the statistical approaches, and drafted the manuscript. SAG designed the study and IRB protocol, collected data, validated data, designed the statistical approaches, and drafted the manuscript. BJM, BZ, DGJ, ELF, LZ, and SAG had unrestricted access to the data. The manuscript was primarily drafted by BJM, BZ, LZ, and SAG with additional feedback from all authors. All authors agreed to submit the manuscript, have read and approved the final draft, and take responsibility for the accuracy of the data and the analyses.

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Declaration of Interests:

Dr. Murdock, Dr. Feldman, and Dr. Goutman are listed as inventors on a patent, Issue number US10660895, held by University of Michigan titled "Methods for Treating Amyotrophic Lateral Sclerosis" that targets immune pathways for use in ALS therapeutics.

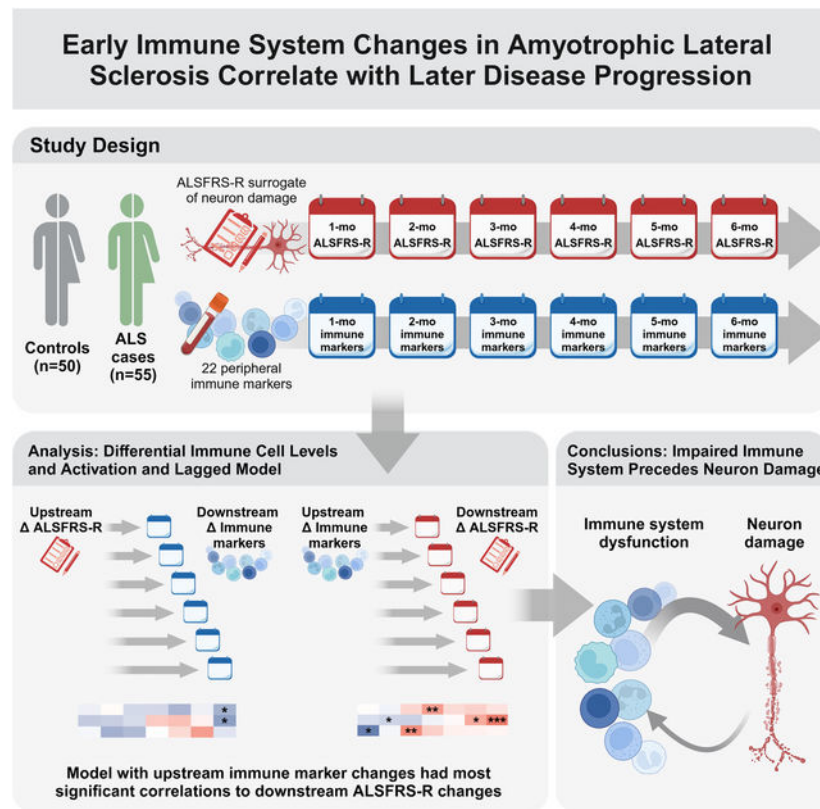
rates and similarly assessed across progression groups. Finally, a novel, lagged linear regression model correlated the rate of immune changes to subsequent, downstream ALSFRS-R changes.

Findings: Numerous immune markers were dysregulated in ALS versus control participants, with altered levels, trajectories, or variability in immune populations and surface markers. ALS participants had increased immune variability relative to control participants; within ALS participants, faster progressors overall had decreased marker variability. Finally, NK cell numbers, NK cell subpopulations, and NK cell surface markers significantly associated with downstream ALS progression.

Conclusions: The immune system is dysregulated in ALS, is more consistently dysregulated in faster ALS progression, and immune dysregulation occurs upstream of clinical changes. These findings suggest the immune system is a causal factor of ALS progression in human patients.

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Graphical Abstract



eTOC Blurp:

Individuals with ALS have greater immune variability in the peripheral blood compared to controls; however, faster progressing individuals with ALS have lower immune variability and consistent dysregulation of these markers. Using a novel lagged analysis model, the study also shows that immune changes occur upstream of clinical changes during disease.

Keywords

Immune system; amyotrophic lateral sclerosis; ALS; NK cells; neuroinflammation

Introduction

Amyotrophic lateral sclerosis (ALS) is an aggressive neurodegenerative disease characterized by motor neuron loss.¹ Individuals with ALS progressively lose voluntary motor control impairing the ability to speak, swallow, use their upper and lower limbs, and breathe^{2,3} ultimately resulting in death from respiratory failure within 2–4 years from onset. Despite intensive research, there are only a handful of FDA-approved ALS treatments, which are only minimally effective.¹ Therefore, new therapies are desperately needed.

There is a universal immune component to ALS,⁴ which has repeatedly been shown to be associated with ALS progression^{5,6} making it an attractive therapeutic target. Indeed, manipulating specific immune populations slows or accelerates disease progression in ALS mouse models.^{7–14} In human patients, a number of peripheral immune cell types have been implicated in disease progression, including neutrophils,^{15–17} natural killer (NK) cells,^{7,14,18} monocytes,^{19–22} CD4 T cells,^{21,23–26} and CD8 T cells.^{27,28} Moreover, immune cells accumulate in the central nervous system (CNS) of ALS patients, further suggesting a role for the immune system in disease.^{14,15,29}

However, it remains unclear whether the immune system contributes to downstream progression in ALS patients or whether dysregulation is merely a result of upstream neuronal damage. Unfortunately, immune studies in human ALS have been largely cross sectional, making it difficult to assess whether observed immune changes are causative or correlative. In our own longitudinal studies, we showed that changes in specific immune populations correlate with disease progression^{5,7} and that immune markers can predict downstream clinical changes.³⁰ Though highly informative, these studies examined immune profiles at six-month intervals, a timeframe too wide to determine which occurred earlier, immune changes or changes in revised ALS functional rating scale (ALSFRS-R). Similarly, although our models built from multiple immune markers can predict future disease course, it is unclear whether these markers represent underlying disease mechanisms or are merely disease biomarkers.

We therefore launched the present study to derive more granular information on peripheral immune and ALSFRS-R changes at shorter intervals during disease progression, to allow us to determine whether immune dysregulation occurs upstream of ALS progression. We collected peripheral blood from ALS and control study participants at monthly intervals and assessed 22 separate peripheral immune markers by flow cytometry and clinical progression via ALSFRS-R scores. First, we compared overall immune marker levels, marker trajectories, and marker variability between ALS and control participants. Next, we repeated these assessments after stratifying the ALS participants by slow, medium, and fast progression rates. Finally, monthly immune marker changes were correlated to subsequent, downstream changes in ALSFRS-R using a lagged model.

We found the immune system was highly dysregulated in ALS versus control participants, with altered individual immune marker levels, trajectories, and variability. Similarly, there were significant differences in these measurements between slow, medium, and fast progressors. The latter group in particular displayed lower marker variability despite higher overall immune variability in ALS participants. Finally, earlier increases in NK cell levels and NK cell subpopulations associated with more rapid downstream disease progression, while earlier increases in NK cell surface proteins correlated with slower subsequent progression. Together these data confirm that the immune system is dysregulated in ALS and suggest it contributes to downstream ALS pathology in human patients.

Results

Study participants

The initial study cohort included 72 ALS and 52 control participants. Comorbidities reflected that of a middle- to late-age population. Past medical history diagnoses present in more than 5 ALS or control participants included: skin cancer, type 2 diabetes, obesity, hyperlipidemia, depression, anxiety, migraine/headache, sleep apnea, amblyopia, hearing loss, hypertension, chronic ischemic heart disease, atrial fibrillation and other arrhythmias, asthma, gastro-esophageal reflux disease, osteoarthritis, calculus of kidney and ureter, benign prostatic hyperplasia, and cardiac murmur. Participants were not included in subsequent analyses unless they provided at least two longitudinal samples, resulting in a final cohort of 55 ALS and 50 control participants. There were no differences in sex and age between the two groups (Table 1). The study was designed to collect blood samples from participants at monthly intervals over the span of a year. ALS participants provided more samples on average than controls because ALS participants continued to provide blood during clinical visits despite COVID-19-related clinical research pauses and restrictions whereas contact with control participants was considered non-essential. ALS participants were 49% male, had a median age of 63 years, and were 31% bulbar onset. The average initial visit occurred 117 days after ALS diagnosis and 506 days after symptom onset; 60% of ALS participants were using riluzole during the initial samples collection while 69% used riluzole at some point during the study. Only one participant used edaravone and none were using investigational agents during the study to our knowledge. The median ALSFRS-R at study start was 35.

Baseline and kinetics of immunity in ALS versus control participants

We first examined peripheral immune marker levels and evolution with time in ALS and control participants. Monthly blood samples were analyzed for 22 separate immune markers by flow cytometry.^{7,30} These included total numbers of immune cell populations (neutrophils, eosinophils, CD16⁺ monocytes, CD16[−] monocytes, CD4 T cells, CD8 T cells, NK cells), NK cell subpopulations (CD27⁺, CD57⁺ CD62L⁺, CD94⁺, CXCR3⁺, KIR2DL1⁺, KIR2DL2⁺), and NK cell surface marker expression (CD11a, CD11b, CD38, CD45RA, CX3CR1, NKG2D, NKp30, NKp46). Using the initial visit only, we first compared immune levels between controls and ALS participants in a cross-sectional analysis and found that several markers differed at this baseline time point. We observed significantly higher neutrophil and CD16[−] monocyte numbers as well as higher levels of CD11a, CD38,

and CX3CR1 NK cell expression in ALS versus control participants (Supplementary Table S1). We also observed similar results when examining all immune marker measurements over the study course, with higher neutrophil levels in ALS versus control participants when correcting for multiple comparisons and reduced CD16+ monocytes, CD4 T cells, and CD27+ NK cells. There was also elevated CD11a, CD38, CD45RA, and CX3CR1 NK cell expression in ALS participants (Supplementary Table S2).

We next used a linear mixed effect models' likelihood ratio test to evaluate whether immune markers exhibited different change rates or variances over time in ALS participants versus healthy controls (Figure 1). To quantify temporal changes in the 22 immune markers, the linear trajectory of each immune marker was calculated for each study participant and then averaged for ALS and control participants (Table 2). We observed decreasing CD4 and CD8 T cell levels in ALS participants relative to controls as well as decreasing CX3CR1 and increasing NKp30 NK cell expression over time. We also observed that expression of CX3CR1 decreased on NK cells while NKp30 expression increased in ALS participants relative to controls. CD16+ and CD16- monocyte levels in ALS participants also decreased relative to controls, though these trends did not reach statistical significance after multiple comparisons. Interestingly, some immune populations previously associated with ALS progression, such as neutrophils, did not significantly change in ALS participants relative to controls over time.

However, the immune system is highly variable, so we next explored whether immune variability might contribute to disease progression rather than linear immune changes over time. Using a linear mixed effect models' likelihood ratio test, we also compared variability in immune markers over time in ALS participants versus controls to assess temporal stability (Table 2). Multiple immune populations, NK cell subpopulations, and NK cell surface markers differed in variability – i.e., were more or less stable – in ALS versus control participants. Eosinophils and CD16- monocytes, for instance, were more variable in ALS participants, while CD16+ monocyte levels were more stable over time. Similarly, NK cell expression of CD11a, CD11b, CD45RA, CX3CR1, NKG2D, and NKp46 was more variable in ALS participants than in controls, while CD27+ levels and NK cell expression of NKp30 was more stable in ALS participants. Together, these data demonstrate that ALS is characterized by multiple dysregulated immune marker trajectories and variability, with ALS participants overall having a higher rate of immune variability.

Correlation of immunity with ALS progression in ALS participants

We next examined whether immune marker levels or changes with time were associated with disease progression by comparing ALS participants with slow, medium, and fast ALSFRS-R progression rates. The linear rate of disease progression was first calculated for each ALS participant as the change in ALSFRS-R over time, and participants were then grouped into tertiles based on the average progression rate (Figure 2A). For each participant, the linear rate was calculated by fitting a least square linear line using ALSFRS-R scores within one year from the baseline. Slow, medium, and fast progressors had an average decline of 0.25, 0.94, and 1.94 ALSFRS-R points per month, respectively (Figure 2B). There were no significant demographic or clinical differences between progression groups, except for a

higher rate of bulbar onset in the fast progression tertile (Supplementary Table S3). Nor were there significant differences in the initial values of the 22 immune markers between tertiles at baseline (Supplementary Table S4); when all measurements were combined medium and fast progressors had significantly fewer eosinophils and CD62L+ NK cells in the peripheral blood compared to slow progressors (Supplementary Table S5).

Next, we examined whether the linear trajectory or the variability differed between progressor groups. Significant trajectory differences were observed in eosinophils between progressor groups, and there was a trend toward altered trajectory of NK cell CX3CR1 expression (Table 3). In contrast, there were highly significant differences in immune stability between slow, medium, and fast progressors. When immune variability was examined over time fast progressors had predominantly greater stability among immune markers (Table 3) including eosinophils, CD8 T cells, NK cell subgroups (CD27+, CD57+, CD62L+, CD94+, KIR2DL1+), and NK cell expression of CD38, CX3CR1, and NKG2D. We also observed trends towards increased stability amongst faster progressors in NK cell levels, CXCR3+ NK cells, and NK cell expression of NKp46. Interestingly, faster progressors showed higher variability in CD16+ monocyte levels. Together these data show that differences in progression rate are more highly associated with the variability of immune markers rather than linear changes over time.

Correlation of immune changes with downstream changes in ALSFRS-R

Finally, it is currently unknown whether immune changes are a cause or consequence of motor neuron damage in ALS. Therefore, we evaluated whether changes in immune markers occurred upstream or downstream of disease progression, *i.e.*, a drop in ALSFRS-R, using a lag linear mixed-effect model (Figure 3A, B). To examine the relative temporal changes in immune markers and ALSFRS-R, we staggered these analyses by up to six months. When we compared ALSFRS-R changes with subsequent immune changes, we observed significant positive correlations to most immune markers at 6 months, *i.e.*, larger ALSFRS-R drop with decreasing subsequent change in immune markers. We also observed ALSFRS-R changes correlated negatively to NK cell CD38 and NKG2D expression at multiple time points, *i.e.*, larger ALSFRS-R drop with increasing subsequent change in immune markers (Figure 3C). When we lagged ALSFRS-R changes to immune changes, we noted more significant positive and negative associations (Figure 3D), suggesting that immune changes may occur upstream of ALSFRS-R changes. Interestingly, CD11a, CX3CR1, CD38, and NKp46 NK cell surface markers associated positively with downstream ALS progression multiple time frames, *i.e.*, decreasing surface markers with larger subsequent ALSFRS-R drop. We found negative correlations between NK cells and CD57, KIR2DL1, and KIR2DL2 NK cell subsets to ALSFRS-R, *i.e.*, increasing cells with larger subsequent ALSFRS-R drop. Together, these data demonstrate immune markers are dysregulated in ALS based on disease progression rate, and that many of these immune changes precede clinical decline by ALSFRS-R.

Discussion

The current study examined longitudinal immune system changes in ALS in granular detail and their associations with subsequent clinical progression. Multiple immune markers were dysregulated in ALS versus control participants, with altered overall numbers, temporal trajectories, and variability. Within ALS participants, immune marker levels, trajectories, and variability also differed by rate of disease progression: slow, medium, and fast, as measured by ALSFRS-R. Lastly, a lagged correlative analysis demonstrated there were more significant correlations between certain immune markers to subsequent changes in ALSFRS-R but few associations between ALSFRS-R changes to subsequent immune changes, implying that immune changes may precede disease progression. Cumulatively, these findings bolster the growing body of data implicating the immune system in ALS pathology and provide evidence that immune dysregulation occurs upstream of clinical decline.

In the initial cross-sectional analysis, we found that neutrophils levels were upregulated in ALS participants, both at baseline and combined across all time points, versus controls. Similarly, we saw a downward trend in CD16⁺ monocytes and downward trend in CD4 T cells in ALS participants when data were analyzed from all time points, consistent with our previous findings.²¹ Multiple NK cell surface markers were also higher in ALS participants in the cross-sectional analysis, both during the single baseline visit and combined across all time points. Consistent with our previous findings, CD11a, CD38, and CX3CR1 were upregulated at baseline and when all time points were combined;⁷ CD45RA was also upregulated when combining all time points. Similarly, there was a trend towards increased CD11b and NKp46 as well when all time points were combined, which align with our earlier results. Our findings are also consistent with previous studies examining the immune system in ALS, finding multiple dysregulated immune cell populations in human ALS patients, including neutrophils,^{16,21} monocytes,^{11,22,31,32} CD4 T cells,^{21,23,31,33} CD8 T cells,²⁷ and NK cells.^{7,14,31} Here we once again find increases in or trends towards increased neutrophils, CD16⁺ monocytes, and NK cell levels when examining using a cross-sectional analysis; we also observed a trend toward reduced CD4 and CD8 T cell levels, further validating previous findings.

Immune system involvement in ALS is supported by many clinical cross-sectional studies as well as ALS mouse studies. In these genetic ALS murine models, manipulating specific immune cell populations alters disease course^{7–12,14,34} suggesting that immune mechanisms actively shape progression rather than occurring merely as downstream biomarkers. However, in human ALS patients, evidence is lacking regarding whether immune dysregulation is a cause or consequence of motor neuron damage. To shed light on the temporal evolution of immune changes relative to disease progression, we next examined changes in the immune system over time. We observed that the trajectory of several immune cell populations significantly differed in ALS participants relative to controls over time, including significantly decreasing CD4 T cells, CD8 T cells, and NK cell expression of CX3CR1 as well as trends towards different trajectories in CD16⁺ and CD16⁺ monocytes. These findings are mostly mirrored in previous longitudinal studies in the literature, which also reported changes in neutrophil, monocyte, CD4 T cell, CD8 T cell, and NK cell

levels,^{7,21,26} as well as our previous study which found decreasing levels of NK cell surface markers despite overall increased levels in ALS participants compared to controls.⁷ Surprisingly, we found that neutrophil levels did not significantly change in ALS versus controls, though overall levels were much higher in ALS participants. Unlike markers that change over the disease course, the impact of neutrophils in ALS may be based on absolute overall numbers and not on rapid changes in the periphery.³⁰ Differences between our findings here in the literature may also have arisen from the stage of ALS samples were analyzed, since at least one study found that neutrophil levels increase during late-stage disease.²⁶

To further support participation of the immune system in ALS, we examined immune dysregulation with disease progression rates. To do so, we stratified participants with ALS by ALSFRS-R progression rate into slow, medium, and fast progressors and examined overall immune levels in these participant groups. Using a cross-sectional analysis of combined sample data, we found that there were few significant differences between progressor groups with regards to immune marker levels using either the initial, baseline value or combined sample data. Previous studies have observed that dysregulated immune markers associate with disease progression rates. For instance, individuals with high NK cell surface marker expression have a faster rate of ALS progression and shorter survival,³⁰ and slowly progressing ALS participants have lower peripheral immune cell numbers than their faster-progressing counterparts.³⁵ Here, the lack of association between these markers and progression rates is likely due to the small number of participants in each progressor group, as we also observed few significant differences in the linear trajectory of immune markers between slow, medium, and fast progressors. However, despite the relatively low number of participants per progression group, we observed significant differences in immune marker variability based on progression rate. Even more interestingly, in most cases where there were significant differences between progression groups, faster progressors had reduced immune variability. These findings indicate that continuous, sustained dysregulation of immune mechanisms may be a key driver of ALS progression. Moreover, they suggest that the variability of the immune system may be a better indicator of disease progression than cross-sectional values or linear trajectories.

Finally, the study provides strong evidence that immune system changes in ALS occur upstream of ALSFRS-R changes, as determine our lagged linear mixed-effect analysis. Increased NK cell levels associated with more rapid downstream progression. Interestingly, decreasing NK cell surface marker expression of KIR2DL1 and KIR2DL2 and effector marker CD57 associated with slower downstream disease progression. While these observations are consistent with our previous report that these markers start high in ALS and decrease over time⁷ it is unclear why this phenomenon is associated with more rapid disease progression. Several possibilities exist. First, immune cell populations may play multiple roles in ALS. For instance, though specific subpopulations of NK cells may contribute to disease progression, Cui et al. found that increased NK cell levels may exert a protective effect in ALS,²⁶ suggesting complex roles of NK cells. They similarly found that specific CD4 T cell subpopulations associated with faster or slower ALS progression rates, further reinforcing the idea that the same cell population may play detrimental and protective roles in disease. Second, the impact of immune cells may differ based on the disease stage. In

our previous study we found that rapidly decreasing CD4 T cells associated with more rapid disease progression, though overall CD4 T cell levels did not significantly change over time.²¹ We later found, in mouse ALS models, that peripheral CD4 T cell levels increase during early disease stages,³⁶ suggesting that disease stage may impact immune kinetics. This is further supported by mouse studies in which the CNS immune environment changes drastically depending on disease stage.³⁷ Further studies will be required to determine the underlying mechanisms contributing to our findings.

The current study reinforces the growing body of literature implicating the immune system in ALS progression. Multiple immune populations, NK cell subpopulations, and NK cell surface proteins were dysregulated in ALS versus control participants, as well as by rate of disease progression. Perhaps most importantly, changes in these markers were significantly associated with downstream clinical changes suggesting that immune system dysregulation occurs early in ALS pathology. Our findings argue that the immune system is a potential viable therapeutic target in ALS, especially tailored to specific immune cell populations, though further examination of the underlying mechanisms and the demographic-specific nature of these mechanisms are necessary.

Limitations of Study

The current study has limitations. Foremost, while the overall study consisted of over 700 measurements for each immune marker, the cohort itself consisted of 55 ALS and 50 control participants, preventing stratification by age and sex, despite their relevance to immune mechanisms in ALS.^{7,15,30} In addition, the study experienced unanticipated disruptions due to the COVID-19 pandemic, which led to restrictions in human subject research causing missed immune samples. This impacted the control participants more than ALS participants, as ALS participant samples continued to be collected in conjunction with clinical visits. In addition, we continued to collect ALSFRS-R scores by trained evaluators at the study intervals, even if an immune sample was not obtained. The increased number of missed samples in the control population may explain why trajectory differences were driven primarily by changes in control subjects, as the linear trajectory may have been impacted by a smaller sample size. Although we employed methods to address missing data, the longitudinal associations of peripheral immune profiles, especially in the control participants, could have been impacted by the COVID-19 pandemic. The current study also did not examine the impact of sex and age on immune dysregulation, as the study was not sufficiently powered to do so; however, our previous studies have shown that immune markers of ALS and the underlying immune mechanisms of disease may vary by age and sex.^{7,15,30} Finally, the study was skewed towards NK cells and NK cell markers; future studies should incorporate markers for other cell types to determine whether similar patterns are present in other cells and their relevant markers. Despite these limitations, the study had numerous strengths. First and foremost, this ALS study is the first of its kind to measure immune markers at frequent intervals in order to assess more granular changes in the immune system with disease progression. Second, the panel of immune markers is more expansive than typical immune studies that measure only total immune cell levels. Finally, to our knowledge, this is the first ALS study to employ a lagged analysis correlating upstream immune changes to downstream clinical metrics.

Resource Availability:

Lead contact

All requests for information or data should be directed to Benjamin J. Murdock (murdockb@umich.edu).

Material availability

This study did not generate any unique reagents.

Data and code availability

All data are managed through our dedicated ALS data management system that is curated by Mr. Adam Patterson and Mr. Sushant Obeja. All data are stored redundantly both on the research team's private server as well as on the University of Michigan Advanced Research Computing (ARC) system in the University's Office of Research. All code and raw data will be supplied by the lead contact upon request.

STAR Methods

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study participants: ALS participants meeting ALS Gold Coast criteria³⁸ were recruited at the University of Michigan Pranger ALS Clinic (n = 77). Control participants were recruited through an electronic database of individuals interested in research participation hosted by University of Michigan Institute for Clinical & Health Research (n = 52). Only participants with two or more visits were included in the final analysis, resulting in a cohort of 55 ALS and 50 control participants. All participants were provided oral and written informed consent. Potential controls were excluded if they had a personal or family history in a first- or second-degree relative of ALS, dementia, or another neurodegenerative disease or had a personal history of an immune-modifying disease (including cancer and autoimmune diseases). All participants were > 18 years of age at the time of first blood draw. Participant demographics were recorded including age and sex (self-reported) and onset segment and diagnostic delay (physician-reported); participants were further categorized as suspected, possible, probable laboratory supported, probable, or definite ALS by revised and/or original El Escorial Criteria to capture disease distribution (physician-reported) (Table 1). Other demographic information was not used in the current study including gender, race, ethnicity, or socioeconomic status. All study participants lived within one hour of the University of Michigan (Ann Arbor, MI) to facilitate off-site sample collection and rapid return of samples for analysis. Control and ALS participants were free of illness and infection at the time of blood draw. Visits were postponed if participants reported illness. Study participants needed to provide at least two separate samples to be included in the analyses. The University of Michigan Institutional Review Board approved the study (HUM00107546). The impact of sex and age on the study results was not assessed, as the study was not adequately powered for data stratification; however, these variables were used to adjust the models where appropriate.

METHOD DETAILS

Sample collection: To isolate immune cells from the peripheral blood, blood was collected by peripheral venipuncture at monthly intervals. Samples were collected primarily by a traveling phlebotomist in the University of Michigan Clinical Research Unit mobile collection service into BD Vacutainer sodium heparin tube (BD Biosciences, San Jose, CA), placed at 4°C, and processed within 2 hours of collection. ALS participants occasionally provided samples on site if they were scheduled for a clinical visit at the time of their monthly collection. All samples were placed on ice following collection and returned to the University of Michigan within two hours. Samples were collected between December 20th, 2016 to January 13th, 2021, and laboratory staff were blinded to the participant's case status. All participants were without fever or infectious illness at the time of blood collection. Participants were followed for one year and provided anywhere from two to twelve samples (Supplementary Figure 1). Sample collection, particularly those from control participants, was impacted by clinical research pauses that resulted from the COVID-19 pandemic.

Clinical metrics: To assess clinical metrics of ALS, ALSFRS-R scores were obtained for ALS participants by a trained evaluator within three days of sample collection. At least two ALSFRS-R scores were required to be included in longitudinal analyses. Clinical visits and assessment of ALSFRS-R were not interrupted by the COVID-19 pandemic.

Flow cytometry: To assess peripheral immune markers, a 1 mL aliquot of whole blood was lysed with red blood cell lysing buffer (0.8% NH₄Cl, 0.098% KHCO₃, 0.1 mM EDTA, 13.8 mM HEPES), washed twice, resuspended in 1 ml of flow buffer (PBS, 4% FBS, 0.1% sodium azide), and leukocytes were counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cells were then plated in 96-well U-bottom plates (Corning, Corning, NY), their Fc receptors blocked with 10 µg/mL human or mouse TruStain FcXTM blocking solution (BioLegend, San Diego, CA) for 20 minutes, and then stained with antibody cocktails for 30 minutes. Twenty-two separate immune markers were profiled, grouped into three broad categories: immune populations, NK cell subpopulations, and NK cell surface marker expression.^{7,30} Cells were then washed and resuspended in 185 µL of BDTM Stabilizing Fixative (BD Biosciences, Franklin Lakes, NJ) until analysis. Myeloid populations were identified using CD3, CD14, CD16, and FSC-SSC profiles (neutrophils = SSC-high, CD16+; eosinophils = SSC-high, CD16-; CD16+ monocytes = SSC-low, CD3-, CD14+, CD16+; CD16- monocytes = SSC-low, CD3-, CD14+, CD16-). Lymphocytes and NK cells were identified using antibodies for CD3, CD4, CD8, CD56, CD16, and FSC-SSC profiles (CD4 T cells = SSC-low, CD3+, CD4+, CD8-; CD8 T cells = SSC-low, CD3+, CD4-, CD8+; NK cells = SSC-low, CD3-, CD56^{mid}, CD16+, CD14-).^{7,30} Within the NK cell population, subpopulation levels and surface markers expression levels were quantitated using BV421-, PE-, or APC-tagged antibodies (BioLegend) to prevent overlap between emission spectra. Samples were analyzed on a BD LSRFortessaTM flow cytometer with FACSDivaTM software (BD Biosciences) and analyzed by FlowJo (FlowJo, Ashland, OR). Surface expression was quantitated as median fluorescent intensity (MFI), and baseline MFI was quantitated using non-specific antibodies. Fold-increase MFI was then calculated by dividing the positive signal MFI by the baseline MFI in order to account for run-to-run variation and instrumentation.

QUANTIFICATION AND STATISTICAL ANALYSES

Demographics: In the demographic tables, all continuous variables are summarized by median and interquartile range (IQR), and all categorical variables are summarized by count (percentage). For continuous variables, Wilcoxon rank sum test was used to compare two groups, and Kruskal-Wallis rank sum test was used to compare three groups or more. For categorical variables, the chi-square test was used when all expected cell frequencies were greater than 5, otherwise Fisher's exact test was used. Though we have previously observed age- and sex-specific differences in ALS immune markers,^{7,15,30} these factors were not examined, as the power of the study did not support stratification of the data.

Immune trajectory and variability analyses: The trajectory analysis examined the linear trajectory of each of the 22 immune markers over 12 months for each participant since the first immune measure. Change rates of markers over time and the variability of markers were first calculated for each participant in the study and then combined and assessed using a linear mixed effect (LME) model. Parametric confidence intervals were used to compute 95% confidence intervals, and the LME models' likelihood ratio test was used to assess significance. To address multiple comparisons, Benjamini-Hochberg correction was applied to obtain the adjusted p-value to control for the false discovery rate. While the statistical assumptions of LMEs, such as homoscedasticity and normality of residuals, were not formally tested, a log-transformation was applied to stabilize the variance of immune markers in these analyses. Additionally, unequal variances for different groups were specified within the models. We fitted an all-participant model and an ALS-only model. The all-participant model was adjusted for age and sex, while the ALS-only model was adjusted for age, sex, onset segment, diagnostic El Escorial Criteria, time between diagnosis and immune sample, and time between symptom onset and diagnosis. In the ALS-only model, participants were categorized into slow/medium/fast progressors (33.3% each) by evaluating the slope of ALSFRS-R scores through a mean square fit. The LME model assumes a random intercept and a random slope for the immune marker trajectory, indicating that participants have separate initial values and different rate of change in the marker value. The model also allows the variance of the measurements around the linear trajectory to be different between case/control groups and slow/median/fast progressors. Variability of immune markers was similarly assessed using LME models. We performed likelihood ratio tests to evaluate 1) whether an immune marker has different change rates across groups, and 2) whether an immune marker has different variability across groups. All models were fitted using the nlme::lme function in R (v4.4.1),³⁹ employing the Maximum Likelihood estimation method. Details on statistics, n values, and definitions can be found in Tables 2 and 3.

Lag analysis using linear mixed effect model: This analysis examined the relationship between the rate of change in each of the 22 immune markers and the rate of change in ALSFRS-R. In this analysis, only participants with at least four measurements were included, with a final cohort of 38 participants for the analysis. First a natural spline was fit for each patient to get a smooth function of the immune marker measurement over time. Then, monthly immune changes were extracted from the spline, which are the predictors in the lagged model. The same technique was applied for the functional scores to

get monthly ALSFRS-R changes, which were the outcomes in the lagged model. With lag = 0 the model associates the two rates in the same month; when lag = 1, the model associated immune rates with the progression rates measured at 1 month later, when lag = 2, the model associated immune rates with the progression rates measured 2 months later, etc. The linear mixed-effect model was used with a compound symmetry correlation structure to account for multiple rates from the participant and adjusted for age, sex, onset segment, El Escorial criteria, and time since symptom onset in the model. The coefficient (beta) represents the association between immune rates and ALSFRS-R changes measured at the same time or X months ahead of time. A positive beta means an increased immune rate slows down the ALS progression speed, while a negative beta means an increased immune rate accelerates the ALS progression speed. A reversed analysis (flipped the predictor and outcome) to analyze the possibility of upstream effect of the disease progression to the immune system was also performed. The mode was fitted using the `lme4::lmer` function in R (v4.4.1).³⁹ Again, the statistical assumptions of LMEs were not formally tested, though a log-transformation was applied. Additional details on statistics, n values, and definitions can be found in the figure legend of Figure 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Peripheral immune markers are more variable in individuals with ALS
- Faster progressing ALS shows less immune variability but more consistent dysregulation
- Changes in peripheral immune markers occur upstream of clinical changes

Context and Significance:

The immune system has been repeatedly implicated in ALS pathology, but it is unclear whether immune dysregulation is a cause or consequence of neuronal damage in individuals with ALS. To address this, Murdock et al. analyzed 22 immune markers in the peripheral blood of ALS participants and associated these markers with clinical metrics of disease. Faster disease progression was associated with lower immune variability but higher levels of overall immune dysregulation. In parallel, using a novel lagged analysis, Murdock et al. show that changes in peripheral immune markers are associated with subsequent changes in disease score. Together, these data suggest that sustained immune dysregulation accelerates ALS progression and that the immune system is contributing to ALS progression.

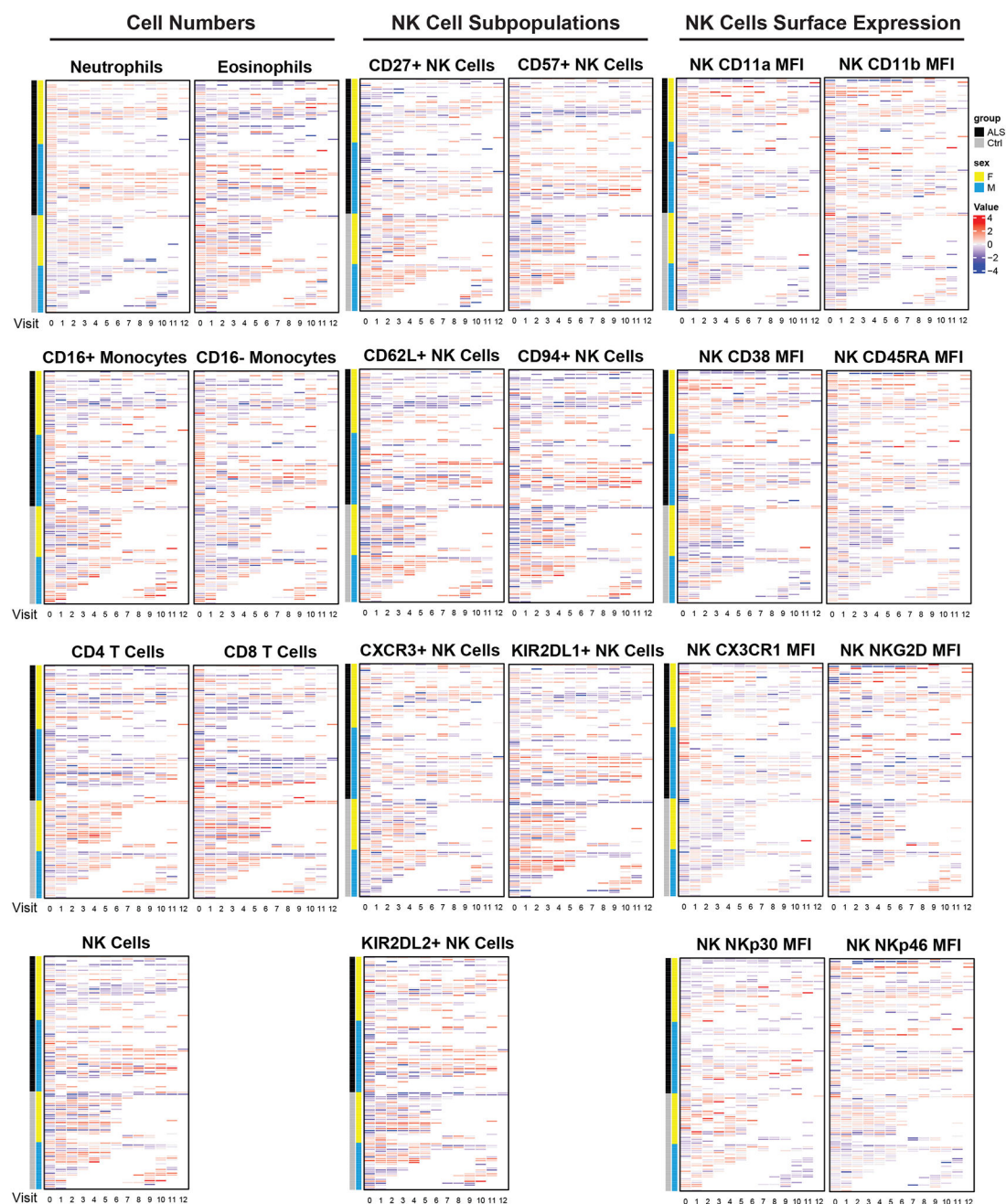


Figure 1. Kinetics of immune markers in individual ALS and control participants.

22 immune markers were assessed in ALS (N = 72 participants; black) and control (N = 52 participants; grey) participants (female, yellow; male, blue) for up to 12 months. Markers included total immune cells, NK cell subpopulations, and NK cell surface markers. For each marker the mean was calculated, and the data were normalized and displayed as a fold-value versus the mean. Legend represents higher (red) to lower (blue). MFI = median fluorescence intensity. Study participants needed to provide at least two samples to be included in subsequent analyses.

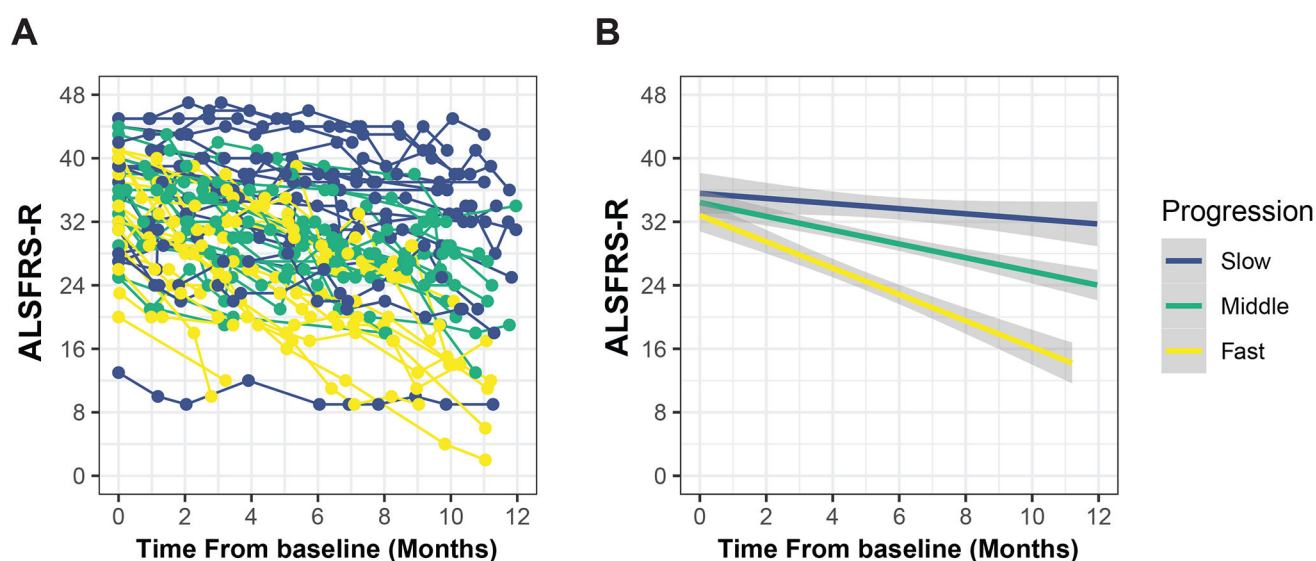


Figure 2. ALS participants stratified by ALSFRS-R progression rate.

ALS participants (N = 55 participants) were stratified into slow (blue), medium (green), and fast (yellow) progression groups by overall change in ALSFRS-R over the study.

(A) Individual study participant ALSFRS-R scores over the study course. (B) Changes in ALSFRS-R for each ALS progression group over the study course. Fitted linear smooth lines represent mean ALSFRS-R of data points in (A) for each progression group, with 95% confidence over the study course (shaded grey). This approach allows visual assessment of the trends of ALSFRS-R within each progression group.

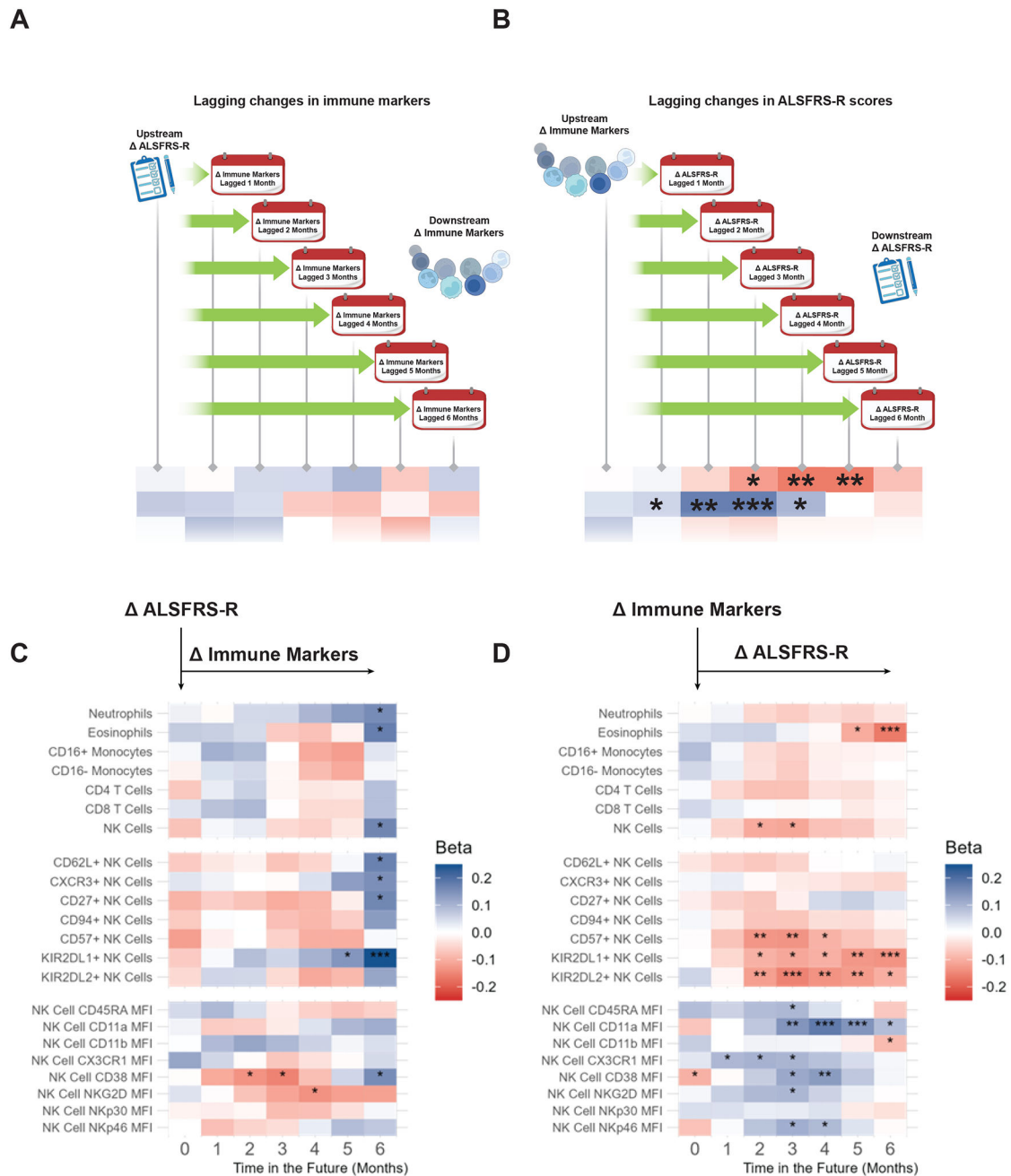


Figure 3. Lagged association between immune markers and ALSFRS-R.

Lagged models were created to assess the association between changes in a first measurement and subsequent, downstream changes in a second measurement occurring 0–6 months later. **(A)** A linear mixed effect model was generated correlating upstream changes in ALSFRS-R (Δ ALSFRS-R) with downstream changes in immune markers (Δ Immune Markers) or **(B)** correlating upstream changes in immune markers (Δ Immune Markers) with downstream changes in ALSFRS-R (Δ ALSFRS-R). Heat maps were then used to visualize the associations between **(C)** upstream changes in ALSFRS-R and downstream immune changes or **(D)** upstream immune changes and downstream ALSFRS-R changes. Colors

represent direction of correlation between changes: red indicates negative correlation (large increase in the upstream variable is associated with decrease in the downstream variable) while blue indicates positive correlation (large increase in the upstream variable is associated with large increase in the downstream variable). For **(D)**, where upstream immune changes were correlated with downstream ALSFRS-R changes, red therefore indicates increases in an upstream immune marker are associated with decreases in ALSFRS-R (more rapid disease progression). n = 38 participants, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; MFI = median fluorescence intensity.

Table 1.

Demographics of ALS and control participants

Characteristic	Control, N = 50 ¹	ALS, N = 55 ¹	p-value ²
Sex			0.751
Female	27 (54.00%)	28 (50.91%)	
Male	23 (46.00%)	27 (49.09%)	
Age (years)	63.36 (60.12, 68.36)	62.68 (57.35, 70.93)	0.878
Age at Onset (years)	NA (NA, NA)	61.73 (54.66, 69.31)	NA
Number of measurements	6.00 (5.00, 7.00)	9.00 (4.50, 11.00)	0.007
Ethnicity			0.105
Hispanic or Latino	2 (4.00%)	0 (0%)	
Not Hispanic or Latino	47 (94.00%)	55 (100.00%)	
Unknown or Not Reported Ethnicity	1 (2.00%)	0 (0%)	
Race			0.799
Asian	1 (2.04%)	1 (1.82%)	
Black or African American	2 (4.08%)	1 (1.82%)	
White or Caucasian	46 (93.88%)	53 (96.36%)	
Unknown	1	0	
Family history of ALS	0 (0%)	8 (14.81%)	0.023
Onset segment			
Bulbar	NA (NA, NA)	17 (30.91%)	
Cervical	NA (NA, NA)	21 (38.18%)	
Lumbar	NA (NA, NA)	17 (30.91%)	
El Escorial initial diagnosis			NA
Definite	NA (NA, NA)	20 (36.36%)	
Possible	NA (NA, NA)	9 (16.36%)	
Probable	NA (NA, NA)	11 (20.00%)	
Probable, lab supported	NA (NA, NA)	15 (27.27%)	
Riluzole use at study commencement	NA (NA, NA)	33 (60.00%)	NA
Riluzole use at any point during study	NA (NA, NA)	38 (69.09%)	NA
Forced vital capacity	NA (NA, NA)	73% (59, 86)	NA
ALSFRS-R at baseline	NA (NA, NA)	35 (29, 40)	NA
Time from symptom onset to diagnosis (days)	NA (NA, NA)	389.00 (256.50, 531.00)	
Time from diagnosis to 1 st immune assessment (days)	NA (NA, NA)	117.00 (50.00, 247.00)	NA

¹N (%); median (interquartile range)²Pearson's Chi-squared test (Sex, Age group); Wilcoxon rank sum test (Age, Number of measurements); Fisher's exact test (Ethnicity, Race, Family history of ALS)

N, number of participants

Table 2. Linear trajectory and variability in immune markers over time in ALS and control participants

Characteristic	Immune Linear Trajectory				Immune Variability			
	Control, N = 50 ¹	ALS, N = 55 ¹	p-value ³	q-value ⁴	Control, N = 50 ²	ALS, N = 55 ²	p-value ³	q-value ⁴
Neutrophils	-0.017 [-0.030, -0.003]	-0.002 [-0.014, 0.009]	0.115	0.243	0.261 [0.238, 0.287]	0.260 [0.241, 0.281]	0.929	0.929
Eosinophils	0.001 [-0.002, 0.004]	0.003 [0.000, 0.005]	0.348	0.588	0.054 [0.050, 0.060]	0.068 [0.063, 0.073]	0.001	0.001
CD16+ Monocytes	0.002 [0.001, 0.003]	0.001 [-0.000, 0.001]	0.025	0.083	0.022 [0.020, 0.024]	0.017 [0.016, 0.019]	< 0.001	0.001
CD16- Monocytes	0.005 [0.003, 0.008]	0.001 [-0.001, 0.003]	0.023	0.083	0.065 [0.059, 0.071]	0.076 [0.070, 0.082]	0.010	0.021
NK Cells	0.004 [0.002, 0.007]	0.001 [-0.001, 0.003]	0.064	0.157	0.047 [0.043, 0.052]	0.045 [0.041, 0.048]	0.396	0.473
CD4 T Cells	0.010 [0.005, 0.014]	-0.001 [-0.004, 0.003]	< 0.001	0.003	0.110 [0.100, 0.120]	0.108 [0.100, 0.116]	0.803	0.841
CD8 T cells	0.005 [0.002, 0.008]	-0.001 [-0.003, 0.002]	0.004	0.022	0.066 [0.060, 0.072]	0.061 [0.056, 0.066]	0.208	0.286
CD27+ NK Cells	0.000 [-0.000, 0.000]	0.000 [-0.000, 0.000]	0.867	0.867	0.006 [0.006, 0.007]	0.004 [0.004, 0.005]	< 0.001	< 0.001
CD57+ NK Cells	0.002 [0.001, 0.004]	0.002 [0.000, 0.003]	0.469	0.661	0.029 [0.026, 0.032]	0.032 [0.029, 0.034]	0.154	0.225
CD62L+ NK Cells	0.002 [0.001, 0.002]	0.000 [-0.000, 0.001]	0.064	0.157	0.021 [0.019, 0.023]	0.022 [0.020, 0.023]	0.409	0.473
CD94+ NK Cells	0.003 [0.001, 0.004]	0.001 [-0.000, 0.002]	0.122	0.243	0.031 [0.028, 0.034]	0.030 [0.028, 0.032]	0.526	0.579
CXCR3+ NK Cells	0.000 [-0.001, 0.001]	-0.000 [-0.001, 0.001]	0.580	0.661	0.024 [0.022, 0.026]	0.022 [0.021, 0.024]	0.320	0.414
KIR2DL1+ NK Cells	0.001 [0.000, 0.002]	0.001 [-0.000, 0.001]	0.313	0.574	0.019 [0.017, 0.021]	0.016 [0.015, 0.017]	0.005	0.012
KIR2DL2+ NK Cells	0.001 [0.000, 0.002]	0.001 [-0.000, 0.002]	0.631	0.661	0.018 [0.017, 0.020]	0.021 [0.019, 0.023]	0.053	0.09
NK Cell CD11a MFI ⁵	-0.009 [-0.026, 0.009]	-0.016 [-0.033, -0.000]	0.522	0.661	0.424 [0.388, 0.464]	0.516 [0.479, 0.556]	0.001	0.002
NK Cell CD11b MFI	0.005 [-0.010, 0.021]	-0.001 [-0.016, 0.013]	0.554	0.661	0.327 [0.300, 0.358]	0.417 [0.384, 0.454]	< 0.001	< 0.001
NK Cell CD38 MFI	-0.026 [-0.045, -0.007]	-0.033 [-0.049, -0.016]	0.629	0.661	0.429 [0.389, 0.473]	0.477 [0.441, 0.515]	0.095	0.149
NK Cell CD45RA MFI	-0.015 [-0.037, 0.007]	-0.001 [-0.022, 0.020]	0.374	0.588	0.427 [0.389, 0.467]	0.556 [0.513, 0.603]	< 0.001	< 0.001
NK Cell CX3CR1 MFI	0.006 [-0.016, 0.027]	-0.041 [-0.060, -0.022]	0.002	0.016	0.431 [0.391, 0.474]	0.496 [0.459, 0.536]	0.024	0.044
NK Cell NKG2D MFI	0.023 [0.009, 0.036]	0.016 [0.003, 0.029]	0.511	0.661	0.270 [0.247, 0.296]	0.345 [0.317, 0.374]	< 0.001	< 0.001
NK Cell Nkp30 MFI	-0.054 [-0.089, -0.020]	0.030 [0.006, 0.053]	< 0.001	0.003	0.739 [0.665, 0.820]	0.571 [0.525, 0.621]	< 0.001	< 0.001
NK Cell Nkp46 MFI	-0.012 [-0.027, 0.004]	0.014 [-0.002, 0.029]	0.026	0.083	0.329 [0.301, 0.360]	0.424 [0.391, 0.459]	< 0.001	< 0.001

¹ Estimated slope and its 95% confidence interval

² Estimated variability and its 95% confidence interval

³ Calculated using linear mixed effect (LME) models' likelihood ratio test

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⁴Benjamini-Hochberg correction for multiple testing
N, number of participants
MFI, median fluorescence intensity

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Table 3. Linear trajectory and variability in immune markers over time amongst the ALS progression tertiles

	Immune Linear Trajectory					Immune Variability				
Characteristic	Slow, N = 19 ¹	Medium, N = 18 ¹	Fast, N = 18 ¹	p-value ²	q-value ⁴	Slow, N = 19 ²	Medium, N = 18 ²	Fast, N = 18 ²	p-value ³	q-value ⁴
Neutrophils	0.001 [-0.013, 0.016]	0.005 [-0.011, 0.021]	-0.016 [-0.037, 0.005]	0.381	0.598	0.243 [0.216, 0.275]	0.273 [0.240, 0.311]	0.278 [0.238, 0.324]	0.387	0.448
Eosinophils	0.007 [0.003, 0.011]	0.003 [-0.000, 0.006]	-0.005 [-0.009, 0.001]	<0.001	0.009	0.080 [0.070, 0.091]	0.061 [0.054, 0.070]	0.061 [0.053, 0.070]	0.005	0.013
CD16+ Monocytes	0.000 [-0.001, 0.001]	0.001 [-0.000, 0.002]	0.000 [-0.001, 0.002]	0.552	0.76	0.017 [0.015, 0.019]	0.018 [0.016, 0.021]	0.018 [0.016, 0.021]	0.454	0.499
CD16- Monocytes	0.002 [-0.002, 0.005]	0.000 [-0.003, 0.003]	0.002 [-0.003, 0.007]	0.703	0.814	0.078 [0.069, 0.088]	0.065 [0.058, 0.074]	0.087 [0.076, 0.099]	0.008	0.019
NK Cells	0.003 [-0.000, 0.006]	0.002 [-0.001, 0.006]	-0.002 [-0.005, 0.002]	0.139	0.598	0.047 [0.041, 0.053]	0.048 [0.042, 0.054]	0.037 [0.032, 0.043]	0.029	0.05
CD4 T Cells	0.001 [-0.004, 0.006]	-0.000 [-0.005, 0.005]	-0.005 [-0.012, 0.002]	0.380	0.598	0.112 [0.100, 0.126]	0.103 [0.091, 0.117]	0.106 [0.092, 0.122]	0.64	0.64
CD8 T cells	0.000 [-0.003, 0.004]	-0.000 [-0.005, 0.004]	-0.004 [-0.008, 0.000]	0.300	0.598	0.051 [0.045, 0.058]	0.078 [0.068, 0.090]	0.046 [0.040, 0.054]	<0.001	<0.001
CD27+ NK Cells	0.000 [-0.000, 0.000]	0.000 [0.000, 0.001]	0.000 [-0.000, 0.000]	0.195	0.598	0.005 [0.005, 0.006]	0.004 [0.004, 0.005]	0.003 [0.003, 0.004]	<0.001	<0.001
CD57+ NK Cells	0.001 [-0.001, 0.003]	0.003 [0.000, 0.006]	-0.000 [-0.002, 0.002]	0.279	0.598	0.028 [0.025, 0.032]	0.041 [0.036, 0.047]	0.021 [0.018, 0.024]	<0.001	<0.001
CD62L+ NK Cells	0.001 [-0.000, 0.002]	0.001 [-0.001, 0.002]	-0.000 [-0.001, 0.001]	0.496	0.727	0.022 [0.020, 0.025]	0.027 [0.023, 0.030]	0.012 [0.010, 0.014]	<0.001	<0.001
CD94+ NK Cells	0.001 [-0.001, 0.004]	0.002 [0.000, 0.004]	-0.001 [-0.003, 0.002]	0.214	0.598	0.033 [0.029, 0.037]	0.030 [0.026, 0.034]	0.024 [0.021, 0.028]	0.016	0.032
CXCR3+ NK Cells	-0.000 [-0.001, 0.001]	0.000 [-0.001, 0.001]	-0.000 [-0.001, 0.001]	0.828	0.868	0.023 [0.020, 0.026]	0.024 [0.021, 0.028]	0.019 [0.017, 0.022]	0.045	0.071
KIR2DL1+ NK Cells	0.001 [0.000, 0.002]	0.001 [-0.001, 0.002]	-0.000 [-0.001, 0.001]	0.268	0.598	0.016 [0.014, 0.018]	0.020 [0.017, 0.023]	0.011 [0.009, 0.012]	<0.001	<0.001
KIR2DL2+ NK Cells	0.001 [-0.000, 0.002]	0.001 [-0.001, 0.002]	0.000 [-0.001, 0.002]	0.656	0.81	0.022 [0.019, 0.025]	0.022 [0.020, 0.026]	0.019 [0.016, 0.022]	0.093	0.133
NK Cell CD11a MFI	-0.021 [-0.045, 0.003]	-0.015 [-0.035, 0.006]	-0.020 [-0.053, 0.014]	0.919	0.919	0.546 [0.486, 0.614]	0.451 [0.378, 0.538]	0.518 [0.447, 0.600]	0.097	0.133
NK Cell CD11b MFI	0.004 [-0.020, 0.028]	-0.002 [-0.024, 0.019]	-0.007 [-0.034, 0.021]	0.753	0.828	0.476 [0.418, 0.543]	0.401 [0.352, 0.456]	0.376 [0.326, 0.435]	0.132	0.171
NK Cell CD38 MFI	-0.037 [-0.062, -0.012]	-0.038 [-0.059, -0.018]	-0.015 [-0.043, 0.013]	0.346	0.598	0.559 [0.495, 0.630]	0.436 [0.386, 0.494]	0.407 [0.352, 0.472]	0.001	0.005

	Immune Linear Trajectory					Immune Variability				
Characteristic	Slow, N = 19 ¹	Medium, N = 18 ¹	Fast, N = 18 ¹	p-value ³	q-value ⁴	Slow, N = 19 ²	Medium, N = 18 ²	Fast, N = 18 ²	p-value ³	q-value ⁴
NK Cell CD45RA MFI	-0.007 [-0.039, 0.025]	-0.011 [-0.043, 0.022]	0.025 [-0.016, 0.065]	0.337	0.598	0.586 [0.518, 0.663]	0.594 [0.512, 0.689]	0.532 [0.460, 0.614]	0.599	0.628
NK Cell CX3CR1 MFI	-0.029 [-0.053, -0.005]	-0.070 [-0.095, -0.045]	-0.011 [-0.038, 0.017]	0.023	0.258	0.539 [0.478, 0.608]	0.546 [0.482, 0.618]	0.405 [0.350, 0.469]	0.005	0.013
NK Cell NKG2D MFI	0.018 [-0.004, 0.041]	0.001 [-0.019, 0.020]	0.040 [-0.015, 0.065]	0.152	0.598	0.405 [0.355, 0.462]	0.319 [0.279, 0.364]	0.303 [0.262, 0.350]	0.014	0.03
NK Cell NKP30 MFI	0.023 [-0.018, 0.065]	0.046 [0.002, 0.090]	0.018 [-0.035, 0.072]	0.663	0.81	0.542 [0.475, 0.618]	0.618 [0.539, 0.707]	0.538 [0.456, 0.635]	0.341	0.417
NK Cell NKP46 MFI	0.032 [0.002, 0.061]	-0.001 [-0.027, 0.026]	0.014 [-0.023, 0.050]	0.276	0.598	0.460 [0.405, 0.523]	0.360 [0.317, 0.409]	0.410 [0.352, 0.477]	0.030	0.05

¹ Estimated slope and its 95% confidence interval

² Estimated variability and its 95% confidence interval

³ Calculated using linear mixed effect models' likelihood ratio test

⁴ Benjamini-Hochberg correction for multiple testing

N, number of participants

MFI, median fluorescence intensity

KEY RESOURCES

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD3 (FITC)	Biolegend	Catalogue #300306
Anti-human CD4 (PerCP/Cy5.5)	Biolegend	Catalogue #357414
Anti-human CD8a (APC/Cy7)	Biolegend	Catalogue #300926
Anti-human CD14 (APC/Cy7)	Biolegend	Catalogue #367108
Anti-human CD16 (PerCP/Cy5.5)	Biolegend	Catalogue #302028
Anti-human CD56 (PE/Cy7)	Biolegend	Catalogue #318318
IgG1 Control Antibody (Brilliant Violet 421)	Biolegend	Catalogue #400157
IgG1 Control Antibody (PE)	Biolegend	Catalogue #400112
IgG1 Control Antibody (APC)	Biolegend	Catalogue #400120
Anti-human CD11b (Brilliant Violet 421)	Biolegend	Catalogue #301323
Anti-human CD45RA (Brilliant Violet 421)	Biolegend	Catalogue #304129
Anti-human CXCR3 (Brilliant Violet 421)	Biolegend	Catalogue #353715
Anti-human NKG2D (Brilliant Violet 421)	Biolegend	Catalogue #320821
Anti-human NKp46 (Brilliant Violet 421)	Biolegend	Catalogue #331913
Anti-human CD11a (PE)	Biolegend	Catalogue #301207
Anti-human CD38 (PE)	Biolegend	Catalogue #356603
Anti-human CD62L (PE)	Biolegend	Catalogue #304805
Anti-human CX3CR1 (PE)	Biolegend	Catalogue #341603
Anti-human KIR2DL1/S1/S3/S5 (PE)	Biolegend	Catalogue #339505
Anti-human/mouse/rat CD27 (APC)	Biolegend	Catalogue #124211
Anti-human CD57 (APC)	Biolegend	Catalogue #359609
Anti-human CD94 (APC)	Biolegend	Catalogue #305508
Anti-human KIR2DL2/L3 (APC)	Biolegend	Catalogue #312611
Anti-human NKp30 (APC)	Biolegend	Catalogue #325209
Biological samples		
Human peripheral blood	University of Michigan Pranger ALS Clinic	De-identified
Deposited data		
Participant ALSFRS-R scores	University of Michigan Pranger ALS Clinic	De-identified
Software and algorithms		
R	R Core Team	v4.4.1