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# CSF-Derived CD4<sup>+</sup> T-Cell Diversity Is Reduced in Patients With Alzheimer Clinical Syndrome

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## Abstract

### Background and Objectives

Patients with Alzheimer dementia display evidence of amyloid-related neurodegeneration. Our focus was to determine whether such patients also display evidence of a disease-targeting adaptive immune response mediated by CD4<sup>+</sup> T cells. To test this hypothesis, we evaluated the CSF immune profiles of patients with Alzheimer clinical syndrome (ACS), who display clinically defined dementia.

### Methods

Innate and adaptive immune profiles of patients with ACS were measured using multicolor flow cytometry. CSF-derived CD4<sup>+</sup> and CD8<sup>+</sup> T-cell receptor repertoire genetics were measured using next-generation sequencing. Brain-specific autoantibody signatures of CSF-derived antibody pools were measured using array technology or ELISA. CSF from similar-age healthy controls (HCs) was used as a comparator cohort.

### Results

Innate cells were expanded in the CSF of patients with ACS in comparison to HCs, and innate cell expansion increased with age in the patients with ACS, but not HCs. Despite innate cell expansion in the CSF, the frequency of total CD4<sup>+</sup> T cells reduced with age in the patients with ACS. T-cell receptor repertoire genetics indicated that T-cell clonal expansion is enhanced, and diversity is reduced in the patients with ACS compared with similar-age HCs.

### Discussion

Examination of CSF indicates that CD4<sup>+</sup> T cell-mediated adaptive immune responses are altered in patients with ACS. Understanding the underlying mechanisms affecting adaptive immunity will help move us toward the goal of slowing cognitive decline.

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## Glossary

**ACS** = Alzheimer clinical syndrome; **AD** = Alzheimer disease; **ADL** = activities of daily living; **A $\beta$**  =  $\beta$ -amyloid; **aMCI** = amnesic mild cognitive impairment; **ATI** = A $\beta$ 42/total tau (hTau) index; **CDR** = clinical dementia rating; **HC** = health control; **MS** = multiple sclerosis; **NfL** = neurofilament light; **TCRB** = T-cell receptor beta; **UTSW** = UT Southwestern Medical Center.

The World Alzheimer Report estimates that the cost of dementia care will reach 2 trillion dollars by 2030, and dementia will affect nearly 75 million people.<sup>1</sup> Alzheimer disease (AD) remains the most common dementia diagnosis,<sup>2</sup> in which accumulation of  $\beta$ -amyloid (A $\beta$ ) plaques and hyperphosphorylated tau tangles in the brain is thought to cause neuronal loss and cognitive dysfunction.<sup>3</sup> According to recent guidelines, a biological diagnosis of AD is verified using a classification system that encompasses both biofluid CSF levels of A $\beta$  and tau and imaging approaches that measure neurodegeneration.<sup>4</sup> Despite advances in diagnostic methods, effective therapies for AD remain unrealized.

In an effort to develop treatments for AD, emerging studies must endeavor to delineate underlying disease mechanisms, including the role of neuroinflammation in pathologic progression.<sup>5-8</sup> Inflammation is mediated by both nonspecific innate and highly specific adaptive immune system components. A typical immune response to an infection, for example, would begin by activating innate immune components to sequester the infectious agent and present components of the infectious agent (antigens) to the cellular components of the adaptive immune system (T cells and B cells). Specific targeting of the infectious agent and its removal from the body are mediated initially by helper CD4<sup>+</sup> T cells, which undergo activation, proliferation, and clonal expansion on exposure to the antigen presented by innate cells. In turn, helper CD4<sup>+</sup> T cells produce cytokines and chemokines that activate cytotoxic CD8<sup>+</sup> T cells to destroy the infectious agent and stimulate CD27<sup>+</sup> memory B cells to develop into antibody-producing B cells that neutralize the infectious agent. This cascade of the immune response is essential to clearance of an infection. In the setting of AD pathology, there is some evidence that components of both the innate and adaptive immune systems are involved. For example, macrophages and neutrophils have been found in the brains of patients with AD,<sup>9</sup> and both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are frequently found at the sites of amyloid deposition in the brain.<sup>10-13</sup> Yet, the role of innate and/or adaptive immune system components in the propagation of neuroinflammatory, regulatory, or even neuroprotective mechanisms remains largely unknown.<sup>14-16</sup> Thus, elucidating the role of the immune system in the development of AD pathology and other dementias is critical to facilitate the development of therapeutics designed to prevent or slow cognitive decline.<sup>5,6</sup>

Our laboratory previously focused on examining early immune profile discordance in patients who have cognitive impairment, but are not experiencing clinical dementia to the

extent that their activities of daily living (ADLs) are affected.<sup>17-19</sup> Such patients are diagnosed with amnesic mild cognitive impairment (aMCI) with an anticipated conversion rate to AD diagnosis of 10% annually.<sup>20</sup> Our previous goal was to determine whether early clinical manifestations of CNS inflammation could be demonstrated in the CSF of patients with aMCI. We investigated CSF-derived immune profiles of patients with aMCI and observed that they have elevated CNS inflammation as measured by a similar frequency and absolute number of CD45<sup>+</sup> leukocytes compared with patients with multiple sclerosis (MS), a well-known CNS inflammatory disease driven by CD4<sup>+</sup> T cells.<sup>17</sup> However, the frequency of total CD4<sup>+</sup> T cells was reduced in patients with aMCI compared with patients with MS despite evidence of A $\beta$  deposition in the CNS of the patients with aMCI by PET imaging.<sup>19</sup> Furthermore, the aMCI cohort demonstrated that as A $\beta$  deposition in the brain increased, the frequency of CD4<sup>+</sup> T cells in the CSF declined with significant correlation.<sup>18</sup> These findings suggested that despite evidence of CNS damage, elevated adaptive immune responses mediated by CD4<sup>+</sup> T cells were not occurring in patients with aMCI. Of interest, the frequency of innate immune cells was increased in the aMCI cohort in comparison to patients with MS, but did not result in expansion of CD4<sup>+</sup> T cells in the CSF of the patients with aMCI.

We first hypothesized that perhaps CNS damage in patients with aMCI has not advanced (either in severity or duration) to incite a detectable disease-driven adaptive immune response. This could be the case as patients with aMCI do not exhibit deficits in cognition severe enough to affect their ADLs. On the other hand, patients diagnosed with Alzheimer clinical syndrome (ACS) who display deficits in cognition severe enough to affect ADLs are further along in their disease course. Thus, in an effort to understand the role of adaptive and innate immune populations, we immunophenotyped CSF-derived cells from patients with ACS with clinically confirmed deficits in ADLs. We used flow cytometry, T-cell receptor repertoire genetics, and brain-specific autoantibody signatures to define the innate and adaptive immune profile of patients with ACS compared with similar-age healthy controls (HCs).

## Methods

### Patient Recruitment

All subjects and/or their legally authorized study partners signed the written informed consent approved by the Institutional Review Board of the UT Southwestern Medical Center (UTSW), in accordance with the Federal Wide

Assurance on file with the Department of Health and Human Services (USA).

The cohort (Table 1) consisted of 26 patients diagnosed with ACS (mean age  $69.2 \pm 9.0$  years) and 12 similar-age HCs (mean age  $70.0 \pm 8.6$  years). Standard clinical evaluations were performed on all participants at the UTSW Alzheimer's Disease Center or Memory Clinic. Clinical diagnoses were based on standard National Institute on Aging and Alzheimer's Association criteria for ACS using a multidisciplinary diagnostic conference format based on history, neurologic examination, clinical dementia rating (CDR) interview, and neuropsychological evaluation in accordance with National Alzheimer's Coordinating Center procedures used in National Institute on Aging-funded Alzheimer's Disease Research Centers.<sup>4</sup> Of the 26 patients with ACS, 17 (identified by black squares in all figures) were confirmed for Alzheimer pathology using the A $\beta_{42}$ /total tau (hTau) index (ATI) and phosphorylated tau 181 (pTau181) tests in a commercial CLIA-certified laboratory. The remaining 9 cases that

were not confirmed for Alzheimer pathology using ATI and pTau181 tests in a commercial CLIA-certified laboratory are identified by gray squares in all figures. In our research laboratory, we tested these 9 subjects for A $\beta_{42}$ , hTau, and pTau181 in the CSF with expected cohort comparison results indicative of increased amyloid burden and tau tangles in the brain (Table 1).<sup>21,22</sup> In addition, we also measured neurofilament light (NfL) levels in the research laboratory as another indicator of neurodegeneration.<sup>23</sup> Subjects were excluded if they did not display deficits in cognition severe enough to affect their ADLs and had a history of stroke, major medical and psychiatric disorders, unstable heart disease, uncontrolled hypertension, diabetes mellitus, or chronic inflammatory diseases. Limited availability of CSF restricted the number of samples used for each assay.

### CSF and Blood Collection, Processing, and Flow Cytometry

CSF collected by lumbar puncture and blood from all subjects in the cohort were processed as previously published.<sup>17</sup>

**Table 1** Cohort Summary

	HC	ACS
<b>Clinical data</b>		
No. of subjects	12	26
M:F <sup>a</sup>	3:8	13:13
Average age (range)	70 (58–84)	69 (55–91)
Average MMSE (range   number of subjects)	29 (28–30   8)	22 (12–29   16) <sup>c</sup>
No. of subjects with clinically confirmed deficit in ADLs	0/12	26/26
No. of subjects with ATI and pTau181 clinical testing <sup>b</sup>	ND	17
Abnormal ATI	ND	17/17
Abnormal pTAU	ND	16/17
<b>Research laboratory data</b>		
No. of subjects	12	26 <sup>d</sup>
CSF-derived A $\beta_{42}$	347 pg/mL	156 pg/mL <sup>e</sup>
CSF-derived hTau	396 pg/mL	736 pg/mL <sup>f</sup>
CSF-derived pTau <sub>181</sub>	46 pg/mL	78 pg/mL <sup>g</sup>
CSF-derived NfL	758 pg/mL	1240 pg/mL <sup>h</sup>
hTau:A $\beta_{42}$	1.7	4.3 <sup>h</sup>
pTau:A $\beta_{42}$	0.19	0.46 <sup>h</sup>
NfL:A $\beta_{42}$	3.2	11.0 <sup>h</sup>

Abbreviations: A $\beta$  =  $\beta$ -amyloid; ACS = Alzheimer clinical syndrome; ADLs = activities of daily living; ATI = A $\beta_{42}$ /total tau (hTau) index; HC = healthy control; MMSE = Mini-Mental State Examination; NfL = neurofilament light; pTau = phosphorylated tau 181.

<sup>a</sup> The sex of 1 HC was not available.

<sup>b</sup> This testing was performed using a commercial CLIA-certified clinical laboratory. There were 9 patients with ACS who were not tested for ATI and pTAU181 using a commercial CLIA-certified clinical laboratory. In those cases, diagnosis was based on clinical diagnosis as described in the methods.

<sup>c</sup> One subject in the ACS cohort had an MMSE of 29.

<sup>d</sup> Innate cell subset data were only available on 5 of the subjects with ACS.

<sup>e</sup> Significantly lower than HCs ( $p < 0.001$ ).

<sup>f</sup> Significantly higher than HCs ( $p < 0.05$ ).

<sup>g</sup> Significantly higher than HCs ( $p < 0.01$ ).

<sup>h</sup> Significantly higher than HCs ( $p < 0.001$ ).

Peripheral blood and CSF cells collected from the same subjects on the same day were stained in parallel using standard protocols established in the Monson laboratory.<sup>24</sup> The sample gating strategy (eFigure 1A, links.lww.com/NXI/A656) and subtype frequencies (eFigure 1B) were based on our previous approach.<sup>17</sup> Of the 26 patients with ACS, 21 were analyzed by flow cytometry to obtain frequencies of innate, CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell, CD19<sup>+</sup> B-cell, and B-cell subsets. An additional 5 patients with ACS were analyzed by flow cytometry to obtain frequencies of innate cell subsets. Of note, the time from sample acquisition to initiation of processing was longer with samples that were not CLIA tested (gray squares in all figures) compared with those that were CLIA tested (black squares in all figures).

### Detection of Analytes in CSF

ELISA kits for A $\beta$ <sub>1-42</sub>, hTau, pTau181 (Innotest, Fujirebio, Malvern, PA), and NfL (Uman Diagnostics, Sweden) were used according to the manufacturer's instructions. The lowest limit of detection for each analyte was as follows: A $\beta$ <sub>1-42</sub> 65 pg/mL, hTau 34 pg/mL, pTau(181) 13 pg/mL, and NfL 13 pg/mL. Samples below the detection limit were excluded from analysis.

### TCR Next-Generation Sequencing

Genomic DNA (gDNA) was isolated from sorted CSF-derived CD4<sup>+</sup> and CD8<sup>+</sup> T cells and subjected to PCR using T-cell receptor beta (TCRB) gene rearrangement primer sets (eTable 1, links.lww.com/NXI/A661) previously published by others.<sup>25</sup> Barcoded libraries were pooled and loaded onto an Illumina MiSeq instrument.

### Repertoire Analysis

The TCR repertoire sequencing data were analyzed on VDJServer v1.1.2<sup>26</sup> and supporting software as detailed in the Supplemental Materials. The raw sequencing data are available at SRA (BioProject: PRJNA625786), and the raw sequencing data,

postprocessed rearrangements, and analysis results are available at VDJServer (UUID: 887088384323808790-242ac116-0001-012). TCRB variable gene distributions are provided in eFigure 2, links.lww.com/NXI/A657. T-cell receptor sequences with the same V gene call, J gene call, and CDR3 sequence were determined to be derived from clonally related T cells for purposes of the clonal abundance and diversity.

### Detection of Antibodies Against A $\beta$ <sub>42</sub>

This assay was performed as previously published<sup>27</sup> with modifications as detailed in the Supplementary Materials.

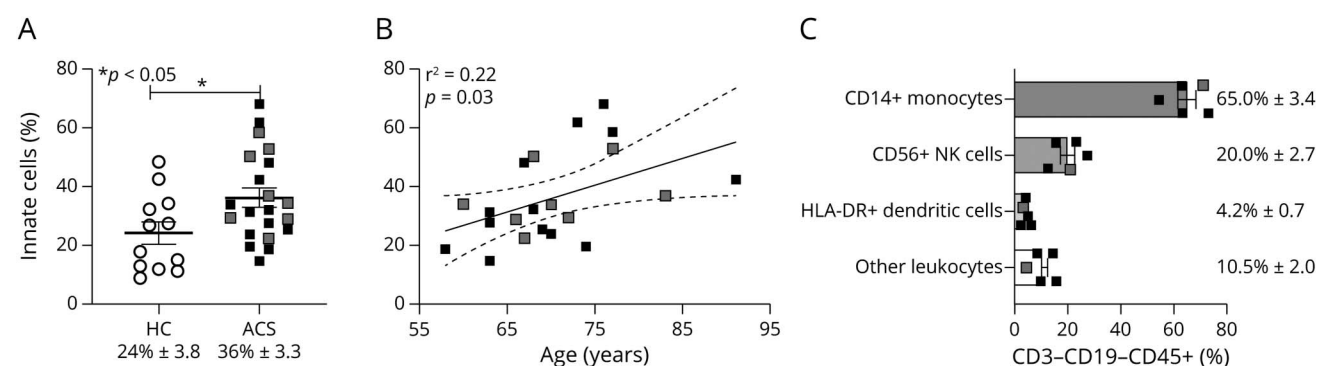
### Brain Antigen Array

The brain antigen array consists of 63 brain-specific antigens (eTable 2, links.lww.com/NXI/A661). Samples were incubated with the arrays for 1 hour with agitation at room temperature, and Cy3-labeled anti-human IgG and Cy5-labeled anti-human IgM were used for detection at 1:1000 dilutions. Images were generated using the GenePix 4000B scanner and analyzed using GenePix Pro 7.0 software (Molecular Devices). The antibody score (Ab score) is detailed in eTable 3.

### Data Analysis

All flow cytometry data were analyzed using FlowJo software (Treestar). The absolute cell numbers were calculated based on the FlowJo data and the hemocytometer counts. All data are presented as mean  $\pm$  SEM. We used GraphPad Prism 8.0 for data analysis unless otherwise specified. All data were first tested for normal distribution using the Shapiro-Wilk test. Data sets passing the Shapiro-Wilk test were then analyzed using the unpaired, 2-sided Student *t* test (parametric). Data sets that did not pass Shapiro-Wilk test were Figure 1B (HC and ACS), 3A (ACS for total CD19), 3B (HC and ACS), 3C (ACS), 3D (ACS), and eFigures 3C, links.lww.com/NXI/A658 (ACS), 5A (HC), 5B (HC and ACS), and 5C (ACS). In these cases, the comparison was made using the

**Figure 1** Innate Cells in the CSF Are Expanded and Positively Correlate With Age in Patients With ACS



(A) The frequency (%) of innate cells in the CSF of 21 patients with ACS was compared with 12 similar-age healthy controls (HCs). (B) Correlation of CSF-derived innate cell frequency with age in the ACS cohort. (C) The relative frequencies of innate cell subtypes in the CSF of an ACS subcohort. CD45<sup>+</sup> leukocytes that did not express any other lineage markers in the panel were categorized as other leukocytes. The mean  $\pm$  SEM of each group is provided in panels A and C. Circles indicate HC donors, solid black squares represent CLIA-tested patients with ACS, and solid gray squares represent non-CLIA-tested patients with ACS. ACS = Alzheimer clinical syndrome. \* $p < 0.05$ .

nonparametric Mann-Whitney test. All correlation analyses were obtained by simple linear regression analysis in Prism. The brain antigen array data were analyzed with RStudio version 1.1.4, using the Wilcoxon test 2-sided hypothesis. A *p* value of less than 0.05 was considered statistically significant.

## Data Availability

Anonymized data will be shared by request from any qualified investigator.

## Results

### Innate Cell Expansion and Correlation With Age

We hypothesized that features of the immune response would be affected in patients with ACS who display deficits in cognition severe enough to affect ADLs. To begin addressing this hypothesis, we used multiparameter flow cytometry to identify innate cells and adaptive (i.e., CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells) leukocyte subtypes in the CSF and blood of 21 patients with ACS and 12 similar-age HCs. We also identified innate cell subsets in the CSF of 5 additional patients with ACS by multiparameter flow cytometry. Patients with ACS in this cohort demonstrated expansion of general innate cell populations in the CSF compared with similar-age HCs by frequency (Figure 1A, *p* < 0.05), but not absolute number (eFigure 3A, [links.lww.com/NXI/A658](https://links.lww.com/NXI/A658)). In addition, the frequency of innate cells in the CSF increased with age in the ACS patients (Figure 1B, *p* = 0.03, *r*<sup>2</sup> = 0.22), but not in the similar-age HCs (data not shown). Of note, the frequency (eFigure 3B) and absolute number (eFigure 3C) of CD45<sup>+</sup> leukocytes in the CSF were similar in both cohorts. In some cases, the absolute number of CD45<sup>+</sup> leukocytes in both the ACS and HC cohorts was nearly undetectable, whereas others reached very high levels. The innate cell subtype in the CSF of patients with ACS consisted mostly of CD14<sup>+</sup> monocytes (Figure 1C). The frequency of CD45<sup>+</sup> leukocytes in the blood was also similar in both cohorts (eFigure 3D), as was the frequency of innate cells in the blood (eFigure 3E). Finally, in contrast to the frequency of innate cells in the CSF, the frequency of innate cells in the blood does not correlate with age in the ACS patients (eFigure 3F). The 11-color panel that allows us to segregate the innate cell subtypes was used on only 5 of the patients with ACS (Figure 1C), and so a more detailed analysis of the innate cell subtypes affecting the frequency and correlation with age could not be performed. Similar-age HCs did not display correlation of age with innate cell frequency in the blood (data not shown).

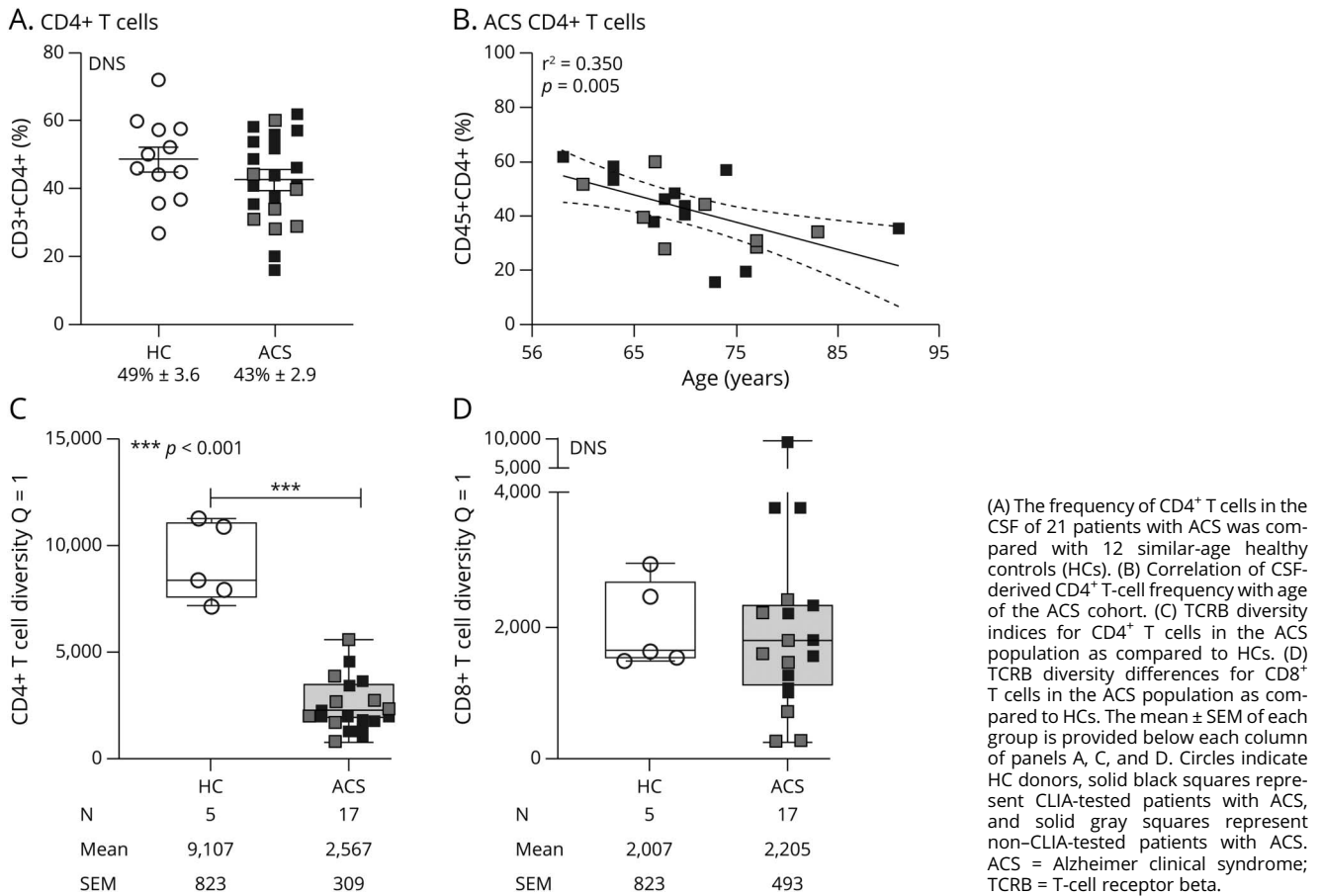
### CSF-Derived T-Cell Features

Next, we focused on T cells, which are a component of the adaptive immune system that we anticipated would be responsive to the expansion of innate cells and the elevation of neurodegeneration in the patients with ACS as evidenced by changes in the CSF of Aβ<sub>42</sub>, hTau, pTau181, and NfL (Table 1). The CD4<sup>+</sup> T-cell frequency (Figure 2A) and

absolute number (data not shown) in the CSF were similar in both cohorts. Nevertheless, the frequency of CD4<sup>+</sup> T cells in the CSF negatively correlated with age in the ACS patients (*p* < 0.005, *r*<sup>2</sup> = 0.35; Figure 2B), but did not correlate in the similar-age HCs (data not shown). The frequency of CD4<sup>+</sup> T cells also did not correlate with the MMSE score or other clinical measures that were available, including markers of AD pathology (i.e., Aβ<sub>42</sub>) or general neurodegeneration (i.e., NfL).

Next, we analyzed the extent of clonal expansion within the TCRB gene repertoires expressed by CSF-derived CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequency of unactivated T cells by TCRB sequence analysis (“NONE”; eFigure 4C, [links.lww.com/NXI/A659](https://links.lww.com/NXI/A659)) and clonally expanded CD4<sup>+</sup> T cells of moderate range by TCRB sequence analysis (eFigure 4D) was reduced in the CSF-derived CD4<sup>+</sup> T-cell population of the patients with ACS compared with similar-age HCs. In contrast, the frequency of advanced clonally expanded T cells by TCRB sequence analysis (greater than 1% representation in the TCRB sequence repertoire) was significantly higher in the CSF-derived CD4<sup>+</sup> T-cell population TCRB sequence repertoire of the patients with ACS compared with the similar-age HCs (61% vs <20%, *p* < 0.01) (eFigure 4E). In parallel, the frequency of CD8<sup>+</sup> T cells in the patients with ACS was comparable to similar-age HCs (eFigure 4A) and did not correlate with age (eFigure 4B). Furthermore, CSF-derived CD8<sup>+</sup> T cells of patients with ACS displayed similar representation of all 3 categories of clonally expanded CD8<sup>+</sup> T cells (eFigure 4F–H) compared with similar-age HCs. T-cell subset clonal expansion in the blood was not examined because the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the blood was similar in both cohorts, and there was no correlation with age in patients with ACS (data not shown).

In addition to specifically analyzing clonal expansion of the CSF repertoires, we also analyzed repertoire diversity by calculating Hill numbers over a range of *q* values.<sup>28</sup> We used Alakazam,<sup>29</sup> which is implemented in VDJSer and uses resampling techniques to account for sample-to-sample variability in sequence numbers. Hill numbers quantify repertoire diversity as a function of both clonal expansion (the relative clonal abundances across all clones) and the overall number of distinct clones in a repertoire. A high diversity index indicates that the repertoire gene distribution has a large number of clones with relatively uniform abundances, as would be the case for repertoires dominated by unactivated or naive T cells. Once a T-cell population responds to its cognate antigen, it becomes activated and undergoes clonal expansion. Subsequently, clonal expansion results in a reduction of diversity within the repertoire, resulting in a low diversity index when T cells are undergoing clonal expansion. CSF-derived CD4<sup>+</sup> T cells of the patients with ACS displayed a reduced diversity index within the expressed TCRB repertoires (Figure 2C). In contrast, the CSF-derived CD4<sup>+</sup> T cells of the similar-age HCs exhibited higher diversity as expected for a T-cell



population dominated by a large number of non-expanded clones (2,567 vs 9,107,  $p < 0.001$ ). CSF-derived CD8<sup>+</sup> T-cell diversity index was similar between the patients with ACS and similar-age HC cohorts (Figure 2D) as a reflection of similar frequencies of advanced CD8<sup>+</sup> T-cell clones.

### Humoral Immune Response Features

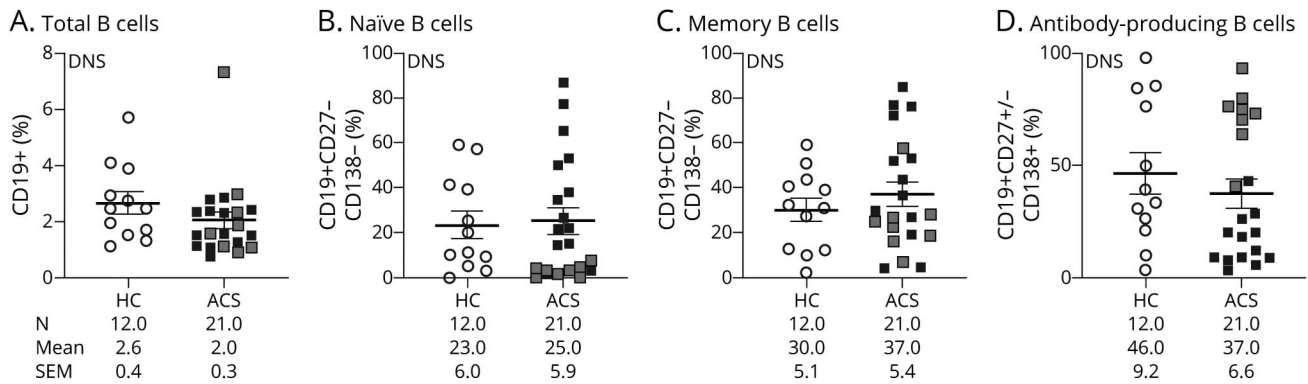
Activated CD4<sup>+</sup> T cells are critical in facilitating B-cell activation and class switching,<sup>30</sup> and the CD4<sup>+</sup> T cells in the CSF of patients with ACS displayed genetic markers of activation including clonal expansion and, in this case, subsequent reduced diversity. Thus, we next examined the impact of this T-cell dynamic on B-cell subsets. The frequency of total B cells (Figure 3A) and the naive (Figure 3B) and memory (Figure 3C) B-cell subtypes was comparable in both cohorts. Naive B cells were not detected in the CSF of 2 patients with ACS and 1 HC. CD138<sup>+</sup> antibody-producing B cells were detected in the CSF of both patients with ACS and HCs at similar frequencies (Figure 3D), whereas an earlier study focused on patients with mild AD could not detect any CD138<sup>+</sup> antibody-producing B cells.<sup>31</sup> Both naive and CD138<sup>+</sup> antibody-producing B-cell frequencies were affected by the covariate of sample handling as noted in the methods. Total IgM and IgG concentrations in the CSF were also similar

(data not shown). Of interest, despite the reduction of Aβ<sub>42</sub> in the patients with ACS compared with similar-age HCs (Table 1), we did not observe a complimentary increase in titers of anti-Aβ<sub>42</sub> of either the IgM or IgG isotype (eFigure 5A and B, links.lww.com/NXI/A660). In addition, the concentration of NfL in the CSF of patients with ACS is elevated in comparison to similar-age HCs (Table 1), yet titers of anti-NfL antibody of either isotype (eFigure 5C and D) were similar in both cohorts. Antibody reactivity to other brain antigens was also comparable in both cohorts (eTable 3, links.lww.com/NXI/A661).

### Discussion

We hypothesized that neurodegeneration in patients with ACS must reach an advanced and critical stage to incite a notable adaptive immune response shift in comparison to similar-age HCs. Our findings indicated increased amyloid burden in the brain (as measured by detection of reduced Aβ<sub>42</sub> in the CSF) and increased neuronal damage (as measured by detection of NfL in the CSF) in patients with ACS compared with similar-age HCs. We anticipated that advancing neurodegeneration in the brain would promote an

**Figure 3** B-Cell Subset Frequencies in the CSF Are Unaltered in Patients With ACS



The frequencies of (A) CD19<sup>+</sup> B cells and subsets including (B) naïve cells, (C) memory cells, and (D) antibody-producing B cells were comparable. The mean  $\pm$  SEM of each group and number of samples (n) included are given below each column. Circles indicate HC donors, solid black squares represent CLIA-tested patients with ACS, and solid gray squares represent non-CLIA-tested patients with ACS. ACS = Alzheimer clinical syndrome; HC = healthy control.

increase in the absolute number of CD45<sup>+</sup> leukocytes in the CSF of the patients with ACS. Instead, the absolute number of CD45<sup>+</sup> leukocytes and each lymphocyte subset remained unchanged compared with similar-age HCs. The broad range of cell counts in the cohort as a whole likely accounted for this, and so comparisons using the frequencies of each CD45<sup>+</sup> leukocyte subset (innate, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD19<sup>+</sup> B cells) were used to evaluate changes to the immune profile.

Indeed, the innate cell frequency was increased in the patients with ACS, just as we had observed in patients with aMCI in our earlier studies. Thus, cognitive decline that affects ADLs is not a necessary driver of innate cell expansion. It is possible that innate cell subtypes are affected differently, particularly as disease progresses. However, the flow cytometry panel we used to segregate the innate cell subtypes was only used on 5 of the subjects (Figure 1C), and so a more detailed analysis of the innate cell subtypes affecting the frequency or correlation with age or neurodegenerative markers such as NfL was not performed for this study.

We anticipated that the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells would also be increased in patients with ACS because we had observed innate cell expansion, which would likely support T-cell activation and proliferation. In addition, others had found T cells in the postmortem AD brain,<sup>10,32</sup> and both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are present at the sites of amyloid deposition in AD brain tissue.<sup>10,33</sup> However, it remains unknown whether their contribution is neurodegenerative, neuroprotective, or of no consequence. Others have shown that CD4<sup>+</sup> and CD8<sup>+</sup> T-cell frequencies in the CSF are similar in patients with dementia and aged controls.<sup>31</sup> However, the cohort of patients with dementia in this earlier article excluded subjects that displayed severe cognitive decline, whereas the dementia cohort presented here focused on patients with dementia with cognitive decline severe enough to affect ADLs. Thus, we anticipated that the frequencies of CD4<sup>+</sup>

T cells in the CSF of patients with ACS would be increased in comparison to similar-age controls. Instead, patients with ACS did not display an increased frequency of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the CSF, indicating that cognitive decline severe enough to affect ADLs is not a sufficient trigger to observe increased CD4<sup>+</sup> or CD8<sup>+</sup> T-cell frequencies in the CSF. However, this degree of cognitive decline may be sufficient to retain a portion of CD4<sup>+</sup> T cells within brain tissue as the frequency of CD4<sup>+</sup> T cells in the CSF decreased with advancing age of disease.

Similar frequencies of T cells do not preclude that the underlying T-cell repertoire is also unchanged. To investigate this, we used TCR repertoire clonality and diversity index to measure CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation status in the CSF of patients with ACS and similar-age HCs. We found significantly increased clonal expansion in CD4<sup>+</sup> T cells within the CSF of patients with ACS compared with controls, which was further verified by substantially reduced diversity index. However, CSF-derived CD8<sup>+</sup> T cells displayed similar clonal representation and diversity index in patients with ACS compared with similar-age HCs. Earlier studies suggested a lack of antigen-triggered clonal expansion of T cells found in postmortem brain tissue of patients with AD,<sup>34</sup> but our observations of T cells within the CSF suggest that clonal expansion and reduced diversity index of CD4<sup>+</sup> T cells are readily identified in patients with ACS. Enhanced clonal expansion of CD8<sup>+</sup> T cells may not be evident in the CSF because activated, proliferating and clonally expanding CD8<sup>+</sup> T cells are retained within the brain tissue where amyloid and tau depositions reside. CD4<sup>+</sup> T cells may migrate more freely between the periphery and brain tissue, which would facilitate detection of clonally expanded CD4<sup>+</sup> T cells in the CSF. Of note, an earlier study demonstrating that CD8<sup>+</sup> T cells from the CSF of a mixed cohort of patients with MCI and AD demonstrate enhanced clonal expansion compared with HCs.<sup>35</sup> However, there were several methodological distinctions between the 2 studies that likely accounts for this



contrast including the use of overnight freeze-thawed CSF cells vs fresh collections, limited availability of cells in the RNAseq platforms instead of total T-cell subset library generation, and lack of diversity calculations to delineate the nature of the clonally expanded population. Further studies are needed to understand the impact of CSF-derived and brain-derived clonally expanding CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the pathology of AD, keeping in mind that their involvement in different processes of AD pathology may be affected by the TCRB genes they express.

Naive<sup>30</sup> and memory<sup>36</sup> B cells require CD4<sup>+</sup> T-cell help to become activated in response to soluble antigen and differentiate to efficient antibody-producing cells. Because CD4<sup>+</sup> T-cell frequencies were reducing with age of the patients with ACS, we expected that the subtype distribution within the B-cell population of the CSF would be similar among the cohorts or that representation of activated antibody-producing B cells would even be reduced in the patients with ACS. Indeed, the B-cell subtype distributions in the CSF were the same in both cohorts, supporting the concept that CD4<sup>+</sup> T-cell help is inadequate to elicit activation and expansion of B cells in patients with ACS. One caveat to this conclusion is that there is a sample handling covariate in the data set, which appears to affect B-cell subsets in particular. This limitation to the data set emphasizes that care must be taken when procuring data sets from multiple clinics or institutions to ensure that the time from sample acquisition to sample processing is comparable, particularly if B-cell subset frequency data are a primary focus of the investigations.

A second caveat to the conclusion that CD4<sup>+</sup> T-cell help is inadequate to elicit activation and expansion of B cells in patients with ACS is that B-cell activation may be occurring within the CNS tissue itself,<sup>37</sup> which is not measurable in a living patient cohort. In addition, it is possible that the frequency of B cells reactive to amyloid or tau is specifically upregulated. We did not test this in our CSF cohort, but others have observed an increase in anti-A $\beta$ <sub>42</sub> antibody-secreting B cells in the periphery compared with controls.<sup>38</sup> In support of our findings regarding the B-cell compartment similarities in the CSF, we also did not observe higher concentrations of total antibody or specifically anti-A $\beta$ <sub>42</sub> or anti-NfL antibodies in the CSF of patients with ACS compared with HCs. Others have demonstrated that patients with probable AD have a 31% decrease in the titer of anti-A $\beta$  antibody in the CSF compared with controls.<sup>39</sup> Increased titers of disease-specific antibodies would likely require robust CD4<sup>+</sup> T-cell help to facilitate B-cell expansion and differentiation to antibody secreting cells. Thus, the lack of increased titers of anti-A $\beta$  antibody in the CSF of this ACS patient cohort is further evidence of limited T-cell activation that may be specific to amyloid reactive T cells matriculating the CSF.

We had anticipated that advancing neurodegeneration in the ACS population would elicit a robust adaptive immune

response in patients with ACS that was discordant from similar-age HCs based on the understanding that a diagnosis of ACS includes cognitive deficits that affect ADLs. Instead, despite the robust presence of biomarkers indicative of CNS damage and expansion of innate cells by frequency in the CSF of patients with ACS compared with similar-age HCs, T-cell clonal expansion and the diversity index were suppressed and antibody titers against A $\beta$ <sub>42</sub> remained similar. These preliminary observations in a limited research cohort further support the continued exploration of potential therapeutic approaches to boost the specific adaptive immune response while limiting off-target effects.<sup>40-42</sup> In light of the finding that only approximately 20% of participants in an initial clinical vaccine trial (AN1792) showed the desired antibody response,<sup>43</sup> future immune-mediated therapeutic approaches would benefit from a better understanding of adaptive immune responses that provide protection from neurodegeneration. Furthermore, the reduced diversity index in the CSF-derived CD4<sup>+</sup> T cells of patients with ACS in comparison to similar-age HCs may provide a unique and effective biomarker for early diagnosis of ACS and provide an avenue to identify those patients with dementia who will advance to develop AD pathology. For example, 1 subject (69M) had an MMSE of 29, but a diversity index that was 7.6 $\times$  lower than the average in the similar-age HCs. This subject was diagnosed with ACS based on cognitive deficits that affected his ADLs, but this very low-diversity index places this subject within the ACS cohort independently. Further studies are warranted to understand whether the immune profile changes we presented here contribute directly to progression of AD pathology.

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## Disclosure

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