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Cerebrospinal Fluid Biomarkers Link Toxic Astrogliosis and Microglial Activation to Multiple Sclerosis Severity

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Abstract

Background: Once multiple sclerosis (MS) reaches the progressive stage, immunomodulatory treatments have limited efficacy. This suggests that processes other than activation of innate immunity may at least partially underlie disability progression during late stages of MS. Pathology identified these alternative processes as aberrant activation of astrocytes and microglia, and subsequent degeneration of oligodendrocytes and neurons. However, we mostly lack biomarkers that could measure central nervous system (CNS) cell-specific intrathecal processes in living subjects. This prevents differentiating pathogenic processes from an epiphenomenon. Therefore, we sought to develop biomarkers of CNS cell-specific processes and link them to disability progression in MS.

Methods: In a blinded manner, we measured over 1000 proteins in the cerebrospinal fluid (CSF) of 431 patients with neuroimmunological diseases and healthy volunteers using modified DNA-aptamers (SOMAscan®). We defined CNS cell type-enriched clusters using variable cluster analysis, combined with *in vitro* modeling. Differences between diagnostic categories were identified in the training cohort (n = 217) and their correlation to disability measures were assessed; results were validated in an independent validation cohort (n = 214).

Results: Astrocyte cluster 8 (MMP7, SERPINA3, GZMA and CLIC1) and microglial cluster 2 (DSG2 and TNFRSF25) were reproducibly elevated in MS and had a significant and reproducible correlation with MS severity suggesting their pathogenic role. *In vitro* studies demonstrated that proteins of astrocyte cluster 8 are noticeably released upon stimulation with proinflammatory stimuli and overlap with the phenotype of recently described neuro-toxic (A1) astrocytes.

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Conclusion: Microglial activation and toxic astroglia are associated with MS disease process and may partake in CNS tissue destruction. This hypothesis should be tested in new clinical trials.

Graphical Abstract

Keywords

multiple sclerosis; CSF biomarkers; microglial activation; toxic astroglia; CNS tissue destruction; MS severity

1) INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), in which immune cells induce demyelination and axonal damage. MS pathogenesis has been partially understood (Mohajeri et al., 2015). The disease course and prognosis are largely unpredictable (Roxburgh et al., 2005; Weideman et al., 2017a), but once MS reaches progressive stage, current disease-modifying treatments (DMTs) have limited efficacy (Hartung et al., 2002; Kappos et al., 2001; Montalban et al., 2016; Weideman et al., 2017b).

These data suggest that cells of the adaptive immunity play a pivotal role in initiating the MS disease process, with T- and B-lymphocytes, and plasma cells found abundant in the early MS lesions (Frischer et al., 2009; Kirk et al., 2003; Lassmann and van Horssen, 2011). But in later stages of MS, formation of new lesions decreases and may completely stop. Furthermore, focal lesions in progressive MS contain generally low numbers of T-cells and activated microglia/macrophages (Frischer et al., 2009; Kroksveen et al., 2015; Revesz et al., 1994). Consequently, inflammation was thought to no longer drive disability accumulation in progressive MS. In fact, primary-progressive MS was considered a non-inflammatory disease (Stys et al., 2012). However, MS patients from all three phenotypical subtypes (i.e., relapsing-remitting [RR-MS], secondary- [SP-MS], and primary-progressive MS [PP-MS]) were found to have identical concentrations of cerebrospinal fluid (CSF) biomarkers specific for B cells (sCD21) or T cells (sCD27) (Komori et al., 2015). The conclusion that patients with progressive MS have identical levels of inflammation that is redistributed outside of MS lesions visible on MRI, e.g. to meninges (Choi et al., 2012; Magliozzi et al., 2007; Reynolds et al., 2011) or normal-appearing CNS tissue (Kutzelnigg et al., 2005), is strongly supported by pathology data.

Nevertheless, the mere presence of the immune cells in CNS does not prove their pathogenicity in progressive MS, where pathologist described alternative processes such as hypoxia (Lassmann, 2016), mitochondrial dysfunction (Mahad et al., 2008) and endoplasmic reticulum stress (McMahon et al., 2012), affecting both neurons and oligodendrocytes. Most

recently, a specific type of astrocyte activation, called “neurotoxic reactive astrocytes (or “A1” astrocytes)” was observed in several neurodegenerative diseases, including MS (Liddel et al., 2017a). If these alternative processes contribute to disability progression, then they must be therapeutically-targeted if we hope to attain greater than 25% decrease of disability progression achievable by current treatments (Montalban et al., 2016) in progressive MS.

However, the inability to measure aforementioned intrathecal processes in living subjects impedes development of treatments that may inhibit them. It is likely that the presence or dominance of such potentially pathogenic processes varies among individual patients, depending on their genetic background and environmental influences. Thus, patients enrolled in clinical trials include subjects with and without targeted process, diminishing the probability of proving efficacy of process-targeted treatment in early clinical testing. We draw an analogy to cardiovascular diseases, where several processes, such as hypertension, hypercoagulable states, hyperlipidemia and diabetes have proven pathogenicity on a group level, but each is present only in a proportion of affected patients. Without biomarker-based pre-selection of suitable patients for clinical trials, the efficacy of process-specific treatments (e.g. anti-hypertensive or diabetic drugs) in cardiovascular diseases would be substantially lower. Furthermore, we could not assemble drugs into logical, highly-efficacious patient-specific combinations in daily clinical practice.

Thus, there is a need to identify and validate biomarkers reflective of CNS cell-specific processes. In a previous publication, we focused on immune cells and defined CSF biomarkers that can reliably quantify intrathecal inflammation while also providing information about its cellular composition (Komori et al., 2015). Here, we sought to take an analogous approach to identify biomarkers that are predominantly secreted by specific CNS cells, assemble related biomarkers into clusters to decrease dimensionality and to investigate whether concentrations of such CNS cell-enriched clusters differ in MS patients during disease evolution. Finally, we asked whether these clusters correlate with clinical measures of disability and MS severity.

2) MATERIALS and METHODS

2.1) Research subjects

All subjects (n = 431) were prospectively recruited under National Institutes of Health (NIH) Central CNS Institutional Review Board-approved protocol (Comprehensive Multimodal Analysis of Neuroimmunological Diseases of the Central Nervous System; [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00794352) Identifier: NCT00794352) and provided informed consent. All subjects underwent detailed neurological examination that included clinician-derived disability scales: Expanded Disability Status Scale (EDSS(Kurtzke, 1983)) and Scripps Neurological Rating Scale (SNRS(Sipe et al., 1984)). Functional disability scales: 25-foot walk (25FW) and nine-hole peg test (9HPT) were collected by non-clinical investigators at the same visits. These four disability measures were then assembled to sensitive Combinatorial Weight-Adjusted Disability Scale (CombiWISE (Kosa et al., 2016)). Additionally, clinical-grade structural MRI images of the brain collected at 1.5T or 3T MRIs under published protocols (Kosa et al., 2015) were semi-quantitatively graded by trained clinical investigators to derive

Composite MRI scale of CNS tissue destruction (COMRIS-CTD(Kosa et al., 2015)). The detailed treatment information (i.e. initiation and termination dates for all MS disease-modifying treatments (DMTs)) were assembled in research database. EDSS-based MS severity scale (Multiple Sclerosis Severity Score (MSSS)) (Roxburgh et al., 2005) and Age Related Multiple Sclerosis Severity (ARMSS) (Manouchehrinia et al., 2017) were calculated using published algorithms. Clinical, MRI and therapeutic history data were used to calculate Multiple Sclerosis Disease Severity Scale (MS-DSS) according to published algorithm (Weideman et al., 2017a) that is available on public website: <https://bielekovalab.shinyapps.io/msdss/>. This website also includes computation of CombiWISE and COMRIS-CTD scales. MS-DSS has significantly enhanced sensitivity and specificity in comparison to MSSS and ARMSS and can predict future rates of disability progression in an independent cohort.

Before analyses, subjects were randomly divided into two cohorts (Table 1): training (n = 217) and validation cohort (n = 214). The cohorts were split in a way that kept gender ratio, age spread, disease diagnosis and disease disability/severity scales comparable for both cohorts. Patients were first binned into groups based on diagnosis and whether CombiWISE scores were available. These groupings that had 10 or more patients were further split by gender. Within each of these groupings, patients were further binned as per whether they were above or below the median measurements of age and CombiWISE (when present). Approximately 50% of patients in each of these final groupings were randomly selected as the training cohort, with the remaining 50% retained as the validation cohort.

2.2) CSF Collection and Processing

CSF were collected by lumbar puncture and processed as per standard operating procedure as described (Barbour et al., 2017). Briefly, research CSF aliquots were placed on ice immediately after collection, assigned an alpha-numeric code, and centrifuged at 335g for 10 minutes at 4°C, at Neuroimmunological Diseases Section (NDS) laboratory within 15 minutes of collection. CSF supernatants were aliquoted and stored at -80°C until further use.

2.3) PBMCs Isolation, Stimulation, and Supernatants Collection

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood using Ficoll separation method (Noble and Cutts, 1967). Briefly, lymphocyte separation media (LSM; Lonza, Walkersville, MD) was underlaid to pre-diluted blood (1:3 dilution with phosphate-buffered saline [PBS]; KD Medical Inc., Columbia, MD) and then centrifuged at 335g for 30 min at room temperature. PBMC were collected, washed twice with ice cold PBS, counted by hemocytometer and cultured in serum-free X-VIVO media (Lonza) at 1×10^6 cells/ml. PBMCs were stimulated with lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich, St. Louis, MO) and CD3/CD28 microbeads (Invitrogen, Carlsbad, CA) at 1:1 cells to beads ratio. After 24h supernatants from stimulated and unstimulated PBMCs were collected by centrifugation at 335g. Stimulation of PBMCs was validated by analyzing pro-inflammatory cytokines in supernatants using Human Pro-Inflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery, Rockville, MD; Supplementary Figure 1). Supernatants were aliquoted and stored at -80°C until further use.

2.4) CNS Cell Cultures and Treatments

Primary human CNS cells or cell lines: human neurons (ScienCell, Carlsbad, CA), human astrocytes (ScienCell), human brain endothelial cell line (HCMEC/D3; provided by Pierre-Olivier Couraud (Weksler et al., 2005)) and human microglia cell line (CHME5; provided by Nazira El-Hage, Florida International University, Miami, FL) were plated (10^5 cells/ml) according to manufacturer's/provider's instructions. These cells were treated with respective culture medium (Control) or inflammatory stimuli (supernatant from LPS- and CD3/CD28 beads-stimulated PBMCs, 50% volume/volume) or mitochondrial-stress stimulus (rotenone, 100 nM). Cell-culture supernatants were collected at 0 and 24 hours after respective treatments and stored at -80°C until further use. Oligodendrocytes were differentiated from human pluripotent stem cells as described (Douvaras and Fossati, 2015); differentiated cultures had 70% of O4+oligodendrocytes.

2.5) SOMAscan

Cell-culture and CSF supernatants were analyzed using Slow Off-rate Modified DNA Aptamers assay (SOMAscan; SomaLogic Inc., Boulder, CO), a multiplex proteomic scan utilizing modified DNA-aptamers (SOMAmers). Cell-free CSF supernatants were analyzed using 1.1K SOMAscan platform (determines expression of 1128 different proteins, available between June 2012 and October 2016), and Cell-culture supernatants were analyzed using 1.3K SOMAscan platform (determines expression of 1310 different proteins, available after October 2016). Binding of SOMAmers to specific proteins enables transforming individual protein concentration into DNA concentrations quantified by hybridization (Barbour et al., 2017; Gold et al., 2010; Gold et al., 2012; Kraemer et al., 2011; Rohloff et al., 2014).

2.6) Identification of CNS Cell Type-Enriched Biomarkers and Biomarker Clusters

Supernatants collected from primary human CNS cells and cell lines (see Methods) at 0 and 24 hours after culturing were analyzed using SOMAscan. If under control culture conditions absolute release of any protein by a specific cell-type (i.e., RFU at 24h – RFU at 0h) was 5-fold higher than any other cell types then that protein was considered specific cell type-enriched. However, human embryonic stem cells-derived oligodendrocyte cultures had only 70% of O4+oligodendrocytes, with astrocytes and neurons representing the remaining cells in these cultures. To circumvent problem of mixed cultures of oligodendrocytes, we first identified cell type-enriched biomarkers for all CNS cell types except oligodendrocytes. This analysis identified neuron-enriched, astrocyte-enriched, microglia-enriched and endothelial cells-enriched biomarkers. To identify oligodendrocyte-enriched biomarkers, we repeated the same analysis including oligodendrocytes, but considered biomarker to be oligodendrocyte-enriched only when the biomarker was not previously identified as enriched for some other CNS cell type. We recognize that the drawback of this sequential process is that some biomarkers identified as enriched for non-oligodendrocyte cell types may in fact be also produced by some oligodendrocytes. However, due to low numbers of embryonic stem cells-derived oligodendrocyte we could not subject these cultures to second isolation procedure.

CNS cell type-enriched biomarker clusters were obtained by performing variable cluster analysis (see Statistical Analyses) for each cell type-enriched proteins on patients' CSF

SOMAscan data. We reasoned that proteins that are secreted together by identical CNS cell types under identical physiological or pathological conditions will have strong correlations in the CSF samples collected from diverse group of individuals.

2.7) Statistical Analyses

2.7.1) Data transformation and outlier Winsorization (the two cohorts combined):

For each cell type-enriched biomarker, Box-Cox transformation was applied, and kurtosis was examined. If the transformed variable had an absolute kurtosis greater than 2, histogram and interquartile range (IQR) were used to identify extreme outliers based on the 3*IQR rule. The outlier(s) was replaced by either the minimum or maximum after said outlier(s) were excluded.

2.7.2) Variable cluster analysis (the two cohorts combined):

For each cell type-enriched biomarkers variable cluster analysis was performed on patients' CSF SOMAscan data (after adjusting or excluding outliers), using the SAS procedure VARCLUS (SAS/STAT® 13.2 User's Guide; SAS Institute Inc., Cary, NC). The maximum second eigenvalue (MAXEIGEN) of 0.7 was used as the criterion for cluster splitting. For each cluster, procedure SCORE was used to calculate the (first principal) component score, which is the weighted average of the variables that explains as much variance in the cluster as possible.

2.7.3) Correlation analysis (separate test for training and validation cohort):

Semi-partial correlation coefficients (Stevens, 2002; Whittaker, 1990) were used to examine for associations between cluster scores and clinical outcomes. These allow for testing of associations between cluster scores and clinical outcomes, after accounting for the association of a confounding variable (i.e. age) with cluster scores. For each cluster, semi-partial Spearman correlation coefficients were calculated using the ppcor R package (Kim, 2015) to evaluate the association between cluster scores (after accounting for potential associations with age) and the clinical outcomes: CombiWISE, EDSS, COMRIS-CTD, MS-DSS, ARMSS and MSSS. In addition, the individual proteins making up two clinically relevant clusters (Astrocyte cluster 8 and Microglial cluster 2) were examined. Only clusters with a statistically significant ($p < 0.01$) correlation coefficients for at least one clinical outcome in the training cohort were tested in the validation cohort. The correlation p-values in the validation cohort were adjusted in R (2018) using the Benjamini-Hochberg False Discover Rate method (Benjamini and Hochberg, 1995).

2.7.4) Examining for differences in disease subgroups (separate test for training and validation cohort):

For each cluster, a linear regression model with diagnostic subgroup and age as independent variables was constructed. Analysis of Variance (ANOVA) was performed to examine for differences in component scores among diagnostic subgroups after accounting for differences in age. When statistically significant ($p\text{-value} < 0.05$) cluster differences were found, pairwise multiple comparisons using Tukey's method were performed. When graphically displayed, cluster scores are adjusted for age by subtracting the respective estimated age effects from these linear regression models.

3) RESULTS

3.1) Identification of CNS Cell Type-Enriched Biomarkers

CNS cell type-enriched biomarkers were identified using cell culture supernatants' SOMAscan data (see Methods for more details). To identify absolute release of any biomarker by a specific CNS cell type, the absolute difference in RFU values between 24h and 0h supernatants was considered to reflect secretion (or consumption) of the measured protein by studied cell type. We arbitrarily defined measured protein as cell type-enriched, if release of a protein by a specific cell type under control culture conditions was 5-fold higher than any other tested cell type. The biomarkers that fulfilled our criteria of >5-fold enrichment by a specific CNS cell type are represented in Supplementary Table 1: 40 neuron-, 73 astrocyte-, 81 oligodendrocyte-, 18 microglia-, and 38 endothelial cell type-enriched biomarkers.

3.2) Identification of CNS Cell Type-Enriched Biomarker Clusters

CNS cell type-enriched biomarker clusters were obtained by performing variable cluster analysis for each cell type-enriched biomarkers (shown in Supplementary Table 1) in patients' CSF SOMAscan data. Variable cluster analysis resulted in cell type-enriched biomarker clusters, represented in Table 2: 7 neuron-, 20 astrocyte-, 19 oligodendrocyte-, 7 microglia-, and 11 endothelial cell type-enriched biomarker clusters.

3.3) Analysis of Cluster Scores Across Disease Diagnosis Subgroups

Cluster scores for each biomarker cluster were calculated using the first principal component. After accounting for associations of age with cluster scores (see Statistical Analyses), cluster scores were compared among disease diagnosis subgroups (healthy donors [HD], clinically isolated syndrome [CIS], non-inflammatory neurological disorder [NIND], RR-MS, SP-MS, and PP-MS patients) in the training cohort (n = 217). Statistically-significant differences were then assessed in the independent-validation cohort (n= 214). Only those statistically-significant results that were reproduced in both cohorts are represented and will be discussed here.

All MS subgroups had statistically significantly elevated astrocyte cluster 8 (MMP7, SERPINA3, GZMA and CLIC1; Figure 1, first row) in comparison to HD and NIND subgroups; while astrocyte cluster 11 (SPARC; Figure 1, second row) showed a decrease in SP-MS subgroup compared to NIND. Microglia cluster 2 (DSG2 and TNFRSF25; Figure 1, third row) behaved similarly to astrocyte cluster 8, that it was increased in all 3 MS subtypes in comparison to HD and NIND. Finally, oligodendrocyte cluster 14 (CXCL13; Figure 1, fourth row) demonstrated elevation in RR-MS compared to HD and NIND, while PP-MS was elevated compared to only NIND subgroup.

3.4) Correlation Analysis Between Cluster Scores and Disability and CNS Tissue Destruction

To determine if CNS cell-specific biomarkers increase with MS progression, we correlated individual subject cluster scores with two MS disability measures: broadly used EDSS (Kurtzke, 1983) and more sensitive CombiWISE (Kosa et al., 2016). Additionally, we also

correlated cluster scores with the amount of CNS tissue destruction measured by COMRIS-CTD (Kosa et al., 2015) using semi-partial Spearman correlations that is generally not driven by outlier values and allow for adjustment for age associations with cluster scores. Only clusters which had reproducible statistically significant correlations ($p < 0.01$) with at least one of the disability measures are represented (Table 3); Correlation p values in the validation cohort were adjusted to account for multiple comparisons (Benjamini and Hochberg, 1995). Only astrocyte cluster 8 and microglia cluster 2 showed reproducible statistically significant correlations with both disability scales and COMRIS-CTD (Table 3 and Figure 2).

3.5) Correlation Analysis Between Cluster Scores and MS severity

Correlation with disability and CNS tissue destruction identifies processes that develop as MS evolves, but not necessarily processes that drive CNS tissue destruction and disability. Instead, correlation with MS severity, which measures how fast individual patients progress is better suited for identification of potentially pathogenic processes, especially if such MS severity scale can predict future rates of MS progression, as MS-DSS scales can (Weideman et al., 2017a). Therefore, we investigated correlations between CNS cell-enriched biomarker clusters and MS-DSS. We also included assessment of correlations with less sensitive, but more broadly used MS severity scales based on EDSS: ARMSS and MSSS.

Astrocyte cluster 8 and microglia cluster 2, which were elevated during all stages of MS had statistically significant ($p < 0.01$) correlations with MS-DSS and ARMSS (Table 3 and Figure 2). No cluster correlated with MSSS.

3.6) Analysis of Individual Proteins of Clinically Important Clusters

For astrocyte cluster 8 (MMP7, SERPINA3, GZMA and CLIC1) and microglia cluster 2 (DSG2 and TNFRSF25), individual protein's SOMAScan RFU values were standardized and accounted for associations with age. Scatter plots for individual proteins were prepared; standardized RFU values (adjusted for age) versus MS disability (EDSS and CombiWISE), CNS tissue destruction (COMRIS-CTD) and disease severity (MS-DSS) measures (Supplementary Figures 2 and 3). Individual proteins of a cluster demonstrated analogous behavior.

3.7) *In Vitro* Secretion of Cluster Proteins by CNS Cells Under Different Culture Conditions

To aid mechanistic interpretation of our results we asked under which conditions the biomarkers constituting most relevant CNS cell-enriched clusters are produced. We assessed 3 different cell culture conditions: native (control or unstimulated) and two different stressors, pro-inflammatory environment modeled by adding 50% volume/volume supernatants from polyclonally-activated PBMCs, and neuro-degenerative environment modelled by adding mitochondrial toxin rotenone (100 nM). For embryonic stem-cell-derived oligodendrocytes, only native protein secretion was tested due to limited numbers of O4+ cells.

Secretion of individual biomarker under different culture conditions (native, pro-inflammatory stimuli, and mitochondrial stressor) was measured by computing standardized RFU differences between start and end of culture conditions (24h – 0h); then the mean of biomarkers for each cluster under respective culture conditions was computed. Results are presented in Figure 3; only data for two clinically important clusters (astrocyte cluster 8 and microglia cluster 2) is depicted. Secretion of astrocyte cluster 8 was robustly elevated after pro-inflammatory stimuli compared to native secretion. In contrast, secretion of proteins constituting microglial cluster 2 was inhibited by inflammation but increased under mitochondrial stressor.

4) DISCUSSION

We consider CSF the body fluid of choice for discovery and development of CNS biomarkers. CNS-derived proteins represent approximately 20% of CSF proteome, and are typically found in higher concentrations in CSF compared to plasma (Reiber, 2003; Thompson, 1995). As CSF is eventually drained to plasma, CNS-derived proteins can be measured in the plasma using highly sensitive assays (Kuhle et al., 2016); however, due to differences in CSF clearance (which is influenced by processes such as cardiac output, sleep, physical activity, and aging) and systemic metabolism, plasma levels explain only 38–60% of the CSF variance (Bielekova and Pranzatelli, 2017). This 40–62% “noise” may require significantly larger cohorts for biomarker discovery and validation. On the other hand, once the simple or complex CNS biomarkers are defined and validated from CSF studies, they may be applicable to large cohorts with matched serum/plasma biological samples (Kuhle et al., 2016). Analyzing clusters of biomarkers is advantageous over analyzing individual biomarkers because it reduces chances of errors in biomarkers identification and provides information regarding probable mechanistic pathways driving disease progression.

In the current study, we have identified CNS cell-enriched protein clusters using *in vitro* models combined with *ex vivo* CSF biomarker measurements. The main limitation of this study is the non-physiological reductionist research approach exemplified by simple *in vitro* culture system(s) of individual CNS cell types. Unfortunately, the environmental influence on CNS cell expression profile is so dynamic (Gosselin et al., 2017), that it is currently impossible to evaluate *in-vivo* secretome of individual CNS cells. Thus, while using reductionist approach is inevitable, one must be mindful of this limitation and search for creative solutions how to mitigate it. In the future, as more human CNS cell-specific *ex-vivo* expression data become available (Gosselin et al., 2017), our results may be further expanded or refined using specific cell-enriched expression profiles, to search for secreted proteins. Additionally, we recognize that it is currently not possible to differentiate secretome of human microglia from other cells of myeloid lineage (e.g., blood-derived macrophages and dendritic cells) that were not studied here. Therefore, we will expand our conclusions to encompass myeloid lineage in general. Finally, while this study focused on CNS cells, we recognize that some of the identified proteins are also produced by lymphocytes (e.g., CXCL13 and GZMA) and their production by CNS cells was either unknown or under-appreciated.

We also acknowledge overlap of patients and, consequently, some results between our previously published SOMAscan-based study (Barbour et al., 2017) and current study. In the previous study we used statistical learning to define “molecular diagnostic tests of MS”, represented by CSF biomarker-based model that can differentiate MS patients from subjects with similar phenotypical features (i.e., clinical symptoms or MS-like CNS imaging) or similar pathophysiology (e.g., other inflammatory neurological diseases [OIND]). In analogous manner, we also defined molecular diagnostic test that can differentiate RR-MS from progressive-MS (Barbour et al., 2017). The methodology employed used diagnostic groups (in the training cohort) to search for biomarkers that differ among diagnostic categories irrespective of their cell specificity. Once the molecular tests were defined and validated in the independent cohort(s), we used *in-vitro* cultures of human immune and CNS cells to interpret the processes/pathways represented by the validated models. Microglial activation and toxic astrogliosis were among processes that help to differentiate RR-MS from progressive-MS, even though statistical learning selected different specific biomarkers reflective of these two intrathecal processes in our previous study (Barbour et al., 2017).

The current project uses opposite methodology of first identifying cell-enriched clusters and then asking whether they differ among diagnostic subgroups. We consider identification of partially overlapping results related to microglial clusters and astroglial clusters highly reassuring.

The most clinically important finding of the current study is the identification of astroglial cluster 8 and microglia cluster 2 as significantly elevated in both progressive- and RRMS patients and reproducibly correlating with measures of MS severity (MS-DSS and ARMSS). These two observations suggest that the aberrant activation of these two glial cell types (and/or other cells of myeloid lineage as described above) represented by these clusters not only happens early in MS disease process but may also contribute to CNS tissue destruction and/or poor clinical recovery. Proteins from both clusters are related to activation of adaptive immune responses. Specifically, TNFRSF25 plays a role in lymphocyte homeostasis by activating NFkB and mediating lymphocyte apoptosis and necroptosis (Bittner et al., 2017). Intriguingly, this receptor was shown to differentially regulate apoptosis of cytotoxic T cells in patients with infectious mononucleosis (IM) than in healthy subjects (Filatova et al., 2016). IM is one of the most potent MS susceptibility factors identified thus far. Thus, it seems plausible that this microglial cluster participates in regulation of cytotoxic CD8+ T cells.

Astrocyte cluster 8 contains metalloproteinase (MMP7), in addition to cytotoxic immune molecule granzyme A (GZMA), chloride intracellular channel 1 (CLIC1) and serpin family A member 3 (SERPINA3), previously linked to neurotoxic reactive astrocyte phenotype induced by pro-inflammatory stimuli (Liddel et al., 2017a; Padmanabhan et al., 2006; Ritchie et al., 2004). Liddel et al. have shown that proinflammatory cytokines, mainly interleukin 1 α (IL1 α), tumor necrosis factor α (TNF α) and complement component 1, subcomponent q (C1q), released by activated microglia causes induction of neuro-toxic, so called A1 astrocytes; they have identified several A1-astrocyte specific markers. By in situ hybridization and immunohistochemistry of post-mortem brain tissue from MS patients they have shown the presence of A1 reactive astrocytes in demyelinating lesions of MS

(Liddel et al., 2017b). We tried to replicate *in vitro* model of reactive astrocytes by treating primary human astrocytes with supernatants from polyclonally-stimulated PBMCs (unpublished studies). Our model has been validated by checking elevated presence of reactive astrocyte-specific markers, serpin family G member 1 (SERPING1) and complement C3, as described by Liddel et al. We observed that serpin family A member 3 (SERPINA3; α -1-antichymotrypsin) is a strong marker of A1 reactive astrocytes (almost 62-fold increase in SERPINA3 SOMAscan RFU was observed); and we validated this finding from the microarray data generated by Zamanian et al. deposited in public database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35338>) (Zamanian et al., 2012). Thus, proteins of astrocyte cluster 8 are highly specific to A1 astrocytes (even though secreted in very low concentration under native condition) and significantly induced under pro-inflammatory stimulation. By demonstrating (and validating) correlation between astrocyte cluster 8 and MS severity, our study expands observation by Liddel et al. postulating important role of astrocyte-specific processes in MS progression. Accordingly, there is a pressing need for further studies regarding detailed role of toxic astrocytes in MS progression to identify therapeutic targets which may block their neurotoxic potential. Only such interventional clinical trials may determine unequivocally whether neuro-toxic astrocytes play pathogenic role in MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MS	multiple sclerosis
CNS	central nervous system
CSF	cerebrospinal fluid
SOMAscan	Slow Off-rate Modified DNA Aptamers assay
MMP7	metalloproteinase 7
SERPINA3	serpin family A member 3
GZMA	granzyme A
CLIC1	chloride intracellular channel 1
DSG2	desmoglein 2
TNFRSF25	tumor necrosis factor receptor superfamily member 25
DMTs	disease-modifying treatments

RR-MS	relapsing-remitting MS
SP-MS	secondary-progressive MS
PP-MS	primary-progressive MS
sCD21	soluble cluster of differentiation 21
sCD27	soluble cluster of differentiation 27
EDSS	Expanded Disability Status Scale
SNRS	Scripps Neurological Rating Scale
25FW	25-foot walk
9HPT	nine-hole peg test
CombiWISE	Combinatorial Weight-Adjusted Disability Scale
COMRIS-CTD	Composite MRI scale of CNS tissue destruction
MS-DSS	Multiple Sclerosis Disease Severity Scale
MSSS	Multiple Sclerosis Severity Score
ARMSS	Age Related Multiple Sclerosis Severity
HD	healthy donors
NIND	non-inflammatory neurological disorder
CIS	clinically isolated syndrome
PBMCs	Peripheral Blood Mononuclear Cells
LPS	lipopolysaccharide
ANOVA	Analysis of Variance
SPARC	secreted protein acidic and rich in cysteine
CXCL13	chemokine (C-X-C motif) ligand 13
OIND	other inflammatory neurological diseases
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
IM	infectious mononucleosis
IL1α	interleukin 1 α
TNFα	tumor necrosis factor α
C1q	complement component 1, subcomponent q

SERPING1

serpin family G member 1

C3

complement component 3

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HIGHLIGHTS

- Microglial/myeloid cell activation markers are present during all stages of MS.
- Toxic astrocyte specific markers increase with MS duration.
- Aberrant activation of glial cells contributes to CNS tissue destruction and enhance MS severity.

Figure 1:

Cluster scores adjusted based on age were compared among diagnosis subgroups. Only clusters which have reproducible statistically significant differences between disease diagnosis subgroups are shown. The y-axis as selected to have better visual assessment of majority of patients' cluster scores. Thus, few of individual patients' points may be missing in these graphs. Data were analyzed using ANOVA, with multiple comparisons using Tukey's method; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ and **** $p < 0.0001$.

Figure 2: Scatter plots for Astrocyte Cluster 8 and Microglia Cluster 2; cluster score (adjusted for age) versus MS disability (CombiWISE and EDSS), CNS tissue destruction (COMRIS-CTD) and disease severity (MS-DSS) measures. Both clusters exhibited reproducible statistically significant (p value < 0.01) correlations with clinical measures.

Figure 3:

CNS cells were cultured either without any treatment (native) or treated with pro-inflammatory stimuli (stimulated PBMCs supernatant, 50% volume/volume) or mitochondrial stressor (Rotenone, 100 nM). Secretion of individual biomarker under these culture conditions was measured by computing standardized RFU differences between start and end of culture conditions (24h – 0h), and then the mean of each cluster was computed. Only data for clinically important clusters is depicted.

Table 1:

All subjects' (n = 431) were divided into two cohorts: Training Cohort (n = 217) and Validation Cohort (n = 214). Gender-ratio, age-, disease diagnostics groups-, disease associated disability scores-range were comparable in the two cohorts.

Diagnosis	Training Cohort(N=217)							Validation Cohort (N=214)						
	HD	NIND	CIS	RRMS	SPMS	PPMS	HD	NIND	CIS	RRMS	SPMS	PPMS	HD	PPMS
N (female/male)	9/10	20/8	8/2	41/25	21/17	24/32	11/12	23/6	7/3	38/23	21/13	28/29		
Average	39.3	43.9	39.7	39.6	51.2	53.6	35.8	49.2	41.0	39.6	53.3	54.3		
Age (SD)	15.4	12.8	14.4	10.9	11.6	9.5	10.5	11.2	11.8	10.5	8.8	9.0		
range	19.4–70.3	18.2–65.8	20.8–60.3	18.0–68.7	22.0–65.4	25.3–65.8	19.7–57.4	21.0–70.6	24.4–57.7	18.3–67.9	36.3–69.6	29.8–74.7		
Average	0.4	2.7	1.4	2.0	5.6	5.3	0.4	2.0	2.0	1.5	5.8	5.0		
EDSS (SD)	0.5	2.3	1.1	1.5	1.7	1.8	0.5	2.0	0.9	1.0	1.3	1.7		
range	0.0–1.5	0.0–6.5	0.0–3.0	0.0–6.5	0.0–8.0	1.5–8.5	0.0–1.0	0.0–6.5	1.0–4.0	0.0–6.0	2.5–7.5	1.5–7.0		
Average	5.4	19.9	11.9	15.2	46.7	41.0	4.9	16.1	15.4	12.5	46.5	39.9		
CombiWISE (SD)	3.6	16.4	7.0	10.3	13.1	15.4	2.9	14.0	5.7	6.7	13.2	13.8		
range	0.4–12.2	1.2–55.6	2.6–22.2	2.2–50.4	17.8–80.6	9.4–84.5	0.7–9.5	2.3–53.2	7.5–24.5	2.4–35.5	20.3–70.8	13.8–66.2		
Average	1.0	5.9	2.1	8.2	16.7	13.8	1.5	6.9	4.3	8.5	15.7	14.0		
COMRIS (SD)	1.7	6.9	1.8	5.5	5.5	6.3	2.7	4.8	3.2	5.4	5.2	6.1		
range	0.0–5.6	0.0–25.5	0.0–5.7	0.0–22.0	6.6–25.7	3.7–31.5	0.0–9.2	0.0–21.2	0.0–9.2	0.0–23.5	8.0–25.6	0.0–26.3		
Average	1.2	1.5	0.9	1.3	2.3	1.9	1.2	1.4	1.0	1.2	2.3	2.1		
MS-DSS (SD)	0.1	0.9	0.2	0.6	1.2	1.1	0.1	0.8	0.3	0.5	1.0	0.9		
range	0.8–1.4	0.5–3.8	0.6–1.3	0.4–3.5	0.3–5.3	0.3–4.8	0.8–1.5	0.5–4.4	0.6–1.6	0.5–2.5	0.7–4.5	0.8–4.4		

Table 2:

CNS cell type-enriched biomarker clusters were obtained by performing variable cluster analysis for each cell type-enriched proteins on patients' CSF SOMAscan data. Table represents list of CNS cell type-enriched biomarker clusters.

Neuron	Cluster 1	IFNG CMPK1	MASPI STK17B	PRSS27	OCIAD1	DKKL1	PPP3R1	KLK14	ING1	DAPK2	SH2D1A
	Cluster 2	FGFR1 DLL1	CYCS	PROC	CNTN1	BCAN	CHL1	ITGAV	RTN4R	NOTCH3	IL27RA
	Cluster 3	LTBR	ARG1	PTPN1	AURKA	KLK6	ATP5B				
	Cluster 4	LTF	ALPL								
	Cluster 5	CAPN1	WISP1	CAMK1	CD84	INS					
	Cluster 6	C1QBP									
	Cluster 7	CDH3	EPB41	HPGD							
Astrocyte	Cluster 1	COL18A1	CXCL16	MMP3	ANGPT1	MRC2	LRIG3	PLAU	BDC	CLU	MAPK11
	Cluster 2	BMP1	TNFRSF4	MMP10	FGF23	ADAM12	ADAMTS1	MMP13			
	Cluster 3	INHBA	THBS1	KDR	MMP2	GSN	OMD				
	Cluster 4	DYNLRB1	TPM2	ZAP70							
	Cluster 5	DCN	FRZB	PTH1H	CLEC11A	ESM1	TNC	CLEC11A	DBNL		
	Cluster 6	ALB	C4								
	Cluster 7	TNFSF13R	IL6								
	Cluster 8	MMP7	SERPINA3	GZMA	CLIC1						
	Cluster 9	ANGPT2	GDF11	ADAM9	PRSS22	NOTCH1	UNC5C	APOE	SPOCK1		
	Cluster 10	NOV	CCL11								
	Cluster 11	SPARC									
	Cluster 12	CAMK2D	FGF1								
	Cluster 13	NOG									
	Cluster 14	TGFB2									
	Cluster 15	F10	SERPINA5	INHBA	TNFAIP6						
	Cluster 16	PRKCD	BPI	GREM1	IL11						
	Cluster 17	PGD									
	Cluster 18	LAYN	MMP8	IL17RA	CA4						
	Cluster 19	CCL2									

Cluster 20		IGFBP5		RARRES2							
Oligodendrocyte	Cluster 1	EFNB3 NTRK2	APOE NRCAM	JAM2 RCB02	L5AMP ICAM5	LRP8 TNFRSF21	HFE2	RGMA	L1CAM	NCAM1	SUTRK5
	Cluster 2	HGF	TNFSF15	CD209	FCGR3B	LY86	CXCL10	CHST6	TFF3	GPNMB	
	Cluster 3	EHMT2	PYY	CKB	FYN						
	Cluster 4	FTH1_FTL	HBA1_HBB								
	Cluster 5	IGFBP3	PROS1	MATN2	KLKB1	TF	DPT	SET	SERPINA3_	Complex	
	Cluster 6	GDF9	COLEC12	PDGFC	MFD1	LRRTM3	FABP5				
	Cluster 7	CTSB	SERP1	NLGN4X	SEMA3E	SPOCK2					
	Cluster 8	CDH1	PLA2G2A	GPC3							
	Cluster 9	ICAM1	APOD	CCL7	TYK2						
	Cluster 10	C4B	C1R	A2M							
Cluster 11	HP										
Cluster 12	ERBB3 GPC5	LAMA1_LA CPE	THBS4	PTPN11	AGT	DKK3	CTSH	IGF1R	SPON1	SPARCL1	
Cluster 13	CX3CL1										
Cluster 14	CXCL13										
Cluster 15	APCS										
Cluster 16	F5										
Cluster 17	SHBG										
Cluster 18	PAFAH1B2 AK1										
Cluster 19	IDUA										
Microglia	Cluster 1	VEGFA	WFIKK2	CHRD1	GPT	IL12RB2	FABP3	GOT1	SEMA6A		
	Cluster 2	DSG2	TNFRSF25								
	Cluster 3	SEMA3A	VEGFA	WNT7A							
	Cluster 4	CTSD									
Cluster 5	IGFBP1										
Cluster 6	PGK1	PPID	IMPDH1								
Cluster 7	MIA										
Endothelial Cells	Cluster 1	TIE1	TGFBR3	VWF	TNFRSF1B	CST6	IL6R	TPI1	CD274	MICB	SCARF1
	Cluster 2	KPNA2	TOP1	C3	EIF4A3	SSRP1					

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Cluster 3	SERPINA7	PGF	CIS	CFB	F9	F2	
Cluster 4	CCL5	IL2RG	XRCC6	CDH6	CDH12	H2AFZ	CSNK2A1
Cluster 5	DKK4	DKK1	MAPK14				
Cluster 6	CST2						
Cluster 7	GNS						
Cluster 8	TK3						
Cluster 9	NAGK						
Cluster 10	MICA						
Cluster 11	C3	MB					

Table 3:

Semi-partial Spearman correlation coefficients between cluster scores (after accounting for association with age) and MS disability measures (EDSS and CombiWISE), CNS tissue destruction (COMRIS-CTD) and disease severity (MS-DSS, ARMSS and MSSS). P values for the correlation coefficients in the validation cohort were adjusted using Benjamini Hochberg False Discovery Rate method.

		Astrocyte Cluster 8		Microglia Cluster 2	
		Training Cohort	Validation Cohort	Training Cohort	Validation Cohort
CombiWISE	Spearman r	0.30	0.26	0.24	0.27
	p value	1.20E-05	1.71E-04	4.00E-04	1.71E-04
EDSS	Spearman r	0.29	0.26	0.23	0.24
	p value	2.24E-05	3.13 E-04	0.001	4.36E-04
COMRIS-CTD	Spearman r	0.38	0.36	0.36	0.36
	p value	8.98E-09	1.08E-07	8.51E-08	1.08E-07
MS-DSS	Spearman r	0.21	0.19	0.19	0.28
	p value	0.003	0.008	0.007	1.34E-04
ARMSS	Spearman r	0.27	0.25	0.21	0.24
	p value	6.91E-05	0.001	0.002	5.79E-04
MSSS	Spearman r	0.19	0.11	0.12	0.12
	p value	0.010	0.157	0.097	0.16