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Comprehensive Immunophenotyping of Cerebrospinal Fluid Cells in Patients with Neuroimmunological Diseases

Sungpil Han,*†‡,1 Yen Chih Lin,*†‡,1 Tianxia Wu,‡ Alan D. Salgado,* Ina Mexhitaj,* Simone C. Wuest,* Elena Romm,*, Joan Ohayon,† Raphaella Goldbach-Mansky,† Adeline Vanderver,* Adriana Marques,† Camilo Toro,† Peter Williamson,** Irene Cortese,* and Bibiana Bielekova*††

We performed unbiased, comprehensive immunophenotyping of cerebrospinal fluid (CSF) and blood leukocytes in 221 subjects referred for the diagnostic work-up of neuroimmunological disorders to obtain insight about disease-specific phenotypes of intrathecal immune responses. Quantification of 14 different immune cell subsets, coupled with the assessment of their activation status, revealed physiological differences between intrathecal and systemic immunity, irrespective of final diagnosis. Our data are consistent with a model where the CNS shapes intrathecal immune responses to provide effective protection against persistent viral infections, especially by memory T cells, plasmacytoid dendritic cells, and CD56bright NK cells. Our data also argue that CSF immune cells do not simply reflect cells recruited from the periphery. Instead, they represent a mixture of cells that are recruited from the blood, have been activated intrathecally and leave the CNS after performing effector functions. Diagnosis-specific differences provide mechanistic insight into the disease process in the defined subtypes of multiple sclerosis (MS), neonatal onset multisystem inflammatory disease, and Aicardi–Goutiéres syndrome. This analysis also determined that secondary-progressive MS patients are immunologically closer to relapsing–remitting patients as compared with patients with primary-progressive MS. Because CSF immunophenotyping captures the biology of the intrathecal inflammatory processes, it has the potential to guide optimal selection of immunomodulatory therapies in individual patients and monitor their efficacy. Our study adds to the increasing number of publications that demonstrate poor correlation between systemic and intrathecal inflammatory biomarkers in patients with neuroimmunological diseases and stresses the importance of studying immune responses directly in the intrathecal compartment. *The Journal of Immunology, 2014, 192: 000–000.

Neuroimmunological disorders of the CNS are an expanding group of diseases affecting all age groups. The most extensively studied disorder is multiple sclerosis (MS). Although the pathogenic role of inflammation is no longer disputed in MS (at least in the most frequent form called relapsing–remitting MS [RR-MS]), neither the antigenic target nor the pathogenic cell populations have been defined. Heterogeneity in pathological MS specimens suggests that diverse mechanisms may be driving the development of CNS lesions in different patients (1). Furthermore, decreased efficacy of immunomodulatory disease-modifying therapies (DMTs) in the later stages of MS raises the possibility that neurodegenerative mechanisms drive disability in primary-progressive (PP-MS) and secondary-progressive MS (SP-MS) (2, 3). This hypothesis is supported by a paucity of contrast-enhancing lesions (CEL) in neuroimaging studies (4) and presence of diffuse CNS tissue injury (5). However, pathological studies also demonstrate continuous presence of inflammatory cells in progressive MS, especially in the meninges (6–8). It is likely that patients with progressive disease who retain a prominent inflammation experience partial benefit from DMTs (9).

Thus, understanding the heterogeneity of intrathecal immune responses and their relationship to disease phenotype is a prerequisite for rational selection of optimal therapy in patients with MS, as well as other neuroimmunological diseases for which pathogenic mechanisms are even less understood. Moreover, when targeted therapies are applied, it often remains unclear whether residual

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immunophenotyping cerebrospinal fluid (CSF) cells by flow cytometry is a powerful tool that provided important insights into the MS. It revealed that RR-MS patients have intrathecal expansion of B cells and plasmablasts, in comparison with non-inflammatory neurologic diseases (NIND) controls (11). Others demonstrated a link between CSF B cells and intrathecal production of CXCL13 (12, 13) and between CSF B cells and CELs (14, 15). Additional cellular abnormalities involved decreased proportion of monocytes (11, 13), an elevated number of dendritic cells (DCs) (16), especially plasmacytoid DCs (17), and an increase in activated T cells (18–20). The latter abnormalities have not been confirmed in independent cohorts. As CSF evaluation of patients with progressive MS is rarely done in contemporary clinical practice, it is unclear if afore-mentioned findings apply also to these patients. The abnormalities summarized above are also found in other inflammatory neurologic disease (OIND) patients and therefore raise the question whether immunophenotyping can reveal differences in the phenotype of inflammatory responses and thus provide insight into disease pathogenesis.

We performed comprehensive immunophenotyping in a sizable cohort of prospectively acquired pediatric and adult patients (n = 221) who presented to the National Institutes of Health for diagnostic work-up of a neuroimmunological diseases. Our goals were to 1) develop a standardized 12-color, single staining flow cytometry immunophenotyping panel that can be applied to CSF specimens containing as few as 5000 CSF cells; 2) define differential immunophenotyping panel that can be applied to CSF studies. When indicated by history or serological studies, CSF work-up was performed after general lysis of erythrocytes. CSF samples were stained according to a previously established protocol (22), which included blocking of Fc receptors by 2% i.v. Ig. Cells were immediately acquired on a BD LSR II with a high-throughput sampler delivery system and analyzed with FACSDiva 6.1 software (all BD Biosciences). Gating was based on isotype controls. Sample acquisition, gating, and sample exclusion (based on the review of quality of the staining and of absolute numbers of acquired events to assess reliability of data) were done on coded samples.

Materials and Methods

Subjects

The study was approved by the National Institutes of Health Institutional Review Board and all patients provided written consent (or assent). Patients were prospectively recruited between February 2011 and August 2013 from multiple National Institutes of Health groups that provide care for patients with neuroimmunological or neuroinfectious disorders. Adult subjects had not received immunomodulatory therapies for a minimum of 3 mo before immunophenotyping. Because untreated pediatric patients could not be readily recruited, all pediatric patients were included irrespective of treatment. All patients underwent a thorough diagnostic work-up evaluating infectious and auto-immune causes, magnetic resonance imaging (MRI) and CSF studies. When indicated by history or serological studies, CSF work-up included serological and quantitative PCR search for infectious etiologies. Diagnosis of MS was based on 2010 revisions to the McDonald diagnostic criteria (21). Patients who presented with clinically isolated syndrome were followed for a minimum of 1 y and reclassified as MS when they fulfilled diagnostic criteria. Patients who presented with clinically isolated syndrome were followed for a minimum of 1 y and reclassified as MS when they fulfilled diagnostic criteria. Patients who did not convert to MS were grouped with OIND subjects, because based on phenotypical and MRI characteristics, these patients were deemed to have low probability of converting to definite MS in the future.

Non-MS patients were grouped into diagnostic categories of adult or pediatric inflammatory (OIND) or NIND. Patients with a defined diagnosis were grouped based on whether the CNS disease was thought to be immune-mediated or not. Patients whose diagnosis remained unclear after diagnostic work-up were classified into the OIND subgroup based on at least one of the following accepted laboratory and imaging markers of intrathecal inflammation: CSF pleocytosis, increased IgG index, CSF oligoclonal bands (OCBs) or presence of CELs on brain or spinal cord MRI. Patients who did not fulfill these criteria were classified as NIND.

Because the National Institutes of Health is a highly specialized international referral center, difficult-to-diagnose patients are overrepresented in our cohort in comparison with regular clinical practice. The demographic data and diagnoses are summarized in Table I.

Sample preparation and flow cytometry

All samples were labeled with a prospectively assigned alpha-numeric code, and personnel performing the studies were blinded to the diagnosis of the subject. Specimen collection, handling, and processing were performed according to written standard operating procedures.

Immunophenotyping of peripheral blood cells was performed on anti-coagulated blood within 60 min of in vivo collection after osmotic lysis of erythrocytes. CSF samples were placed on ice immediately after collection. Within 15 min the CSF (usually 20 ml) was spun and cell pellets were resuspended in 400 µl ice-cold X-Vivo media (Lonza). Concentrated CSF cells were counted by a Neubauer hemocytometer (Hauser Scientific) at high magnification to allow differentiation of erythrocytes from nucleated cells. To calculate concentration of CSF leukocytes per 1 ml CSF was calculated by dividing the total number of CSF leukocytes by volume of collected CSF.

The 12-color immunophenotyping panel is described in Table II. A minimum of 10^6 blood cells and 5000 CSF cells were stained according to a previously established protocol (22), which included blocking of Fc receptors by 2% i.v. Ig. Cells were immediately acquired on a BD LSR II with a high-throughput sampler delivery system and analyzed with FACSDiva 6.1 software (all BD Biosciences). Gating was based on isotype controls. Sample acquisition, gating, and sample exclusion (based on the review of quality of the staining and of absolute numbers of acquired events to assess reliability of data) were done on coded samples.

Statistical analysis

Appropriate transformations were applied to the 66 markers based on the results of the Box–Cox method. To evaluate the association of the markers with the factor of diagnosis, analysis of covariance (ANCOVA) with unequal variance model was performed with gender as covariate. Because age was related to the factor of diagnosis, it could not be used as a covariate. To assess the effect of age, ANCOVA with both age and gender as covariates was applied to a subset of four patient groups (PP-MS, SP-MS, OIND, and NIND) in which there was no significant difference in age. To distinguish age-related from the disease-related effects in the pediatric group, we searched PubMed for articles describing age-related effects on the immune system in healthy donors (HDs) and compiled data from these articles into Supplemental Table I. We also performed analysis of correlations between measured markers and age within three age-homogeneous cohorts in our study (i.e., pediatric, young adult, and older adult cohorts; Supplemental Table I). When congruency in correlation between the marker and the age was observed within several cohorts (including published data), we attributed the observed difference to age-related changes and associated statistical annotations by gray shading in the relevant figures.

To evaluate the relationship between markers, pairwise Spearman correlation coefficients were calculated for each cohort. To visualize the combined marker effect on diagnosis, a heat map was created from cluster analysis (Ward method) based on the markers with p value of F test (df = 6) <0.015 in ANCOVA.

To examine the difference between CSF and blood, repeated measures ANOVA was performed with two factors, that is, diagnosis (between-subject factor) and type (within-subject factor), and their interaction in the model. Statistical analyses were performed using SAS version 9.2.

Because we were able to recruit only five HDs, this group was too small to use for statistical analysis. Instead, we plotted mean ± 2 SD for the HD group as an approximate reference range, with the understanding that the SD may be artificially inflated owing to small cohort size.

Results

Development of a 12-color flow cytometry immunophenotyping panel

In pilot experiments we have optimized the combination of commercially available fluorochrome-conjugated Abs (Table II) that allowed us to reliably quantify 14 subpopulations of immune cells (see gating strategy in Supplemental Fig. 1) and assess their in vivo activation. We used several activation markers (HLA-DR, activated effector T cells; CD25, activated T cells, B cells, monocytes, and DCs; and CD80, activated monocytes, DCs, and B cells) combined with cell-specific measurements of size and granularity.
Two hundred twenty-one patients were prospectively recruited between February 2011 and August 2013 from the Neuroimmunological Diseases Unit Natural History Protocol 09-N-0032 that provides diagnostic work-up for untreated patients with putative neuroimmunological disorders. Statistically significant differences in demographic and clinical parameters are noted in the table. Median (range); a, b, and c with different letters are significantly different with 0.001.

A, I, no bands in CSF and serum, normal profile; II, oligoclonal bands in CSF only, consistent with intrathecal IgG synthesis; III, partially identical oligoclonal bands in CSF and serum, consistent with systemic disease; IV, identical oligoclonal bands in CSF and serum, consistent with systemic inflammation.

We observed significant differences between blood and CSF samples in proportion and activation status of virtually all immune cells analyzed. These changes were seen across all diagnostic categories. In Fig. 1 we highlight markers for which there was interaction between sample type and diagnosis. In Supplemental Fig. 2 we provide plots of differences between diagnostic categories for the highlighted markers. Diagnosis-specific changes are discussed later; here we focus on global changes within the entire cohort and highlight only statistically significant changes.

Compared to blood (Fig. 1A), the proportion of innate immune cells is lower in the CSF; monocytes were decreased by 45.18 ± 7.51%, granulocytes by 71.73 ± 1.69%, and basophils by 53.89 ± 36.83%. Subsets of DCs and NK cells were differentially represented: myeloid DCs (MyDCs) and CD56dim NK cells were underrepresented in the CSF compared with blood (MyDCs by 3.89 ± 1.22% and CD56dim NK cells by 47.85 ± 2.99%), whereas plasmacytoid DC (PIDs) and CD56bright NK cells were overrepresented in the CSF (PIDs by 143.57 ± 0.18% and CD56bright NK cells by 109.93 ± 40.09%).

Several innate immune subsets in the CSF had phenotypes consistent with recent activation: monocytes were significantly smaller and dramatically degranulated (side scatter [SSC] decreased by 17.32 ± 9.15%). Granulocytes were also smaller in the CSF but had comparable granularity to blood cells (SSC smaller by 0.94 ± 1.58% in the CSF compared with blood), and basophils did not have altered size or granularity. In contrast, DC and NK cells were significantly larger in the CSF; indicating their activated status, but only those DC and NK cells that were overrepresented in the CSF were also significantly degranulated in comparison with blood; that is, PIDs were degranulated by 18.64 ± 6.49% and CD56bright NK cells were degranulated by 12.50 ± 2.19%.

Among adaptive immune cells (Fig. 1B), proportions of all T cell subsets were significantly increased in the CSF, whereas B cells were dramatically reduced (by 75.79 ± 37.18%). CD4+ T cells were proportionally more expanded in the CSF than CD8+ T cells (by 38.30 ± 8.14% for CD4+ and by 13.03 ± 4.72% for CD8+ T cells), which resulted in an increased CD4/CD8 T cell ratio in the CSF, consistent with a previous report (23). Compared to blood, T cell populations had activated phenotypes in the CSF: CD8+ T cells, 3.07% granularity; HLA-DR/CD8+ T cells, 2.09% granularity). These effects were dramatically expanded in the CSF (Fig. 1B). We also noted overrepresentation of “cytotoxic” T cells, CD4+ and CD8+ T cells expressing NK marker CD56 in the CSF, which were also larger than their blood counterparts. However, only CD8+/CD56+ T cells were degranulated in the CSF.

B cells were significantly larger in the CSF (28.90 ± 3.09%) and were the only cellular subpopulation with higher granularity in the CSF (4.3 ± 0.91%). This was likely due to the fact that our immunophenotyping panel could not differentiate B cells from plasmablasts, which are larger and more granular than B cells, but still express CD19 (14).

Differences in the immune cells among diagnostic categories

Overall, we observed prominent overlap between diagnostic categories for both blood and CSF markers (Figs. 2–5). In general, the two pediatric cohorts were more dissimilar compared with the
adult subgroups. Although we show all significant differences in Figs. 2–5, we only highlight those that we consider to be biologically meaningful based on consistency and high levels of significance. Differences that could be age-related (and thus were not considered to be disease-specific) are highlighted by gray shading of the statistical annotations within Figs. 2–5.

Whereas no differences among adult subgroups were observed for blood T cells, in the CSF the OIND patients had significantly higher absolute numbers of all T cells (CD3+, CD4+, CD8+), including HLA-DR+ effector CD4+ T cells (Fig. 2), in comparison with NIND patients and the two progressive MS subgroups. Additionally,

### Table II. Optimized combination of 12 commercially available fluorochrome-conjugatedAbs to reliably quantify 14 subpopulations of immune cells

<table>
<thead>
<tr>
<th>Conjugation</th>
<th>Name</th>
<th>Company (Clone)</th>
</tr>
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<tbody>
<tr>
<td>FITC</td>
<td>Anti-human CD56 Ab</td>
<td>BD Biosciences (MEM188)</td>
</tr>
<tr>
<td>PE</td>
<td>Anti-human CD80 Ab</td>
<td>BD Biosciences (M-A712)</td>
</tr>
<tr>
<td>PE</td>
<td>Mouse IgG1 isotype control</td>
<td>BD Biosciences (G18-145)</td>
</tr>
<tr>
<td>PE</td>
<td>Anti-human CD123 Ab</td>
<td>eBioscience (7G3)</td>
</tr>
<tr>
<td>PE-Cy5.5</td>
<td>Anti-human CD11c Ab</td>
<td>eBioscience (3.9)</td>
</tr>
<tr>
<td>V450</td>
<td>Anti-human CD45 Ab</td>
<td>BD Biosciences (HI30)</td>
</tr>
<tr>
<td>AmCyan</td>
<td>Anti-human CD8 Ab</td>
<td>BD Biosciences (SK1)</td>
</tr>
<tr>
<td>eFluor 605 nanocrystal</td>
<td>Anti-human CD19 Ab</td>
<td>eBioscience (HIB19)</td>
</tr>
<tr>
<td>eFluor 655 nanocrystal</td>
<td>Anti-human CD3 Ab</td>
<td>eBioscience (OKT3)</td>
</tr>
<tr>
<td>Qdot 705</td>
<td>Anti-human CD4 Ab</td>
<td>Invitrogen (S3.5)</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>Anti-human CD25 Ab</td>
<td>BD Biosciences (M-A251)</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>Mouse IgG1 isotype control</td>
<td>BD Biosciences (MOPC-21)</td>
</tr>
<tr>
<td>Alexa Fluor 700</td>
<td>Anti-human CD14 Ab</td>
<td>BioLegend (HC14)</td>
</tr>
<tr>
<td>Allophycocyanin-Cy7</td>
<td>Anti-human HLA-DR Ab</td>
<td>eBioscience (LN3)</td>
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**FIGURE 1.** The differences between the blood and CSF samples in the proportion and activation status of immune cells for all patients. (A) Differences in the cells of the innate immune system: monocytes, granulocytes, PlDCs, MyDCs, and CD56dim and CD56bright NK cells and basophils. (B) Differences in the cells belonging to adaptive immune system: CD4+ and CD8+ T cells and their subsets (HLA-DR+ effector cells and CD56+ cytotoxic cells) and CD19+ B cells. Left panels in each row demonstrate differences in the proportions of specified cell population among all CD45+ leukocytes between blood (red) and CSF (blue). The next two panels in each row show representative raw FACS images of the size (forward scatter [FSC] on x-axis) and granularity (SSC on y-axis) for specified cell population from MS patient. The right two panels in each row represent group comparisons between blood and CSF of the size and granularity for specified subpopulation of immune cells. *p < 0.05, **p<0.001 < p < 0.05, ***p < 0.001. Mean values are shown ± SD. Red edge highlights those markers for which statistical interaction was identified between sample type and diagnosis. For these markers, the diagnosis-specific plots can be found in Supplemental Figs. 2A and 2B.
RR-MS patients had significantly higher absolute numbers of most CSF T cell subpopulations (CD3⁺, CD4⁺, CD8⁺, and CD4⁻/CD8⁻ double-negative T cells; Fig. 3) in comparison with PP-MS patients, but not SP-MS patients.

Multiple differences were observed between pediatric and adults subgroups. In the blood, pediatric patients had elevations in absolute numbers of T cells (Fig. 2), especially CD8⁺ and CD4⁻/CD8⁻ double-negative T cells (Fig. 3), as compared with adult patients. Based on literature review and our age-homogenous subgroup analysis (Supplemental Table I), these results reflect physiological elevations of lymphocytes in pediatric subjects.

The increases in CD4⁻/CD8⁻ double-negative T cells in both pediatric cohorts compared with adults was also seen in the CSF. The uniformity of this change and its concordance with our age-homogenous subgroup analysis suggest that this difference is likely age-related. Both pediatric cohorts also had elevated absolute numbers of CD3⁺ and CD8⁺ T cells in the CSF in comparison with NIND, PP-MS, and SP-MS subjects. For CD4⁺ T cells, the difference was only significant for absolute numbers between pediatric OIND (pOIND) cohort and NIND, PP-MS, and SP-MS patients, which was likely driven by the overall CSF pleocytosis observed in the pOIND cohort. The pOIND patients had actually significantly lower percentage age of CD4⁺ T cells in the CSF. Because this change is inconsistent with the physiological enrichment of T cells in pediatric patients, it is likely disease related. The proportional decline in CSF T cells in the pOIND patients can be explained by substantial enrichment of monocytes and granulocytes in the CSF of this cohort (see below), consisting mostly of NOMID patients. Our interpretation that adaptive immunity does not play a dominant role in this disorder is further supported by additional observations: the pOIND cohort had decreased proportions of HLA-DR⁺ T cells (Figs. 2, 3) and it was the cohort that was driving the statistical interactions with the diagnosis identified in Fig. 1. Specifically, in comparison with all other cohorts, pOIND patients had the smallest enrichment of T cells in the CSF in comparison with blood (Supplemental Fig. 2) and, in fact, for CD8⁺ T cells, the CSF/blood ratio was inverted (Supplemental Fig. 2B). Only absolute numbers of cytotoxic CD56⁺/CD8⁺ T cells were significantly enriched in the CSF of the pOIND cohort, but these cells cannot be distinguished by our immunophenotyping panel from NKT cells, which are part of the innate immune system.

We did not observe significant differences in the remaining activation markers, such as size, granularity, and CD25 expression among other patient subgroups (data not shown).

**Differences in B cells**

In adults, we did not observe any B cell–related differences in the blood. In contrast, B cells were overrepresented in the CSF of RR-MS and SP-MS (but not PP-MS) patients in comparison with NIND controls (Fig. 4, top panels). When considering absolute numbers of CSF B cells, RR-MS patients had significantly higher numbers in comparison with both NIND and PP-MS (but not SP-MS) patients. Similarly, OIND patients had elevated CSF B cell numbers in comparison with NIND and PP-MS cohorts.

We also observed a significant increase in CD80 expression on CSF B cells in RR-MS patients as compared with NIND subjects (p = 0.0225, data not shown).

B cells were dramatically enriched in the blood of pediatric NIND (pNIND) patients in comparison with all adult subgroups,
both as a proportion and as absolute numbers. This change is consistent with physiological enrichment of lymphocytes in the pediatric subjects (Supplemental Table I). However, we cannot rule out disease-related contributions, because the pNIND group exhibited dramatic enrichment of blood B cells in comparison with CSF B cells among all diagnostic categories, including HDs (Fig. 4, upper panels, Supplemental Fig. 2B). In contrast, no statistically significant differences from adult cohorts were observed in pOIND patients. Pediatric patients had increased absolute numbers of CSF B cells in comparison with NIND and PP-MS adult patients, which is likely age related.

Differences in monocytes

RR-MS patients had a significantly lower CSF proportion of monocytes in comparison with NIND and PP-MS, but not SP-MS patients (Fig. 4, second row). Due to relative CSF pleocytosis in the RR-MS cohort, this change was no longer significant when considering absolute numbers of monocytes.

pNIND patients had a robust decrease in the proportion of monocytes in the blood in comparison with all adult patients. Because this difference disappeared when absolute numbers were considered, we think that this proportional decrease of monocytes in the blood is linked to robust proportional enrichment of lymphocytes (especially B cells) in the same patients. In the CSF, pOIND patients exhibited a strong increase in the absolute numbers of monocytes compared with all subgroups, including pNIND.

Differences in NK cells

Among adult patients, the OIND group had significantly higher absolute numbers of NK cells in the CSF in comparison with NIND and PP-MS patients (Fig. 4, last two rows). No significant differences were noted among adult subjects in the blood.

In the blood, pediatric patients had overall higher numbers of NK cells in comparison with adult patients; this difference was more pronounced for CD56\textsuperscript{bright} NK cells and especially in the pNIND cohort. Although this is consistent with physiological enrichment of lymphocytes in the blood of pediatric patients, the difference between pNIND and pOIND subjects in numbers of CD56\textsuperscript{bright} NK cells suggests the possibility of disease-related change.

Absolute numbers of CD56\textsuperscript{dim} NK cells were increased in the CSF of pediatric patients in comparison with all adult subgroups, except OIND. Absolute numbers of CD56\textsuperscript{bright} NK cells were increased only in pOIND subgroup and only in comparison with NIND, PP-MS, and SP-MS cohorts. Because of the lack of normative pediatric data, it is unclear whether this represents a physiological or disease-related difference.

Differences in DCs

The more prevalent MyDCs were proportionally underrepresented in the blood of pediatric cohorts (Fig. 5, upper row); this difference was significant only in pNIND in comparison with adult subgroups. The difference was less robust for the pOIND group and disappeared when absolute numbers of MyDCs were considered. However, both pediatric cohorts had significantly higher absolute numbers of MyDCs in the CSF in comparison with NIND, PP-MS, and SP-MS subgroups. In contrast, no differences in the proportion, activation status, or absolute numbers of MyDCs were observed among adult patients.

PDCs were conspicuously elevated in the CSF of the pediatric cohort, both in terms of proportions and absolute numbers (Fig. 5, upper row).
The prominent increase in the proportion and absolute numbers of CSF PlDCs in a subgroup of pNIND patients (in Aicardi–Goutières syndrome [AGS] patients; Supplemental Fig. 2A) strongly suggests a disease-related process.

In contrast, among adult subjects, only OIND and RR-MS patients had higher absolute numbers of PlDCs in the CSF in comparison with the NIND group.

Differences in granulocytes and basophils

Granulocytes were proportionally decreased in the blood of pNIND cohort in comparison with all adult patients (Fig. 5), consistent with age-related normative data (Supplemental Table I). In contrast, pOIND cohort did not have a physiological proportional decrease in blood granulocytes. Furthermore, pOIND patients had prominent expansion of absolute numbers of granulocytes in the CSF, in comparison with adult subgroups and the pNIND cohort. There was similarly robust expansion of absolute numbers of basophils in the CSF of the pOIND cohort as compared with all adult subgroups, except OIND. Overall these data indicate that the primary immune alteration in the pOIND cohort resides in innate immune cells, such as monocytes, granulocytes, and basophils. This interpretation is supported by the observation that most statistical interactions with the diagnosis identified in Fig. 1A were driven by pOIND NOMID patients. Specifically, these patients had an inverted blood/CSF ratio of monocytes (i.e., had a higher proportion of monocytes in the CSF than in the blood) and had also inverted ratios of monocyte size and granulocyte granularity (Supplemental Fig. 2A). In other words, CSF monocytes of NOMID patients were larger and CSF granulocytes were significantly more degranulated in comparison with their counterparts in the blood.

No significant differences in the proportion, numbers, or activation status of granulocytes and basophils were identified among adult patients, with the exception of an increased proportion of granulocytes in the CSF of the PP-MS cohort in comparison with the RR-MS cohort.

Correlations between immune subpopulations in the blood and CSF

Virtually all nonphysiological differences between diagnostic categories were related to CSF and not blood biomarkers, suggesting that immune cells in the CSF are not simply recruited from the periphery. To support this interpretation, we analyzed correlations between blood and CSF biomarkers using a Spearman correlation coefficient $r$ as an indication of a biologically meaningful correlation (i.e., explaining $>25\%$ of variance).

We found significant correlations between immune cell subsets in the blood and CSF only in two noninflammatory cohorts: the NIND and pNIND (Table III). In NIND, the strongest correlations were observed for double-negative T cells ($r = 0.72$, $p < 0.0001$), CD8$^+$ T cells ($r = 0.55$, $p = 0.0035$), and their effectors (HLA-DR$^+$ CD8$^+$ T cells; $r = 0.68$, $p = 0.0001$) and for B cells ($r = 0.67$, $p = 0.0002$). Within the same cohort, the activation status of B cells, as measured by levels of CD80, also correlated strongly between blood and the CSF compartment ($r = 0.84$, $p = 0.0001$). In the pNIND cohort we observed significant correlations between blood and CSF B cells ($r = 0.61$, $p = 0.0358$) and between subsets of CD4$^+$ T cells (CD56$^{-}$CD4$^+$, $r = 0.73$, $p = 0.0065$; HLA-DR$^+$ CD4$^+$, $r = 0.66$, $p = 0.0202$) and MyDCs ($r = 0.59$, $p = 0.0446$). Additionally, although monocyte numbers in the CSF did not correlate with monocyte numbers in the blood for any cohort, we observed statistically significant correlation between CD80...
expression on monocytes in the blood and CSF for both OIND \((r = 0.62, p = 0.0036)\) and pOIND \((r = 0.53, p = 0.0432)\) patients.

**Unsupervised clustering based on CSF immunophenotyping data**

Because the biggest challenge for clinicians is to determine the extent of intrathecal inflammation in diagnostically uncertain cases, we wanted to assess the relationship between immunophenotyping data and the diagnostic categories by unsupervised clustering (Fig. 6).

On the cellular level, the algorithm clustered absolute numbers of adaptive immune cells, such as T and B cell subsets. Interestingly, CD56\textsuperscript{dim} NK cells and MyDCs also clustered with this large group. Based on this largest discriminatory group, patients could be separated into three clusters: A, low; B, medium; and C, high absolute numbers of immune cells. A smaller cluster contained absolute numbers of monocytes, granulocytes, and basophils. The next cluster consisted of proportions and numbers of PlDCs and double-negative T cells, which contain a high proportion of gd T cells (Y.C. Lin and B. Bielekova, unpublished observations). Proportions of B cells represented a unique cluster, but with close proximity to the double-negative T cell cluster. The final two clusters consisted of proportions of granulocytes and monocytes, and proportions of granulocytes and monocytes, and proportions of PlDC and CD3\textsuperscript{+} and CD4\textsuperscript{+} T cells and proportions of granulocytes and monocytes, and these subdivided patient categories with moderate to high CSF pleocytosis (i.e., groups B and C) into two distinct subgroups: B1 and C1 had proportional predominance of monocytes and granulocytes, and basophils. The next cluster consisted of proportions and numbers of PIDCs and double-negative T cells, which contain a high proportion of γδ T cells (Y.C. Lin and B. Bielekova, unpublished observations). Proportions of B cells represented a unique cluster, but with close proximity to the double-negative T cell cluster. The final two clusters consisted of proportions of CD3\textsuperscript{+} and CD4\textsuperscript{+} T cells and proportions of granulocytes and monocytes, and these subdivided patient categories with moderate to high CSF pleocytosis (i.e., groups B and C) into two distinct subgroups: B1 and C1 had proportional predominance of monocytes and granulocytes, and basophils. The next cluster consisted of proportions of CD3\textsuperscript{+} and CD4\textsuperscript{+} T cells and proportions of granulocytes and monocytes, and these subdivided patient categories with moderate to high CSF pleocytosis (i.e., groups B and C) into two distinct subgroups: B1 and C1 had proportional predominance of monocytes and granulocytes, and basophils. Thus, the unsupervised clustering actually reflected the biology of the immune responses by clustering in proximity those elements that are usually activated together.

On the patient level, group A, characterized by low numbers of immune cells and relative dominance of innate immunity, contained most patients with noninflammatory etiology. Most HDs (80%) and NIND (46.7%) patients fell into this group. More than a third of progressive MS patients also fell into this group (42.31% of PP-MS and 33.33% of SP-MS).

Group B contained intermediate numbers of immune cells. RR-MS patients clearly dominated this group (65.96%), followed by a large proportion of SP-MS (61.90%) and PP-MS (50.00%) patients. Although 43.3% of NIND patients and 20% of HDs also fell into this group, all of them were classified into group B1, with relative predominance of innate immune cells. Similarly, out of 40.6% of OIND patients who clustered here, three fourths clustered to the B1 subgroup. Likewise, pNIND and pOIND patients clustered exclusively to the B1 subgroup. In contrast, MS patients, especially RR-MS and SP-MS, clustered preferentially to the B2 subgroup.

Finally, group C was characterized by high numbers of immune cells. Not surprisingly, the largest proportions of patients with intrathecal inflammation (40.63% of OIND, 76.92% of pOIND, but also 25.53% of RR-MS and 75.00% of pNIND patients) clustered here, whereas NIND patients, as well as patients with progressive MS, were almost completely excluded. As would be expected for NOMID disease process, most pOIND patients (61.54%) clustered to C1. Interestingly, a large portion of pNIND patients (66.7%) also clustered to C1. In addition to AGS patients, who had the highest proportions and absolute numbers of PIDCs with relative lack of monocytes and granulocytes (and thus clustered at the R edge of the C1 category; Fig. 6), the pNIND category contained mostly children with autoinflammatory syndromes other than NOMID.

Whereas OIND patients were equally distributed between C1 (21.88%) and C2 (18.75%), almost all RR-MS patients clustered to...
group C2, consistent with predominance of adaptive immunity in this disease.

Discussion
Results of the current study can be conceptually divided into two categories. The first relates to the physiological relationship between systemic and intrathecal immune responses. CSF leukocytes differ from those from blood in a surprisingly uniform manner, irrespective of patient diagnosis. Most of innate immune cells (i.e., granulocytes, monocytes, MyDCs, basophils, and CD56dim NK cells) and B cells are proportionally underrepresented in the CSF, whereas T cells (especially CD4+), immunoregulatory CD56bright NK cells, and PIDsCs, are overrepresented. We confirmed that T and B cells in the CSF have a more activated phenotype than do analogous cells in the blood, an observation that was previously attributed to selective ability of activated lymphocytes to cross the blood brain barrier (BBB) (24). We expand these findings by demonstrating that CSF T cells not only express more activation markers such as HLA-DR, but they are also significantly and uniformly degranulated in comparison with their blood counterparts. Our data argue that CSF immune cells do not simply reflect cells recruited from the periphery. Instead, they represent a mixture of cells that are recruited from the blood, are activated intrathecally, and leave the CNS after performing effector functions.
functions. This explanation is consistent with our observation that the only biologically meaningful correlations between numbers of immune cells in the blood and CSF were observed in patients with noninflammatory CNS processes, where selective expansion and/or retention of immune cells in the intrathecal compartment was minimized. Finally, this explanation is also consistent with observations from animal models, which demonstrated that activated T cells enter the CNS compartment irrespective of their Ag specificity, but only those T cells that recognize Ags expressed in the CNS are retained and expanded (25, 26). Although most T cells that expand in CNS tissue undergo apoptotic death leading to termination of the intrathecal immune responses, some of them leave the CNS compartment to become memory T cells, as evidenced by increased precursor frequency of autoreactive T cells in the blood several weeks/months after experimentally induced stroke (27).

The uniformity of the phenotypical changes affecting immune cell subsets in the CSF supports the notion that the CNS microenvironment shapes intrathecal immune responses; for example, we observed simultaneous activation and degranulation of the PlDCs and CD56^{bright} NK cells in the CNS, the two cell populations that are also selectively enriched in the CSF compared with blood. In contrast, MyDCs, CD56^{dim} NK cells, but also granulocytes and basophils (all cell types that are underrepresented in the CSF) had comparable granularity in both compartments. It is likely that recruitment to the CNS and local activation of these cells is physiologically restricted, because they can be highly destructive for CNS tissue (28), others have shown that under physiological conditions monocytes promote repair of CNS tissue, including remyelination (29–31).

In contrast to constrained innate immune responses, CSF is highly enriched for memory/effector T cells, especially CD4^{+} T cells. It is known that T cells are important for immunosurveillance against persisting neurotrophic viruses (32, 33). Physiological intrathecal immunity controls inadvertent activation of such opportunistic pathogens and memory T cells in conjunction with PlDCs (34), and NK cells (35, 36) are uniquely suited to play this role. However, the humoral part of the adaptive immune responses is profoundly underrepresented in the CSF. One wonders whether an abundance of B cells poses a special threat for CNS tissue. It is informative to recall that transgenic animals, in which most T cells (or B cells) recognize CNS autoantigen, rarely develop spontaneous CNS autoimmunity. However, when they are crossed to animals, in which transgenic T and B cells recognize the same autoantigen, spontaneous CNS inflammation is frequent (37, 38). Thus, autoreactive T cells need help from autoreactive B cells to mediate CNS tissue injury, explaining the high efficacy of B cell–depleting therapies in MS (39).

However, one cannot forget that CSF immunophenotyping reflects only the “mobile” pool of immune cells that reside in the CNS. For example, it has been demonstrated previously that despite predominance of CD4^{+} T cells in the CSF, CD8^{+} T cells actually represent most T cells infiltrating the CNS tissue (28). This explanation is supported by our observations that in NOMID patients, with genetically determined aberrant activation of the inflammasome pathway, granulocytes (and monocytes) in the CSF are both abundant and degranulated.

Out of the common innate effectors, monocytes behave uniquely in their decreased granularity in the CSF, indicating intrathecal engagement of effector functions. Interestingly, although some studies have highlighted a pathogenic role of monocytes in CNS tissue destruction (28), others have shown that under physiological conditions monocytes promote repair of CNS tissue, including remyelination (29–31).

FIGURE 6. Unsupervised clustering of diagnostic codes based on CSF immunophenotyping data. A two-way, unsupervised hierarchical clustering dendrogram of subjects and biomarkers based on selection of CSF immunophenotyping markers that were discriminatory in subgroup analyses is shown. Red indicates relatively high expression; blue, relatively low expression. The proportions of patients from different diagnostic categories that are classified to distinct clustering subgroups are outlined below the dendrogram. Color-coded diagnostic categories are identical for both panels.
the existence of physiological regulation of intrathecal immune responses, which likely aim to provide immunity against persistent pathogens and to enhance immune-mediated repair while limiting the potential for immune-mediated destruction of CNS tissue by enhanced presence of regulatory immune cells such as Foxp3+ regulatory T cells (41, 42) and CD56dim NK cells (22), as well as by limited recruitment of granulocytes, CD56dim NK cells, MyDCs, and B cells.

Nevertheless, our data also indicate that despite physiological regulation, in situ inflammation-driven changes shape cellular composition of CSF in a disease-specific manner. Referring back to previously mentioned “mobile” versus “static” pools of intrathecal inflammatory cells, we recognize that CSF immunophenotyping may be significantly underrepresenting disease-specific differences, especially as they relate to immune cells infiltrating CNS tissue.

With this drawback in mind, we reproduced previously validated enrichment of CSF B cells and relative decrease of CSF monocytes in RR-MS patients in comparison with NIND controls, leading to a profound decrease in the monocyte/B cell ratio in the RR-MS cohort (Supplemental Fig. 3A). We have put unvalidated reports of other CSF abnormalities observed in MS patients into the perspective of properly controlled large datasets, analyzed in a blinded fashion using identical standard operating procedures. Thus, we conclude that the most conspicuous and reproducible intrathecal immune abnormality in RR-MS resides in abnormal adaptive immune responses, including humoral immunity, in accordance to previously described presence of OCBs and a high IgG index (43), high intrathecal concentrations of CXCL13 (12, 44, 45), and high CSF numbers of B cells and plasma cells. MS has been traditionally viewed as a T cell–mediated disease, and our data do not disprove this notion. Instead, they indicate that both parts of the adaptive immune responses, that is, T cells and B cells, play an important role in MS disease process, likely through potentiation of each other’s functions. In contrast, we reason that the scarcity of CSF monocytes in RR-MS is likely secondary to their preferential recruitment to actively demyelinat- ing lesions, where they clear myelin debris and potentially promote remyelination (30). This explanation is consistent with ob- servations that RR-MS patients have higher (not lower) CSF levels of IL12-p40 (the cytokine preferentially released by activated monocytes/macrophages) and that levels of IL12-p40 peak after development of MRI CELs (46). The rapid filling of MS plaques with i.v. contrast likely signifies that the tissue integrity inside the CELs has already been damaged; otherwise, the fluid would slowly propagate along the white matter tracks, as it does in vasogenic edema associated with brain tumors. It is into this damaged tissue that monocytes are recruited to phagocytose myelin and are activated to produce IL12-p40.

We also provide a comprehensive comparison between patients with different MS subtypes: our data show decidedly that on a group level, SP-MS patients are immunologically closer to RR-MS patients than are PP-MS patients. RR-MS patients have significantly higher numbers of CD4+ and CD8+ T cells and B cells, in comparison with PP-MS only. Both RR-MS and SP-MS (but not PP-MS) patients have elevated proportions of CSF B cells in comparison with NIND. Finally, PP-MS patients have higher proportions of monocytes and granulocytes as compared with RR-MS, but not SP-MS subjects. Unsupervised clustering also grouped SP-MS closer to RR-MS in comparison with PP-MS. Having said this, we also observed substantial overlap between the three MS groups, supporting the notion of heterogeneity of disease mecha- nisms across clinical diagnostic categories. We will get back to this point later in the discussion.

Genetically confirmed NOMID and AGS patients represent clear examples of the potential of CSF immunophenotyping to provide insight into disease processes. Despite the fact that the vast majority of studied NOMID patients were treated with anakinra, we found significant elevations in their intrathecal levels of monocytes and granulocytes, even in comparison with the pNIND cohort. Furthermore, intergroup comparison in the size and granularity of monocytes and granulocytes provided strong evidence for their intrathecal activation in NOMID patients. Thus, we conclude that therapy with anakinra does not completely normalize intrathecal immune abnormalities in this cohort, perhaps due to persistent activation of the IL-18 arm of the inflammasome pathway (47) or inability of anakinra to access intrathecal compartment after therapeutic closure of the BBB.

Similarly, we observed homogeneous abnormalities in AGS patients, which were grouped into the pNIND cohort based on the lack of CSF abnormalities on clinical laboratory tests. However, research laboratory CSF counts were consistently elevated in this group, in accordance with published reports (48). We observed prominent expansion of PIDCs in their CSF (Supplemental Fig. 2A), consistent with the proposed disease mechanism, where a genetic defect in the metabolism of nucleic acids leads to activa- tion of innate immune system and intrathecal production of IFN-α (49). PIDCs are the best known cellular producers of IFN-α (50). AGS might be more appropriately classified into the pOIND group despite lack of clinical laboratory biomarkers of CNS inflammation. AGS may be considered another autoinflammatory disorder, especially because AGS-associated genetic defects can also aberrantly activate the inflammasome pathway (49). This brings us to the final topic of the discussion.

We acknowledge that our study has important limitations: we consider the lack of normative data on HDs the most imperative. We were able to collect only five HDs. The concentration of CSF cells (median, 2472.73; range, 866.67–4740.64) obtained from this small cohort is slightly higher than the only other published cohort of HDs we are aware of (median, 968 cells/ml CSF; range, 413– 2616) (51). Our strict standard operating procedures designed to limit CSF cells loss are the likely explanation for the higher numbers of CSF leukocytes in our cohort. Accordingly, we ob- served a higher proportion of granulocytes and monocytes, which are most susceptible to lysis or adherence to plastic in CSF samples that remain unprocessed for an extended time. If this small cohort is truly representative, then all patient cohorts differ from HDs in many aspects (Figs. 2–5).

In this regard, inclusion of a broad range of patients with putative neuroimmunological disorder represents both a strength and noteworthy challenge of the current study. In contrast to previous studies, where control groups were clearly different from MS (e.g., patients with normal pressure hydrocephalus), NIND and OIND patients included in this study were all referred for evaluation of possible neuroimmunological disorders. Patients classified as OIND have infectious, autoimmune, and autoinflammatory CNS diseases with diverse phenotypes of the intrathecal immune responses. This diversity contributes to the broad spread of immunophenotyping values and diminishes statistical significance of the intergroup comparisons. Furthermore, because of our strict adherence to the recently approved CSF laboratory tests in diagnostic classification, it is likely that the OIND cohort includes patients with a history of past intrathecal inflammatory process, but without active CNS inflam- mation. This is due to the fact that the IgG index or OCBs may remain elevated for years after the intrathecal inflammatory process has subsided, because of the longevity of plasma cells (52), and that contrast enhancement on brain MRI represents opening of the BBB, which may or may not be due to inflammatory process.
A similar degree of heterogeneity and diagnostic uncertainty applies to NIND patients. Classification of the NIND category was based on negative systemic work-up for neuroimmunological disorder, the absence of CELs on MRI, and a benign CSF profile. Unfortunately, these standard markers, developed decades ago, are not of sufficient sensitivity to unambiguously exclude intrathecal inflammation. For example, counting in a Neubauer hemocytometer is (according to the manufacturer’s insert) unreliable under cell concentrations <250,000 cells/ml. This represents the vast majority of unspun CSF samples. In contrast, our research laboratory effectively concentrated CSF cells 50-fold and thus increased the reliable range of hemocytometer counts to specimens with >5000 cells/ml CSF. This represents most CSF samples processed in this study. Not surprisingly, therefore, we observed poor, albeit statistically significant ($r = 0.4636, p < 0.001$; Supplemental Fig. 3B) correlations between CSF cell counts generated in the National Institutes of Health clinical laboratory versus our research laboratory. Most importantly, this enhanced counting of CSF cells demonstrated that many patients from the NIND category had absolute counts $>2$ SD of HD range. Although we do not dare to reclassify patients from NIND to OIND category solely based on the CSF counts obtained in our research laboratory, we remain open to the possibility that current diagnostic processes are insensitive to intermediate levels of intrathecal inflammation, which may nevertheless be pathophysiologically important.

This is the openness with which we also interpret results of unsupervised clustering. By itself, CSF immunophenotyping cannot represent a diagnostic test. However, our data on NOMID, AGS patients, and MS subtypes indicate that CSF immunophenotyping captures the biology of the immune process extremely well, probably better than clinical diagnostic classification of polygenic diseases. Therefore, the question we should be asking is “What kind of disease characteristics do patients who cluster together have in common?” Can clustering based on immunophenotyping data identify those patients with progressive MS who have a remaining intrathecal inflammatory process amenable to therapy with current DMTs? Will NIND patients, who cluster with OIND patients, show abnormal levels of other neuroinflammatory biomarkers? Undoubtedly, these questions are beyond the scope of the present study. Nevertheless, each clinical collaborator plans to address in future studies whether CSF immunophenotyping relates to the phenotype, genotype, or severity of the disease process. Our anecdotal observations suggest that this may be the case; for example, when the immunophenotyping profile of PP-MS patients clusters with RR-MS patients, such patients may have phenotypical aspects of disease that are more typical for RR-MS, such as CELs or a large MS lesion load in the brain, as opposed to predominant involvement of the brainstem or spinal cord, which is more typical for PP-MS. However, we clearly need to perform this analysis in an unbiased way, where rating of the imaging and clinical disease characteristics is done by an evaluator blinded to the immunological data and by using predefined, reproducible outcomes (53).

Lastly, our study adds to the increasing list of publications that demonstrate poor correlation between systemic and intrathecal immune responses in patients with neuroinflammatory diseases. For example, soluble inflammatory markers do not correlate between blood and CSF and sometimes may have even opposing trends (45, 54, 55). Similarly, a study that evaluated B cell exchange between peripheral blood and CSF by deep sequencing of IgG H chain V region genes (56) identified on average <5% sharing of B cell clonotypes between these two compartments. Together with present data, these studies indicate that the CSF represents an unique window into CNS pathology (57) and that assessment of the phenotype or severity of neuroinflammatory process from blood biomarkers may lead to unreliable conclusions, especially when such studies use methodology susceptible to biases (58). At best, the signature of the intrathecal process is extremely “diluted” in the systemic circulation and until we fully understand what we are looking for, we should focus our search for mechanistic insight into CNS diseases by studying CSF or, noninvasively, CNS tissue.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplementary figure 1: Gating strategy. Live leukocytes were first identified based on expression of CD45. Mononuclear cells were differentiated from granulocytes based on forward (size) and side scatter (granularity) characteristics. T cells were identified as mononuclear cells expressing CD3, and were further subdivided based on expression of CD4 and CD8 into CD4+ T cells, CD8+ T cells and double negative T cells (DnT). Expression of HLA-DR and CD56 identified proportion of “effector” T cells (HLA-DR+) and “cytotoxic” T cells (CD56+). Monocytes were gated as mononuclear cells that do not express CD3 but express CD14 (and also HLA-DR and intermediate levels of CD45). NK cells were gated as mononuclear cells that do not express CD3, CD14, but express CD56. These were further subdivided into CD56dim and CD56bright NK cells, identified as distinct subpopulations in CD56 mean fluorescent intensity (MFI) histogram. B cells were identified as mononuclear cells that did not express CD3, CD14, CD56, but expressed CD19 (and HLA-DR). Dendritic cells were identified as mononuclear cells that expressed HLA-DR, but lacked expression of lineage markers (CD3, CD4, CD8, CD14, CD19, CD56). They were further subdivided into myeloid DCs (expressing CD11c) and plasmacytoid DCs (expressing CD123). Cells that lacked lineage markers and HLA-DR, but expressed CD123 were gated as basophils.
Supplementary figure 2: Diagnosis-specific data for biomarkers that demonstrated significant interaction between sample type and the diagnosis. (a) the innate immune cell: monocyte, granulocyte, myeloid dendritic cell (MyDC), plasmacytoid dendritic cell (PlDC), and CD56dim NK cell; (b) the adaptive immune system: CD4+ T, CD8+ T, HLA-DR+ CD4+ T, CD56+ CD8+ T, and B cell. The biomarker measured in the blood is represented in red color, while the biomarker in CSF is represented by blue color. Statistically-significant differences between blood and CSF for each diagnostic category are highlighted. * P<0.01.
Supplementary figure 3: (a) The ratio of CSF monocytes and CSF B cells in different diagnostic groups. (b) The correlation coefficient between CSF cell counts generated by NIH clinical laboratory, versus Neuroimmunological Diseases Unit (NDU) research laboratory. (3a): Each diagnostic category is represented by one vertical box blot, while data from healthy donors (HD) are depicted as grey shading, with horizontal line representing mean, dark shade of grey representing +/- 1SD and lighter shade of grey representing +/- 2SD of HD cohort. Each box plot shows median, 25-75%-tile and whisker blots represent minimum-maximum-tile for each diagnostic category. *, P<0.05, **: 0.01<P<0.005, ***: P<0.001. (3b): Cell count by NIH clinical laboratory was based on unspun CSF sample. In contrast, NDU laboratory based CSF count on spinning on average of 20ml of CSF and re-suspending the cell pellet in 0.4ml of media, achieving 50 fold concentrations.
Supplementary table 1: Age-related changes in the immune subpopulations. To distinguish age-related effects on the immune system from the disease-related effects in pediatric group, the summary of articles describing age-related effects on the immune system in healthy controls is shown in this table (Upper panels: left for proportions and right for absolute numbers). Such data were available only for blood biomarkers. Additionally, correlation coefficients of age with immunophenotyping biomarkers were calculated within each one of three age-homogeneous cohorts in our study. (Pediatric: 0.80-18 years-old, Young adult: 18.00-65.80 years-old and Older adult: 19.80-74.86 year-old). + means positive coefficient (green); - means negative coefficient (light orange), no means no coefficient, and **Bold** signifies $P < 0.0001$.

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