High dimensional mass cytometry analysis unravels distinct profiles of peripheral blood mononuclear cells in patients with neuromyelitis optica spectrum disorder and myelin oligodendrocyte glycoprotein associated disease or in health

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Research Article

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Abstract

Background

Neuromyelitis optica spectrum disorders (NMOSD) and myelin oligodendrocyte glycoprotein-antibody associated disease (MOGAD) are rare demyelinating diseases of the central nervous system but can cause severe disability. Although these two diseases share inherent similarities, they show different pathogenesis, clinical features, and treatment response. Investigation performed by a more powerful approach is needed.

Methods

Cytometry by time-of-flight mass spectrometry (CyTOF) was used to cluster and phenotype the immune cell subsets in peripheral blood mononuclear cells (PBMCs) isolated from patients with NMOSD or MOGAD and healthy controls (HC). RNA sequencing was used to screen pivotal genes. The obtained algorithm-based data were further verified through traditional flow cytometry and qPCR analysis.

Results

We identified 29 cell clusters and found several immune cluster changes between NMOSD and MOGAD. Interestingly, no significant differences were found in the B cells fraction between patients and HC group. Immunity dysfunction was mainly attributed to changes of diverse subsets in T cells and mononuclear phagocytes (MNPs). Similar properties of two distinct CD56+ natural killer (NK) cell phenotypes and transcription factor T-bet + or chemokine receptor CCR2 + subsets were shown between patients and health.

Conclusions

Our results show an overview of the circulating PBMCs landscape of NMOSD and MOGAD patients compared to that of HC. Our data reveal that different immune phenotypes may involve in the distinct pathogenesis of NMOSD and MOGAD and highlight T cells or MNPs as a potential target for precision treatment.

Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is a relapsing inflammatory central nervous system (CNS) disorder that primarily affects the optic nerves and spinal cord, about 80% of patients harbor antibodies directed against the water channel aquaporin-4 (AQP4-IgG) expressed on astrocytes. In another subset of patients with AQP4-IgG-seronegative NMOSD, serum antibodies against myelin oligodendrocyte glycoprotein (MOG-IgG) may be detected. MOG-IgG-associated disease (MOGAD) has
been termed a distinct CNS demyelinating disorder, with manifestations mainly including optic neuritis, transverse myelitis, and acute disseminated encephalomyelitis. Despite different studies have focused on the clinical and preclinical characteristics in recent years, many questions still remain unanswered.

Although patients with AQP4-IgG-seropositive NMOSD (AQP4 + NMOSD) and MOGAD often present with similar clinical symptoms, it is difficult to differentiate them at the time of onset. Detection of autoantibodies such as AQP4-IgG and MOG-IgG in serum by cell-based assay (CBA) is fundamental and regarded as the gold standard for disease diagnosis and treatment decisions, however, the serum antibody levels are fluctuating by therapeutic drugs and unpredictable at different disease stages [1]. To better understand and identify new biomarkers potentially useful in clinical practice for diagnostic and treatment purposes, a panel of 65 serum cytokines, chemokines, and related molecules were analyzed and compared by bead-based multiplex assays. They found that significantly increased peripheral inflammatory responses in NMOSD and MOGAD compared with another demyelinated disease multiple sclerosis (MS)[2]. Immunosuppressive therapy can reduce relapse in most autoimmune diseases, but, unlike AQP4 + NMOSD, some MOGAD patients treated with rituximab experience relapses despite a complete B-cell depletion [3]. In order to further understand the pathogenesis of NMOSD and MOGAD and describe the circulating immune cell landscape totally, a powerful approach to phenotype the immune cell subsets in peripheral blood mononuclear cells (PBMCs) is needed.

Cytometry by time-of-flight mass spectrometry (CyTOF) enables the simultaneous measurement of more than 30 parameters per single cell using metal isotope-conjugated antibodies with minimal overlap, which maximizes the information obtained from each individual sample [4]. Here, we use a panel of 36 cell surface or intrinsic markers expressed on or inside in PBMCs and characterize their phenotypes among NMOSD, MOGAD patients, and healthy individuals. Subsequently, we further verified the distinct population of three subsets in monocytes, a major component of human peripheral blood, which accounts for ~10% of all circulating leukocytes by fluorescence cytometry. The results obtained from this study will unveil distinct signatures of peripheral blood immune cells of patients with NMOSD or MOGAD, and provide a reference for diagnostic and therapeutic intervention in neuroinflammatory diseases.

Materials and methods

Participants

We collected two parts of patients at the Department of Neuroinfection and Neuroimmunology, Beijing Tiantan Hospital affiliated of Capital Medical University, according to the 2015 International Panel for Neuromyelitis Optica Diagnosis criteria or previously proposed MOGAD diagnostic criteria respectively [5, 6]. In the first part, 33 patients were diagnosed as NMOSD (16 in relapse and 17 in remission), 12 patients were diagnosed as MOGAD (11 in relapse and 1 in remission), between January 2021 and March 2022, and 15 age-matched healthy individual controls were enrolled in this study. The key clinical characteristics of all participants were shown in Table 1. The other part, 6 NMOSD (all in relapse) and 3 MOGAD (all in relapse) patients vs. 4 healthy individuals were performed, patients in hospital between
March 2023 and June 2023. The key clinical characteristics of participants who participated in Flow cytometric were shown in Table 2. The study was approved by the Ethical Committee of Beijing Tiantan Hospital (No. KY2019-050-02), and written informed consent was obtained from all participated subjects.
Table 1
Demographic data and clinical characteristics of patients who participated in mass cytometry

<table>
<thead>
<tr>
<th></th>
<th>NMOSD Acute (n = 16)</th>
<th>NMOSD Remission (n = 17)</th>
<th>MOGAD n = 12</th>
<th>HC n = 15</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>13/3</td>
<td>17/0</td>
<td>5/7</td>
<td>12/3</td>
<td>a0.04</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>47.6 ± 12.7</td>
<td>44.5 ± 15.2</td>
<td>34.1 ± 16.6</td>
<td>45.4 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>Age (years), mean (SD)</td>
<td>4.5 (2.5-8)</td>
<td>3 (0-7)</td>
<td>2.8 (0-5)</td>
<td>-</td>
<td>b0.02</td>
</tr>
<tr>
<td>EDSS, median (range)</td>
<td>13 (81.3)</td>
<td>15 (88.2)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Seropositivity for AQP4-Ab, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>12 (100)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Seropositivity for MOG-Ab, n (%)</td>
<td>1:32 (1:10 - 1:100)</td>
<td>1:32 (1:10 - 1:320)</td>
<td>1:32 (1:10 - 1:100)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Serum antibody titer</td>
<td>6.7 (2.7–12.1)</td>
<td>-</td>
<td>7.1 (2.8–12.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>QAlb (×10⁻³), median (range)</td>
<td>0.50 (0.41–1.21)</td>
<td>5 (1–48)</td>
<td>18 (6–84)</td>
<td>-</td>
<td>b0.01</td>
</tr>
<tr>
<td>IgG index, (range)</td>
<td>1.25 (1.5)</td>
<td>-</td>
<td>1 (0.34)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Disease duration (months), median (IQR)</td>
<td>1.25 (1.5)</td>
<td>-</td>
<td>1 (0.34)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: EDSS = Expanded Disability Status Scale; AQP4-Ab = aquaporin-4 antibody; MOG-Ab = myelin oligodendrocyte glycoprotein antibody; QAlb = albumin quotient, presented as CSF-to-serum albumin ratio; IQR = interquartile range.

aMOGAD vs. HC

bNMOSD-acute vs. MOGAD

Table.2 Demographic data and clinical characteristics of patients who participated in flow cytometric
Blood sample collection and PBMCs isolation

Fresh blood samples were collected into ethylenediaminetetraacetic acid (EDTA) anticoagulation tubes and then centrifuged at 800 g for 5 min with minimal braking to remove the plasma. None of the patients during the acute phase were given any treatments before sampling.

PBMCs were immediately isolated from fresh blood within 2 hours after sample collection using SepMate tubes containing Ficoll (catalog no.86450, STEMCELL Technologies). The samples were centrifuged at 1,200 g for 10 min and then transferred into a new centrifuge tube. Mix with 3 mL sterile cold PBS, centrifuge at 300 g for 8 mins, and discard the supernatant. Dissolve with 100 µL PBS, and transfer the sample into a new 1.5 mL EP tube. The cells were washed with red blood cell (RBC) lysis buffer (catalog no. 555899, BD Biosciences). Then, the cells were washed twice with a complete RPMI medium and then stored in liquid nitrogen. Each tube of PBMCs was divided into 2 parts, one portion was resuspended in staining buffer and fixed for mass cytometry, and another portion was mixed with TRIzol Reagent (Thermo Fisher, 15596018) for RNA-sequence.

Mass Cytometry

Remove frozen PBMC samples from liquid nitrogen and transport them to a lab on dry ice. Samples were thawed rapidly in a 37°C water bath and washed twice in RPMI 1640 (HyClone®, Thermo Scientific) supplemented with 10% FBS in 15 mL Falcon tubes (BD Biosciences), penicillin and streptomycin and 10 U/mL benzonase (Sigma), resuspended in PBS, and kept on ice for further use. PBMC samples were counted and checked for viability using a Vicell counter (Beckman Coulter). PBMC viability was typically > 95%.

Firstly, PBMCs were stained with anti-CD45 antibody conjugated with 89Y. Then, cells were stained with cell surface antibodies for 30 min at room temperature. Subsequently, the samples were permeabilized overnight at 4°C and stained with intracellular antibodies for 30 min at room temperature. All metal-conjugated antibodies were titrated for optimal concentration before staining with cells. The antibody-labeled samples were washed and incubated in 0.125 nM intercalator-Ir (catalog no. 201192B, Fluidigm) diluted in phosphate-buffered saline (PBS, catalog no. 806544, Sigma-Aldrich) containing 2% formaldehyde and stored at 4°C until CyTOF examination. Prior to the acquisition, samples were resuspended in deionized water containing 10% EQ Four Element Beads (catalog no. 201078, Fluidigm), and the concentration of cells was adjusted into 1 × 10^6 cells/mL. The samples were then examined by mass cytometry (Fluidigm).

A panel of 36 antibodies designed to distinguish a broad range of immunocytes was used. Antibodies were either purchased in a pre-conjugated form from Fluidigm or purchased in a purified form from Biolegend or Abcam and conjugated in-house using the Maxpar® X8 Multimetal Labeling Kit (catalog no. 201300, Fluidigm) according to the manufacturer’s recommendations. The antibodies and reporter isotopes are listed in Supplementary Table 1.

CyTOF analysis
Data were obtained as fcs files and data analysis were performed according to the previous study [7]. The CyTOF data were analyzed with Cytobank (www.cytobank.org). The cell types were identified based on the following parameters: T cells, CD45+CD3+; B cells, CD45+CD20+; natural killer (NK) cells, CD45+CD3-CD16+CD56+ [8, 9]; monocytes, CD45+CD14+CD16+ [10]; macrophages cells, CD45+CD11b+CD3-CD19- [11]. Monocytes and macrophages constitute mononuclear phagocytes (MNPs) [12]. The identified cell types were shown in Supplementary Table 2. Manual gating was applied to indicate the cell types as previously reported [13]. ViSNE [14] algorithms were used on the indicated gated cells. The viSNE analysis of T cells or MNPs was performed for patients of samples with more than 500 cell counts. Then, the automatic cluster gate functionality was used for the hierarchical cluster analysis. Heatmaps were generated by R software (version 4.2.0).

**Flow cytometric analysis**

Before staining, PBMCs samples first were blocked in Human TruStain FcX™ (catalog no. 422302, BioLegend), 5 µL per million cells in 100 µL staining volume, mixed and incubated at room temperature for 5–10 minutes. Then, Samples were labeled by incubating with a mixture of different monoclonal antibodies for 20 min in the dark at 4°C. Before Flow cytometric examination, samples were incubated with the 7-AAD Viability Staining Solution (catalog no. 420403, BioLegend), 5 µL per million cells for 5–10 minutes in the dark. Surface protein expression was detected on CytoFLEX (Beckman). CytExpert software (version 2.3) was used to determine median fluorescence intensity.

The following antibodies were used: Alexa Fluor® 700 anti-human CD3 Antibody (catalog no. 317340, BioLegend), PE anti-human CD19 Antibody (catalog no. 392506, BioLegend), Brilliant Violet 650™ anti-human CD14 Antibody (catalog no. 301836, BioLegend), APC/Cyanine7 anti-human CD16 Antibody (catalog no. 302018, BioLegend), APC anti-human CD192 (CCR2) Antibody (catalog no. 357208, BioLegend).

**RNA-sequencing**

An RNA-sequencing (RNA-seq) study of PBMC in 6 NMOSD and 3 MOGAD patients vs. 3 healthy individuals were performed. Total RNA from PBMC was extracted using commercial kit (Tianmo#TR205-200). Nanodrop and Agilent Bioanalyzer (Agilent 2100 bioanalyzer) were used to quantify the RNA concentration and control the RNA quality before RNA-seq experiment.

RNA sequencing service was performed at Sinotech Genomics Co. Ltd., Shanghai. Briefly, poly-A pull-down to enrich mRNAs from total RNA samples were used and proceeded on library preparation. The library was validated using Agilent Bioanalyzer to ensure the size and purity of the sample by observing a band at approximately 260 bp. Libraries were then sequenced using an Illumina NovaSeq 6000 instrument.

**qPCR identification**

As previously described [15, 16], the total RNA was isolated from PBMCs using TRIzol™ reagent (Invitrogen). A NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA)
was used to determine RNA purity and concentration. RNA (1 mg) was used to synthesize cDNA with the Roche Transcriptor First Strand cDNA Synthesis Kit (Roche Holding AG, Basel, Switzerland) using anchored oligo (dT) and random hexamer primers. SYBR Green-based qPCR was performed to measure the relative mRNA expression of CCR2, which was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primers were designed and ordered from BGI Genomics Co., Ltd. The primer sequences are as follow: CCR2 forward, 5′-CAGGTGACAGAGACTCTTGGGA-3′, reverse, 5′-GGCAATCCTACAGCCAAGAGCT-3′; GAPDH forward, 5′-GTCTCCTCTGACTTCAACAGCG-3′, reverse, 5′-ACCACCTGTTGCTGTAGCCAA-3′. Data were analyzed using the 2−ΔΔCt method.

Data analysis

For the CyTOF experiments, PBMCs samples from 33 NMOSD, 12 MOGAD patients and 15 healthy controls were analyzed; for flow cytometry and RNA-sequence experiments, data were analyzed from 3–6 patients in each group and paired controls. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, United States) and IBM SPSS Statistics (version 20.0). The significant differences between the groups were determined by a 2-tailed unpaired Student’s t-test or Mann-Whitney U test when the samples were not distributed normally. Count data were detected by the chi-squared test. All data are represented as mean ± standard error of the mean (SEM). The statistical significance level was assumed for p values < 0.05.

Results

Comparation of PBMCs phenotypes among NMOSD, MOGAD patients and HC group

To identify the properties of immune-cell subsets of PBMCs in single-cell levels from patients with NMOSD or MOGAD compared to HC, CyTOF analysis was performed. Schematic experimental strategy is represented in Fig. 1. The initial gating strategies used for CD45+ cells are provided in supplementary Fig. 1, and the gating strategies used for the indicated immune cells are summarized in supplementary Table 2. We focused our analysis on average of 120,000 CD45+ PBMCs per sample and identified 29 clusters as shown superimposed on the t-distributed stochastic neighbor embedding (t-SNE) map in Fig. 2A. Proportion of each cluster was shown in Fig. 2B. The cluster percentage of all participants were visualized in a heatmap (Fig. 2C). The viSNE map of collected CD45+ PBMCs showed differential abundances of immune cell populations. All samples were mapped using viSNE, and surface marker expression was visualized using spectrum maps (Fig. 1D) and heatmaps (supplementary Fig. 2). Furthermore, we compared each lineage subset including T, B, NK cells and MNPs. Although no significant difference in the proportion of B cells was observed among 3 studied groups, the proportion of T cells was significantly increased (p < 0.05), while the proportion of NK cells was significantly decreased (p < 0.05) in NMOSD patients (Fig. 2E&F). The proportion of MNPs was significantly increased in MOGAD as compared to HC group (p < 0.05). Column graphs showed that the percentages of T cells and MNPs
were statistically significantly different between patients with NMOSD and MOGAD (Fig. 2E&F), suggesting the distinct pathogenesis of the two similar demyelinated diseases.

**T lymphocytes changed significantly in the peripheral blood of NMOSD and MOGAD patients**

ViSNE map showed a similar distribution of CD3+ T cells in NMOSD and MOGAD patients to that in the HC group analyzed by phenograph algorithm (Fig. 3A). According to the surface markers, CD3+ T lymphocytes in PBMCs could be subdivided into 22 subgroups. The proportion of each cluster showed in Fig. 3B. In comparison to health controls, similar changes were observed in NMOSD and MOGAD patients. NMOSD and MOGAD patients had a higher abundance of 7 clusters (including cluster 2,4,14,16,17,20,21) and lower abundance of 3 clusters (including cluster 5,6,10) (p < 0.05, Fig. 3C). The expression profiles of the T cell clusters were visualized in a heatmap (Fig. 3D). Compared to HC group, the fraction of CD4+ T cells was higher in NMOSD patients; and the fraction of CD3+CD20+ T cells was higher in MOGAD patients (Fig. 3E). The box charts showed marked decreased of CD4+CD8+double-positive T cells (DPT), naive T cells and CD3+CD33+ T cells in both NMOSD and MOGAD patients, compared to that of the HC group (Fig. 3E). The frequency of NKT cells was significantly different between NMOSD and MOGAD patients. There was no significant alteration between the percentage of CD8+ T cells and CD4-CD8- double-negative T cells (DNT) (Fig. 3E).

As we known, T-bet was identified as a key transcript factor which can direct naïve T cells to differentiate into Th1 subset [17] and associated with cell-mediated immunity [18]. In the present study, T-bet + T cell subset analysis were performed. ViSNE map showed T-bet + T cells distributions in NMOSD, MOGAD patients and HC group (Fig. 4A). The violin plot showed the T-bet expression from NMOSD and MOGAD patients decreased significantly compared to HC (Fig. 4B). Cluster 14 characterized by T-bet + CD4+ and Cluster 1 characterized by T-bet + CD8+ both decreased significantly in NMOSD and MOGAD patients compared to HC (Fig. 4C). Cluster 2 characterized by T-bet + CD4-CD8- DNT cells decreased markedly only in NMOSD patients and a reduction tendency in MOGAD patients (Fig. 4C).

*Lower monocytes and higher macrophages in the proportion of NMOSD and MOGAD patients compared to HC group*

Monocytes, DCs, and macrophages are regarded as MNPs, which play a major role in inflammatory diseases [12, 19]. By CyTOF, we differentiated CD45+CD11c+CD68+ MNPs into 23 clusters based on the patterns of expression markers (Fig. 5A). The proportions of each cluster varied among 3 detected groups (Fig. 5B). Eight clusters (including cluster 3, 9, 10, 11, 15, 18, 20, 21) increased and 4 clusters (including cluster 5, 13, 16, 17) decreased significantly in patients with NMOSD and MOGAD, compared to HC group. Heatmap of abundance of cell events per cluster (Fig. 5C) and each representative surface marker expressions: CD14, CD16, CD86, HLA-DR, CD11b and CD11c (Fig. 5D). Compared to HC group, we found a lower proportion of monocytes and a higher proportion of macrophages in patients with NMOSD and MOGAD groups (Fig. 5E, F and G). The lower proportion of classical monocytes subset may be the
main cause of monocyte decrease, and the higher proportion of macrophages attributed to CD163 + macrophages increase (Fig. 5F and G).

**Identification of key genes for MNPs subsets differentiation**

We used scatter and heatmap to identify the UP or DOWN differentially expressed genes (DEGs) among datasets. There were 563 Up and 331 DOWN of DEGs in NMOSD compared to HC group, meanwhile there were 742 UP and 476 DOWN DEGs in MOGAD patients compared to HC. The fold changes (Log [Fold change] > 1.5, \( p < 0.05 \)) distribution of DEGs obtained from RNA sequencing of PBMC samples show similar trends in NMOSD and MOGAD groups (Fig. 6A&B). We observe a significant higher expression of CCR2 as one of the up-regulated genes in samples of patients with NMOSD and MOGAD, compared to HC by using Venn diagram software (https://jvenn.toulouse.inrae.fr/app/example.html) (Fig. 6C). CCR2 gene is mainly expressed in MNPs and activated NK cells [20, 21]. q-PCR analysis further verified the CCR2 elevation (Fig. 6D). t-SNE maps show CCR2 + MNPs distribution and proportion of HC, NMOSD and MOGAD patients (Fig. 6E).

**Validation monocytes subset changes by fluorescence cytometry**

Violin plots show CCR2 + macrophages elevation and CCR2 + monocytes decline significantly in patients with NMOSD and MOGAD, compared to HC (Fig. 6F). Monocytes are innate blood cells and three distinct human monocyte subsets could be defined as classical (CD14++CD16–), intermediate (CD14++CD16+), and nonclassical (CD14-CD16+) based on the expression of CD14 and CD16 surface antigens [22]. For monocytes, CCR2 is the crucial receptor responsible for their migration out of bone marrow following the binding of chemokine CCL2. Violin plots show CCR2 + classical monocytes decreased significantly in NMOSD patients, CCR2 + nonclassical monocytes decreased markedly in both NMOSD and MOGAD patients. No significant changes were observed in CCR2 + intermediate monocytes among HC, NMOSD and MOGAD groups (Fig. 6F).

Moreover, we verified the percentage of CCR2 + monocytes and measured each subset of monocytes in PBMCs from HC, NMOSD and MOGAD patients by traditional flow cytometry (Fig. 6G). The proportion of total monocytes was significantly decreased in both NMOSD and MOGAD patients compared to that of HC. The relative proportions of the three monocyte subsets showed that the proportion of classical monocytes decrease, while nonclassical and intermediate monocytes increase significantly (Fig. 6H). Consistent with above, the expression of CCR2 was decreased on monocytes. CCR2 + nonclassical monocytes decreased markedly in NMOSD and MOGAD patients, CCR2 + intermediate monocytes decreased significantly in MOGAD patients. No significant changes were observed in CCR2 + classical monocytes among HC, NMOSD and MOGAD groups (Fig. 6I).

**Decreased CD56 + NK cells in PBMC of patients with NMOSD and MOGAD, compared to HC**
Human NK cells comprise approximately 15% of all circulating lymphocytes [23]. They can be subdivided into different populations with distinct phenotypic properties based on the relative expression of the surface markers CD16 and CD56 [24]. The two major subsets are CD56^{bright} (CD16-) and CD56^{dim} (CD16+), respectively. A significant decrease of CD56^{bright} NK cell in PBMCs samples of NMOSD and MOGAD patients compared to HC (Fig. 7A). Especially lower T-bet expression of the CD56^{bright} NK cells in NMOSD and MOGAD patients than in the HC group (Fig. 7B). No significant differences of CCR2 expression in the CD56^{bright} NK cells were observed among above 3 groups (Fig. 7C). However, there was no significant difference in the proportions of CD56^{dim} NK cells in the 3 studied groups (Fig. 7D). T-bet and CCR2 expressions on CD56^{dim} NK cells were both significantly decreased in patients with NMOSD and MOGAD compared to HC group (Fig. 7E&F).

**Paired comparation of PBMCs specific phenotypes between the acute and remission phases in NMOSD patients**

We selected four-pair matched samples from the same patients with NMOSD in the acute and remission phases for comparison of PBMCs phenotypes. Through paired samples between acute and remission comparison, we found that the proportion of NK cells in the acute phase was significantly higher than that in the remission phase. No significant changes were observed in B cells, T cells, and MNPs between the acute and remission phases (Fig. 8A). In T-cell subsets, the percentage of DNT cells was significantly higher in NMOSD patients during the acute phase than in remission. In MNP subsets, the proportions of non-classical monocytes were significantly higher than those in the remission phase, while the proportions of classical monocytes and CD163+ macrophages in the acute phase were significantly lower than those in the remission phase (Fig. 8B).

**Discussion**

Here, we applied high-dimensional single-cell mass cytometry in combination with phenograph-based algorithms to provide an overview of the circulating immune cell landscape in patients with NMOSD or MOGAD compared to healthy individuals. Using a panel of 36 markers expressed on or intrinsic PBMCs by CyTOF analysis, we identified T, B, NK cells and MNPs as separate populations by extensive surface protein phenotyping. We noted that obvious differences in T cells, NK cells and MNPs between patients and health. Unexpectedly, no significant difference was observed in the proportion of CD20+ B cells, a subset producing autoantibodies. Furthermore, RNA-sequencing, qPCR and fluorescence cytometry were performed in parallel to identify and characterize multiple distinct immune populations and genes within MNPs.

Autoantibodies are a hallmark of NMOSD and MOGAD. MOGAD cannot be considered as equivalent to AQP4-IgG-seronegative NMOSD [25]. Retrospective studies suggest that targeting CD20+ B cells treatment strategies that work well in NMOSD are not similarly effective in MOGAD [26]. To characterize
the heterogeneity of CD3+ T cells in response to autoantibodies-mediated pathogenesis, CD4 and CD8 T cells, DPT and DNT cells, NKT cells, naïve T cells, CD20 T cells and CD11c T cells were further profiled (Fig. 2). When compared to those in HC group, we found that the proportions of CD4 T cells and CD20 T cells were increased significantly in patients with NMOSD and MOGAD, respectively, whereas DPT cells, naïve T cells and CD3 + CD33 + T cells were generally decreased in demyelinated patients. The frequency of NKT cells was significantly different between NMOSD and MOGAD patients. CD4 + T cells outnumber CD8 + T cells perivascular infiltrated in the brain parenchyma lesion of patients with NMOSD and MOGAD, while CD8 + T cells predominate in MS [27]. In periphery, AQP4 peptides were recognized by T cells, which then polarized to a helper T-cell subtype 17 (Th17) and provided help to B cells differentiation into plasmablasts that secrete AQP4-IgG [28]. CD20 T cells are a population of CD3 + T cells co-expressing CD20 that make up to 3–5% of the CD3 + T-cell compartment in the peripheral blood. Emerging studies have shown a pathogenic behavior of CD20 T cells in autoimmune diseases and patients with these diseases may benefit from anti-CD20 immunotherapy. Meanwhile, CD3 + CD20 + T cells may also play a protective role in ovarian cancer and HIV infection for their strong propensity to IFN-γ production [29]. Patients with MOGAD treated with rituximab experience relapses and appeared to be less robust than NMOSD despite a complete B-cell depletion. Severe infections and hypogammaglobulinemia occurred in a significant proportion of MOGAD patients [30]. In MOGAD, depletion of CD20 T cells by rituximab administration may lead to reduction of protective function, disease relapse and severe infections in patients. Although NMOSD and MOGAD are now recognized as separate disease entities, these disorders share inherent similarities, DPT cells, naïve T cells and CD3 + CD33 + T cells decreased synchronously.

Distinct T cell subsets could be associated with resistance to different classes of pathogens, aberrant T cell activity contributes to inflammatory and autoimmune conditions [18]. T-bet, defined as a Th1-specific T box transcription factor, controls the expression of the hallmark Th1 cytokine, IFN-γ and expresses in Th1 and NK cells [17]. T-bet positive T cells or their CD4+ and CD8+ subsets decreased in both NMOSD and MOGAD patients (Fig. 3). T-cells or its subset dysfunction lead to an inflammatory environment in the peripheral blood, which allows auto-antibodies production and penetration into CNS inducing destructive attack [31]. Compared to HC group, the proportions of T-bet + DNT cells were significantly decreased in NMOSD patients, suggesting widespread dysfunction of T cell in NMOSD.

Using a combination of clustering algorithms, we identified three monocyte subsets and two macrophage subsets within human blood of healthy individuals and in subjects with NMOSD or MOGAD (Fig. 4). We found significant decreases in proportions of monocytes and its classical subset in demyelinated patients. However, nonclassical and intermediate monocytes defined by CD14 and CD16 are frequently increased in patients. In addition to CD14 and CD16, CCR2, CD36, HLA-DR and CD11c are the most informative markers and improve monocyte subset definition [32]. RNA-sequencing and qPCR approaches were performed to screen and identify CCR2 expression elevation in demyelinated patients. We further verified the proportion of CCR2 + monocytes subset in other patients (Fig. 5). Nonclassical monocytes have been shown to play a protective role of vascular endothelium and patrol in the blood vessel, clear dying endothelial cells and maintain vascular homeostasis [22]. We found a significant decrease of CCR2 + nonclassical monocytes in demyelinated disease, with an average of approximately
9.92% and 11.00% for NMOSD and MOGAD, respectively, which may also contribute to the pathogenesis of disease. The phagocytic mononuclear cells are capable of taking up pathogens and debris. We found the proportion of macrophages increased significantly in patients compared to HC, especially CD163+ macrophages (Fig. 4). We observed higher CCR2 expression on macrophages and lower on monocytes in disease groups (Fig. 5).

Cytotoxic immune cells play a crucial role in inflammation and immune surveillance. There are two types of cytotoxic cells: one is NK or NKT cells, the other is CD8+ T cells. NK cells were considered as innate immune cells by their strong cytotoxicity against virus-infected cells and important effector functions in anti-tumoral immune responses [33]. As T-bet and CCR2 were both expressed on NK cells, we further measured the proportion of T-bet or CCR2 expression in two NK subsets, CD56bright and CD56dim NK cells (Fig. 6). T-bet expression decreased significantly either on CD56bright or CD56dim NK cells, while expression of CCR2 decreased only in CD56dim NK cells, suggesting dysfunction of the cytotoxicity and immune surveillance in CD56dim subset.

To identify circulating immune cells that may play much more important roles for trigger acute disease processing, we profiled paired PBMCs fractions obtained from 4 NMOSD patients during acute attacks and remission periods. The proportions of immune cells and their subsets including DNT cells, classical monocytes, nonclassical monocytes and CD163+ macrophages were measured and compared (Fig. 7). DNT cells and nonclassical monocytes increased significantly during acute attack compared to remission phase, suggesting immunological and inflammatory homeostasis destruction in peripheral blood of NMOSD patients. Meanwhile, we found that classical monocytes and CD163+ macrophages decreased in acute phases. Given classical monocytes highly expressed CCR2, massive classical monocytes migrate into CNS under the chemotaxis of CCL2 derived from astrocytes induced by NMO-IgG damage in NMOSD [16], which may cause a significant decrease in classical monocytes in periphery. CD163, a scavenger receptor expressed on macrophage, may shed from macrophages massively in response to acute inflammation and demyelination, leading to the decreased frequencies of CD163+ macrophage during acute phase.

There are several limitations of this study. First, the sample size was small and patients were from a single-center, the study may have selection bias. Second, our study did not further verify the function of immune cells through in vitro or in vivo functional experiments. Third, sera AQP4-IgG levels do not always directly correspond with B-cell depletion in patients with NMOSD, indicating heterogeneous properties of B-cell populations. In the present study, we cluster B cells through regular markers such as CD19 or CD20, specific antibody-secreting cells (ASCs) markers such as CD27, MS4A1, MK67, SDC1 [34] should be further identified in future. Fourth, comorbidities, such as hypertension, hypercholesterolemia, and diabetes may affect PBMCs. All control subjects enrolled in the current study were healthy, and only age and sex matched, and they may have few comorbidities.

Conclusions
Taken together, our data systematically characterized the circulating PBMCs landscape in HC, NMOSD and MOGAD patients, and reveal the dysfunction of circulating immune cells may play potential roles in the development of demyelinated diseases, such as NMOSD or MOGAD.

**Abbreviations**

NMOSD: Neuromyelitis optica spectrum disorders; MOGAD: myelin oligodendrocyte glycoprotein-antibody associated disease; CyTOF: Cytometry by time-of-flight mass spectrometry; PBMCs: peripheral blood mononuclear cells; HC: healthy controls; NMPs: mononuclear phagocytes; NK: natural killer; CNS: central nervous system; AQP4-IgG: antibodies directed against the water channel aquaporin-4; MOG-IgG: antibodies against myelin oligodendrocyte glycoprotein; CBA: cell-based assay; MS: multiple sclerosis; EDTA: ethylenediaminetetraacetic acid; RBC: red blood cell; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DPT: positive T cells; DNT: double-negative T cells; DEGs: differentially expressed genes.

**Declarations**

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Availability of data and materials

The original data are available from the corresponding author upon request.

Data availability

The raw CyTOF data used and analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

References


**Figures**
Workflow. The study included peripheral blood from 33 patients who were diagnosed as NMOSD, 12 patients diagnosed as MOGAD, and 15 age-matched healthy individual controls. Mass cytometry was performed on all the patients and healthy individual controls, and some of these patients and healthy individual controls (6 NMOSD and 3 MOGAD patients vs. 3 healthy individuals) were performed for RNA-seq analysis. In addition, 6 NMOSD and 3 MOGAD patients vs. 4 healthy individuals, were confirmed using flow cytometry. The workflow of the study is depicted in this figure.
Figure 2

Single-cell mass cytometry profiling of PBMCs in HC, or patients with NMOSD and MOGAD. (A) According to the relative expression markers, high-dimensional mass cytometry clustering reveals distinct cell type subpopulations. CD45+ PBMCs were divided into 29 clusters. About 4,000 CD45+ PBMCs per sample were included in the viSNE analysis. (B) Cluster abundances in HC and patients with NMOSD or MOGAD. (C) A heatmap shows the cluster percentage of all participants in the 29 t-SNE clusters. (D) ViSNE plots of
complete immune systems according to the relative expression of CyTOF markers in all samples. The cell populations are also indicated (left). (E) Composition of the CD45+ PBMCs showing immune lineages in HC, NMOSD and MOGAD groups. (F) Column graphs showing the frequencies for each subset of CD45+ PBMCs in HC, NMOSD and MOGAD. Data were presented as mean ± SEM; *p < 0.05.
T lymphoid lineage profiles of patients with NMOSD or MOGAD and HC in peripheral blood by CyTOF. (A) A total of 22 clusters were identified in all participants plotted on bivariate viSNE plot. (B) The percentage of total event count per cluster was shown. (C) The SPADEVizR method was used to analyze differences in immune composition among HC, NMOSD and MOGAD, and the results were presented on a volcanic map. (D) Heatmap of abundance for cell events per cluster and each surface marker were shown. (E) Column graphs showed significant differences in clusters of NMOSD and MOGAD patients compared to HC. Data were presented as mean ± SEM; bars represent maximum and minimum values, and dots represent individual samples. *p < 0.05; ***p < 0.001.

Figure 4

T-bet positive T cell subset analysis. (A) Statistical t-SNE map obtained from patients with NMOSD or MOGAD and HC for differential abundance using phenograph analysis. (B) Violin plot shows the T-bet expression level of HC, NMOSD and MOGAD. (C) The column graph shows mean frequencies of each subset as % of total T cells. Data were presented as mean ± SEM; bars represent maximum and minimum values, and dots represent individual samples. **p < 0.01; ***p < 0.001.
Figure 5

Differential abundances of monocytes and macrophages subsets in patients with NMOSD or MOGAD compared to HC. (A) t-SNE map highlights monocytes and macrophages population and identifies 23 clusters. (B) The bar graph shows mean cluster frequencies as % of total monocytes and macrophages. (C) Heatmap and cluster analysis from all samples on the basis of the mean marker expressions on monocytes and macrophages. (D) The representative t-SNE plot shows CD14, CD16, CD86, HLA-DR,
CD11b, CD11c, CD163, IRF4, and IRF8 expression levels of monocytes and macrophages subsets. (E) Ring charts show the relative frequency of monocytes and macrophages subset distributions. The column graph shows mean frequencies of the monocytes (F) or macrophages (G) subset as % of total mononuclear phagocytes. Data were presented as mean ± SEM; bars represent maximum and minimum values, and dots represent individual samples. *p < 0.05; **p < 0.01; ***p < 0.001.
Deeply identify subsets of MNPs according to CCR2 gene screening from RNA-sequencing datasets. (A) Scatter plot showing DEGs between peripheral blood of NMOSD and MOGAD patients and HC. (B) Heatmap displaying the expression pattern of differentially expressed genes in A. (C) Venn diagram showed the overlap of the detected DEGs in PBMCs of HC, NMOSD and MOGAD patients. (D) q-PCR analysis identified the alterations of CCR2 expressions in PBMCs of NMOSD and MOGAD patients. (E) t-SNE maps show the distribution of CCR2+ MNPs in HC, NMOSD and MOGAD groups. (F) Violin plots show the volume of all samples at each point by width and lines correspond to the 25th percentile, median, and 75th percentile. (G) The gating strategy in flow cytometry. a: CD14-CD16++ monocytes (nonclassical), b: CD14++CD16+ monocytes (intermediate), c: CD14++CD16- monocytes (classical). (H) The proportion of total monocytes in PBMC and three subsets. (I) The column graphs show percentage of the CCR2 positive monocytes of total monocytes in PBMC and three subsets in monocytes. Bar charts show the percentage of monocytes and each subpopulation. Percentage data were presented as mean ± SEM; bars represent maximum and minimum values, and dots represent individual samples. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 7

Percentages of CD56+ NK cell and its subsets in patients with NMOSD or MOGAD compared to HC. (A) Percentage of CD56\textsuperscript{bright} NK cells in PBMCs samples. Violin plots show the distribution of CD56\textsuperscript{bright} T-bet...
(B) and CD56\textsuperscript{bright} CCR2 (C) subsets, respectively. (D) Percentage of CD56\textsuperscript{dim} NK cells in PBMCs samples. Violin plots show the distribution of CD56\textsuperscript{dim} T-bet (E) and CD56\textsuperscript{dim} CCR2 (F) subsets, respectively. Percentage data were presented as mean ± SEM; bars represent maximum and minimum values, and dots represent individual samples. **p < 0.01; ***p < 0.001.

**Figure 8**

Percentages of PBMCs phenotypes and their subsets in patients with NMOSD during acute and remission phases. Data show the change of percentages of each PBMCs phenotypes (A) and subsets in PBMCs (B). *p < 0.05; **p < 0.01.

**Supplementary Files**

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- SupplementaryTable1.docx
- SupplementaryTable2.docx
• FigureS1.pdf
• FigureS2.pdf