

CD21^{lo} B cell subsets are recruited to the central nervous system in acute neuromyelitis optica

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Abstract

Neuromyelitis optica (NMO) is an acute inflammatory demyelinating disease of the CNS. The presence of astrocyte-targeted AQP4-immunoglobulin G (IgG) in peripheral blood is a major factor in its diagnosis. Previous studies show that AQP4-IgG directly contributes to CNS inflammation, and B cells play a central pathogenic role in NMO. However, where and how B cell response is altered remains controversial. In this study, we comprehensively analyzed with high-parameter flow cytometry the immune cell populations in the CSF samples obtained from first-episode acute-phase NMO patients, and compared to those from patients with acute-phase Multiple Sclerosis (MS) and other neurological diseases (OND). Among ten immune cell populations defined in the analysis, only the frequency of B cells and antibody-secreting cells (ASC) were higher in the CSF of acute-phase NMO compared to OND. Detailed assessments of B cell and ASC subsets in the CSF revealed differences in the dominant subsets between NMO and MS. In NMO, a series of CD21^{lo} B cell subsets ranging from “activated” naïve B, double negative, and switched memory, thus subsets considered as ASC-precursors, were dominant. A majority of these CD21^{lo} B cell subsets expressed CD69 and CXCR3, suggesting their CNS residency. An increase of CD21^{lo} B cell subsets was also observed in the CSF of treatment-refractory NMO patients. Furthermore, two B helper T cell subsets, T peripheral helper type1 and T follicular helper type1 cells, both highly expressing CD69 and CXCR3, were enriched in the CSF of NMO patients, suggesting their interactions with ASC-precursors in the CNS. In vitro culture experiments using blood samples from patients with NMO showed that CD21^{lo} B cells contained AQP4-IgG-producing cells and displayed a high propensity to differentiate into ASCs. We further found that

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CD21^{lo} B cell subsets in NMO upregulated the expression of C5a receptors, and C5a signals promoted their differentiation into ASCs. ASCs derived from CD21^{lo} B cells expressed high levels of CXCR3 and CD138. The increase in CD21^{lo} B cell subsets significantly correlated with the annual relapse rate. Collectively, our study highly suggests that the mechanism to promote the generation of CD21^{lo} B cells, likely via the extrafollicular pathway, becomes activated during the acute phase of NMO, and the generated CD21^{lo} B cell subsets contribute to the pathogenesis. Targeting CD21^{lo} B cell subsets might be useful for the development of novel therapeutic approaches.

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Introduction

Neuromyelitis optica (NMO) is an inflammatory disease of the central nervous system (CNS) that has historically diverged from multiple sclerosis (MS).^{1,2} The diagnostic criteria for NMO have been well-established, and the presence of serum IgG specific for AQP4 (AQP4-IgG) is one of the most important factors for the diagnosis.³ AQP4 is highly expressed by astrocytes, and AQP4-IgG recognizes its conformational epitopes and mediates AQP4-directed antibody-dependent cellular cytotoxicity and complement-mediated lysis.⁴ Pathological assessment shows perivascular deposition of antibody-complement complexes in the brain.⁵ Accordingly, therapies with monoclonal antibodies targeting the surface proteins of B cells (CD19, CD20) and the complement C5 have been very effective in preventing NMO relapse.⁶⁻⁸ Thus, AQP4-IgG directly contributes to CNS inflammation in NMO, and B cells play a central pathogenic role.

In NMO, antibody-secreting cells (ASC) increase in peripheral blood, and serum AQP4-IgG titers are higher in the serum than in the CSF.⁹ While AQP4-IgG titers also increase in CSF during an attack,¹⁰ a recent study showed that NMO patients develop another auto-antibody against GRP78, which contributes to the disruption of the blood-brain barrier and promotes the entrance of AQP4-IgG into the CNS.¹¹ Therefore, it is generally considered that AQP4-IgG is primarily generated in peripheral lymphoid organs¹² rather than in the CNS. On the other hand, there is evidence that AQP4-IgG is produced in the CNS by ASCs¹³ differentiated from their precursors *in situ*, based on the B cell repertoire comparison between the CNS and peripheral blood.¹⁴⁻¹⁶ Thus, where and how B cell response is altered remains controversial. Furthermore, the mechanisms accelerating the differentiation of ASCs in NMO remain unclear.

1 In addition, mouse models for NMO showed that circulating AQP4-IgG in the blood itself is
2 insufficient to cause the disease.¹⁷ To induce sufficient inflammation in the CNS,⁴ intrathecal
3 administration of AQP4-IgG together with complement¹⁸ or co-induction of encephalitogenic T-
4 cells¹⁹ are required. Also, in the clinic, AQP4-specific T cells responses are amplified in NMO.²⁰
5 Some individuals remain asymptomatic even with the presence of AQP4-IgG in the serum.²¹
6 Furthermore, successful treatment with prednisolone or B cell-depleting treatments rarely results
7 in a substantial decrease in serum AQP4-IgG levels in NMO patients.^{22,23} Several reports
8 demonstrate that AQP4-IgG *per se* has a low predictive value for disease recurrence.²⁴ Such
9 evidence in mice and humans suggests that factors other than serum AQP4-IgG are also involved
10 in NMO pathogenesis.

11 NMO is more prone to cause pleocytosis than MS, especially in the acute phase^{10,25}. However,
12 what immune cells constitute in the CSF and how they contribute to excessive ASC generation
13 remain largely unknown. Analysis of CSF is understudied in NMO, largely due to the lack of
14 necessity to examine CSF for diagnosis. Treatment is often initiated promptly with a provisional
15 diagnosis, and collecting CSF samples of acutely untreated patients is extremely challenging.

16 In this study, to gain insights into the mechanisms of B cell alterations in the CNS of NMO, we
17 comprehensively analyzed the immune cell population with high-parameter flow cytometry
18 (FCM) using paired CSF-blood samples from first-episode acute-phase patients. We also analyzed
19 CSF and blood samples obtained from patients with treatment refractory NMO. Our study shows
20 that the CSF of NMO was enriched with ASCs and a series of CD21^{lo} B cell subsets, thus are ASC
21 precursors. Comparison of the B cell subsets between CSF vs. blood in NMO and between CSF in
22 NMO vs. CSF in MS provided evidence that at least a fraction of ASCs is differentiated *in situ* in
23 the CNS in NMO. Consistently, we also found that the dominant B-helper T cell subsets present
24 in the CSF of NMO shared the phenotype with B cells, suggesting their interactions *in situ*.
25 Furthermore, we show that the complement C5 contributes to B cell differentiation into ASCs in
26 NMO.

27

Materials and methods

Subjects

The study involved 38 patients with NMO, 10 with MS, and 40 with Other Neurological Diseases (OND, including Parkinson's disease, Alzheimer's disease, mild cognitive impairment, epilepsy, spinal cord tumor, recurrent ischemic stroke, CIDP, intracranial hypertension, chronic fatigue syndrome, and bipolar disorders), who presented at specialized neurological disease centers from May 2020 to November 2024, and eight healthy subjects (HS). For Spectral FCM analysis, paired fresh CSF and peripheral blood samples were obtained from untreated first-episode patients with NMO (n=6) or MS (n=9). Blood samples from one NMO and one MS patient were excluded because they were collected post-treatment initiation. CSF samples were collected from two patients with relapsing NMO (on low-dose prednisolone alone or in combination with tacrolimus) and a patient with relapsing MS (on dimethyl fumarate). CSF samples were collected by clinicians for clinical practice. Patients with NMO met specific criteria for NMO spectrum disorders with AQP4-IgG (IPND).³ We confirmed that patients with MS were negative for AQP4-IgG and MOG-IgG, and met the McDonald's criteria.²⁶ Paired CSF and PBMC samples were simultaneously analyzed for the global immune cell populations with spectral FCM. All of these samples were obtained at a single institution (Kyoto University Hospital), and patients who received immunotherapy within a year were excluded from this analysis. Another set of blood samples was collected from 30 NMO patients under maintenance treatment at multiple clinical centers, and used for the experiments with conventional FCM. Treatments included oral steroids (30 patients), azathioprine or tacrolimus (21 patients), and eculizumab (1 patient). Of the 30 patients, five patients with NMO (subject number NMO30-34 in supplementary table2) were started on treatment with eculizumab within one week of the first blood sample collection and had another blood sample taken two months after induction. Additional paired CSF and blood samples were obtained from 4 non-acute treatment-refractory NMO patients at 1–7 months after the last attack: Two cases with no recurrent lesions on MRI but with fluctuating symptoms (one of which had a minor imaging recurrence on MRI 2 months after CSF collection), and two cases with repeated recurrences and admitted for evaluation prior to the introduction of a biologic agent. Written informed consent was obtained from all subjects and samples were used with approval by the

Ethics Committee of Kyoto University Hospital. This study was conducted in accordance with the declaration of Helsinki. The patient characteristics are shown in the supplementary table 1,2.

Cell isolation

Fresh CSF was centrifuged at 3000 rpm for 5 minutes, and the cells were used for the experiments, whereas the supernatant was used for clinical testing. Blood samples paired with CSF samples were collected from the same patients on the same day or the following day. Peripheral Blood Mononuclear Cells (PBMCs) were isolated using lymphocyte separation solution (Nacalai) and either used immediately or frozen in LN₂ for long-term storage.

Flow cytometry analysis

Two types of FCM, spectral and conventional, were used in this study. The information of fluorochrome-conjugated antibodies is described in Supplementary Table 3A, B.

Spectral FCM

A spectral FCM Cytex Aurora was used for the analysis of freshly obtained samples. PBMCs and CSF cells were simultaneously analyzed with a 33-parameter flow panel, including basic cell information (FSC, SSC, dead cell) and cell lineage markers. To reliably analyze few cells present in the CSF samples, PBMC and CSF samples were first labeled separately with anti-CD45 conjugated with different fluorochrome and then stained together for the 33-parameter flow panel. This strategy permits a direct comparison of immune cell subsets between the CSF and blood samples by applying the same gating strategies (Fig.1A). In more detail, both PBMCs and CSF cells were first treated with Fc blocking. This was followed by a 5-minute staining procedure with a dead cell stain, then a 5-minute staining with either CD45-PerCP (blood) and CD45-APC/Fire810 (CSF). After a separate wash, PBMC and CSF samples were mixed and then incubated for 20 minutes at 4°C in the dark with 100 µl of antibody mix. After a wash, the mixed cells were resuspended in 1 ml of FACS buffer and analyzed with Cytex Aurora. CSF sample from the OND group was analyzed together with a healthy subject stock PBMC.

1 Conventional FCM

2 FACS BD Aria Fusion was used for the analysis of frozen/thawed PBMC samples and in some
3 culture experiments. Fc-blocking, dead cell staining, and antibody staining were performed as
4 described above.

5 Analysis of FCM data

6 The data from four NMO, eight MS, and 22 OND were analyzed using FlowSOM and X-Shift
7 clustering. In the spectral FCM data analysis, we first excluded doublets and dead cells, and
8 separated the cells into CSF- and PBMC-derived cells based on different anti-CD45 fluorochrome.
9 Next, we concatenated all datasets for the comprehensive immune cell analysis. To avoid the
10 strong influence of specific samples, 30,000 cells from PBMC of each NMO case, 25,000 from
11 PBMC of each MS case, and up to 10,000 from CSF samples of each case were randomly extracted
12 and concatenated. Unsupervised clustering was performed with the FlowSOM²⁶ and X-shift²⁷
13 provided as add-on packages in FlowJo. For dimensionality reduction, UMAP (Uniform Manifold
14 Approximation and Projection) was employed. Defined clusters were further analyzed with the
15 Cluster Explorer in FlowJo. These tools were used for the visualization of the defined cell clusters.
16 This was followed by statistical analysis and representation of the results, including cluster
17 frequencies and marker expression levels. To validate the results obtained with FlowSOM and X-
18 shift clustering, the subsets of interest were defined by manual gating to determine their frequency.
19 The detailed gating strategy is presented in Supplementary Figure 1A. Because CSF samples
20 contained only a small number of cells, the gating was determined by referring to the FMO and
21 simultaneously analyzed blood samples (Supplementary Figure 1A, B).

22 Cell culture

23 Frozen/thawed PBMCs were allowed to rest for 3 hours in RPMI complete medium (Gibco). The
24 cells were stained with CFSE to assess cell proliferation and were seeded at a density of 100,000
25 cells per well in U-bottom 96 well plates (TPP). PBMCs were cultured for 5 days in RPMI medium
26 supplemented with 10% fetal bovine serum supplemented with soluble CD40L (100 ng/ml,
27 BioLegend) and IL-21 (10 ng/ml CD40L (100 ng/ml, BioLegend). In the culture experiment of
28 sorted B cells, thawed PBMCs were sorted with FACS BD Melody to isolate CD3⁻ CD14⁻ CD19⁺

B cells. After sorting, B cells were washed, stained with CFSE, counted, seeded at 30,000 cells per well in U-bottom 96 well plates, and cultured in RPMI complete medium containing 10% FCS with the addition of soluble CD40L (100 ng/ml) and IL-21 (30 ng/ml). L cells stably transfected with human CD40L (CD40L-L cells) were generated by transducing the CD40L gene into L cells using a lentiviral vector purchased from the DNASU Plasmid Repository and transfected into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). CD40L-expressing L cells were selected by sorting from spin-infected L cells. For culturing CD21^{lo/+} B subsets, PBMCs were first magnetically separated into IgD-positive and IgD-negative fractions using IgD microbeads (Miltenyi), and six subfractions, CD21^{lo/+} NAV, CD21^{lo/+} SWM, and CD21^{lo/+} DN, were FACS-sorted from IgD-positive and IgD-negative fractions. Sorted B cell subsets were cultured at 1,000 cells per well with IL-21 (50 ng/ml, BioLegend) for 7 days in flat-bottom 96 well plates pre-seeded with CD40L-L cells. In some experiments, recombinant human C5a (100 ng/ml, R&D Systems) was also added to the culture. FBS used for culture was heat-inactivated prior to usage (56°C for 30 min). The absence of human C5a in the culture medium was confirmed by using C5a ELISA kit (Abcam). Each experiment was performed in triplicate. After the culture period, the supernatants were collected for ELISA, and the cultured cells were analyzed with FCM. The detailed information on the reagents used in the experiments is described in Supplementary Table 3C.

Measuring total IgG and AQP4-IgG in Cell Culture Supernatants

The cell culture supernatants were diluted appropriately to fall within the standard curve range. The experiment utilized 96-well plates pre-coated with anti-human IgG antibody, as provided in the Invitrogen IgG (Total) Human ELISA Kit. Plates were washed twice, and the assay buffer, diluted standards, and final prediluted samples were added in duplicate to the designated blank wells. HRP-conjugated anti-human IgG detection antibody, as supplied in the kit, was added to each well. The plates were incubated for 60 minutes. After washing, substrate solution was added to each well and incubated for 30 minutes. The reaction was terminated by adding a stop solution to each well. Absorbance was measured at 450 nm using a SpectraMax iD3 ELISA plate reader (Molecular Devices). The concentration of IgG in the samples was calculated based on a standard curve generated from the provided standards. To assess AQP4-IgG levels, supernatants were concentrated with Amicon Ultra (50kDa), re-diluted to 1x, 4x, and 16x, and measured semi-

quantitatively with a live immunofluorescent cell-based assay (CBA) validated previously (Cosmic Co. Ltd).²⁸ In CBA, imaging was performed using EUROStar III Plus (EUROIMMUN) under the conditions of a 40x objective lens and a 10x eyepiece lens, and the final visual assessment was performed blind to the culture condition.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v10.0.2 (GraphPad Software). Considering that the samples were obtained from NMO, MS, and OND groups with heterogeneous populations in terms of disease status, nonparametric tests were used to compare disease groups. CD20+ B cells and ASC in the OND group were extremely low in cell counts and thus were excluded from the analysis of subclusters. Comparisons between two groups were performed using the Mann-Whitney U test (unpaired), and comparisons between multiple groups were performed using the Kruskal-Wallis test followed by Dunn's test. Comparisons of the CSF and blood, including samples from the CSF OND group, were performed using the Mann-Whitney U test (unpaired). Comparison of CSF-blood paired samples from NMO and MS cases were statistically analyzed using the paired t-test, considering the homogeneity of the biological background, the fact that the value of paired samples was measured simultaneously, and statistical distribution. Correlation analysis was performed using Pearson correlation. For all calculations, P values less than 0.05 were considered statistically significant.

Results

Global immune cell composition in the CSF of acute-phase NMO

Paired fresh CSF and peripheral blood samples were obtained from untreated first-episode patients with NMO (n=4) or MS (n=8). Paired CSF and PBMC samples were simultaneously analyzed for the global immune cell subsets with spectral FCM. To this end, the samples were first labeled separately with anti-CD45 conjugated with different fluorochrome and then stained together for an FCM panel. This strategy permits a direct comparison of immune cell subsets between the CSF and blood samples by applying the same gating strategies. CSF samples were also obtained from

1 patients with other neurological diseases (ONDs) with no history of immune-modulatory
2 treatments as controls (n=22).

3 To comprehensively analyze immune cell subsets in the CSF and blood, the data of CD45⁺
4 hematopoietic cells from 34 CSF and 11 blood samples were concatenated (total 375,243 cells)
5 and analyzed with UMAP for dimensional reduction. X-shift and FlowSOM were used to define
6 cell clusters (Fig. 1A). Ten immune cell clusters were identified, including CD20⁺ B cells (CD20⁺
7 B), antibody-secreting cells (ASC), CD8⁺ T cells, CD4⁺ and double negative T cells (CD4⁺ & DN
8 T), NK cells, dendritic cells (DC), CD14⁺ monocytes, and CD14⁺CD16⁺ intermediate
9 monocytes²⁹, neutrophils, and basophils (Fig. 1B, C). Intermediate monocyte uniquely present in
10 the CSF displayed the phenotype of HLA-DR^{hi} CD38^{lo}.

11 We compared the composition of cell clusters among the CSF samples of NMO, MS, and OND.
12 We found that the frequency of CD20⁺ B cells and ASCs was higher in NMO and MS than in
13 OND (Fig.1D, E, Supplementary table 4). Furthermore, ASCs were more enriched among the B
14 lineage cells in the CSF than in the blood of NMO and MS patients (Fig.1F). This suggests the
15 preferential migration of ASCs toward CSF and/or their *de novo* generation in the CNS.
16 Intermediate monocytes were absent both in the CSF of NMO and MS. The frequency of
17 monocytes and DC were lower in the CSF of MS than in NMO (Fig. 1E). Other cell clusters were
18 not substantially different among the CSF samples of NMO, MS, and OND (not shown).

19 To strengthen our findings on CSF immune cells, we added four additional cases of acute-phase
20 NMO (two of which were relapse cases), two cases of acute-phase MS, and five cases of ONDs to
21 the 34 cases already analyzed and determined the frequency of the subset of interest using a manual
22 gating strategy (Supplementary Fig. 1A). NMO and MS exhibited a higher frequency of CD20⁺ B
23 and ASCs than OND, while DCs were more common in NMO than in MS (Fig.1G). Of note, one
24 NMO case showed massive granulocyte infiltration, consistent with NMO brain pathology reports
25 (Fig.1G, Supplementary Fig. 1A).⁵

26 Thus, B cells and ASCs were enriched in the CSF of untreated patients with NMO and MS.

27

1 **Increased CD21^{lo} B cell subsets in the CSF of acute-phase NMO**

2 Next, we defined subclusters within CD20⁺ B cells using X-Shift. CD20⁺ B cells were divided into
3 three naïve (Nav. IgD⁺ CD27⁻) and four memory (Mem) subsets (Fig. 2A, B). Memory B cells
4 were more abundant than naïve B cells in the CSF of all the patient groups (Fig. 2B).

5 Nav2 was conventional naïve B cells, whereas Nav1 was “activated” naïve B cells with the
6 phenotype of CD21^{lo} CD38^{hi} CD11c^{dim} ^{30,31} (Fig. 2B, C). Nav3, characterized by low integrin α 4
7 (INT α 4), was a minor subset within the naïve B cells in the CSF. Strikingly, whereas Nav2 was
8 dominant among naïve B cells in the CSF of MS, Nav1 was dominant in NMO (Fig. 2E, F,
9 Supplementary Fig.2A, B). A similar trend was also observed in naïve B cell subsets in the blood
10 (Fig. 2E). Notably, a fraction of Nav1 cells in NMO CSF expressed a tissue-resident marker CD69
11 (Fig. 2D), suggesting their CNS residency.

12 Mem1 displayed the phenotype of CD21^{lo} CD27⁻ IgD⁻ CD11c⁺ (Fig. 2B, C), a population referred
13 to as double-negative 2 (DN2) and one of the ASC-precursors.³² Mem1 highly expressed CXCR3
14 (Fig. 2B) and a fraction of Mem1 in the CSF also expressed CD69 (Fig. 2D). Mem2, a population
15 with CD21^{hi} CXCR3⁻, was absent in the CSF (Fig. 2E). Mem3 and Mem4 were the dominant
16 memory B subsets in the CSF (Fig. 2E) and highly expressed CD69 (Fig. 2D), thus likely represent
17 CNS-resident B cells. Mem3 was characterized by high expression of INT β 1, an integrin important
18 for brain homing.^{33,34} Mem4 contained CD21^{lo} CXCR3⁺ CD11c⁺ cells (Fig. 2B), a subpopulation
19 similar to Mem1 yet positive for CD27, switched memory (SWM) cells, and enriched within the
20 CSF (Fig.2F). The frequency of Mem4 was significantly higher in NMO CSF than in MS CSF
21 (Fig. 2G).

22 ASC were divided into four subsets with X-shift (Fig. 2H). ASC1 and ASC2 highly expressed
23 INT β 1, and ASC2 co-expressed high CXCR3 and CD69 (Fig. 2I, J). ASC2 showed clear
24 enrichment in the CSF and was almost absent in the blood (Fig. 2K, L). ASC3 expressed high
25 INT β 7, and ASC4 expressed high INT α 4 and CXCR3 (Fig. 2I, J). The composition of ASC subsets
26 was also largely different between NMO and MS in the CSF (Fig.2K). Whereas ASC2 and 4,
27 subsets expressing CXCR3 and CD69, were dominant in MS, ASC1 and 2 were dominant in NMO
28 (Fig.2K). In particular, ASC1 absent for CXCR3 expression was more abundant in NMO CSF than
29 in the MS CSF (Fig.2M).

To validate our findings on CSF B-cell subsets, we determined the frequency of the subset of interest using a manual gating strategy (Supplementary Fig. 2C) in additional acute phase NMO, MS, and OND cases as in Figure 1G. CD21^{lo} SWM, CD21^{lo} DN, and CD21^{lo} NAV B subsets were elevated in NMO-CSF compared to those in MS-CSF, including additional cases (Fig. 2N). Compared to blood, CSF contained more CD69⁺CXCR3⁺ CD21^{lo} SWM/ASCs (Fig. 2O).

In summary, various B cell subsets were increased in the CSF of NMO, ranging from CD21^{lo} “activated” naïve B cells, CD21^{lo} DN2-like cells, and CD21^{lo} SWM cells to ASCs. Furthermore, ASCs lacking the expression of CXCR3 (ASC1) were more abundant in NMO CSF than MS CSF, indicating the presence of ASCs accumulated independent of CXCR3 functions. An increase of a series of CD21^{lo} B cell subsets is also observed in the blood of patients with active systemic erythematosus, and these B cells are shown to represent ASC precursors³⁵. These results suggest that B cell differentiation from activated naïve to ASC might occur in the CNS of NMO. Although increased ASCs themselves are commonly found in NMO and MS, differences in the composition of B cell subsets in the CSF between NMO and MS suggest the difference in B cell differentiation mechanisms.

Increased CD21^{lo} B cell subsets in the CSF of non-acute phase NMO

We were intrigued by the observations that B cells in the CSF of acute-phase NMO contained various subsets ranging from CD21^{lo} “activated” naïve B cells to ASC. To examine whether such a broad range of B cells are present only in the acute phase, we analyzed CSF samples freshly obtained from four treatment-refractory NMO patients at 1–7 months after the last attack using conventional FCM (Supplementary Fig.3). We also simultaneously analyzed the paired PBMC samples in three of the four patients. Although lower than at the acute phase, the frequency of ASCs and B cells in NMO CSF at the non-acute phase was still higher than in the control CSF (Fig. 3A, B). Furthermore, the frequency of CD21^{lo} SWN and CD21^{lo} DN was higher in the CSF than in the blood, and CD21^{lo} activated naïve B cells were enriched in the CSF (Fig. 3C). Thus, B cell subsets in the CSF remained altered in treatment-refractory NMO patients and enriched with ASCs and their precursors.

Increased Tph1 and Tfh1 cells in CSF of acute-phase NMO

Next, we examined whether CSF samples of acute-phase NMO contained B-helper T cells, such as T peripheral helper (Tph)^{36,37} and T follicular helper (Tfh)^{37,38} cells. The CSF CD4⁺ T cells were highly enriched with memory CD4⁺ T cells (CD4⁺ CD56⁻ CD45RA⁻) than their blood counterpart (Fig. 4A). Twelve subsets were defined within memory CD4⁺ T cells with X-shift (Fig. 4A, Supplementary Fig. 4A, B). Two out of the twelve subsets, CD69⁺ Tph type 1 (Tph1: CXCR5⁻ PD1^{hi} CXCR3⁺ CCR6⁻)^{36,39,37} and CD69⁺ Tfh type 1 (Tfh1: CXCR5⁺ PD1^{hi} CXCR3⁺ CCR6⁻)^{38,40}, was significantly higher in frequency in the CSF than in the blood (Fig. 4B, C, D). Furthermore, CD69⁺ Tph1 was more frequent in NMO CSF than in MS and OND CSF (Fig. 4E). By contrast, the fraction of Tfh17 (CXCR5⁺ CXCR3⁻ CCR6⁺) was high in MS CSF (Fig. 4C,E).

To validate our findings on CSF T-cell subsets, we determined the frequency of the subset of interest using a manual gating strategy (Supplementary Fig. 4C). CD69⁺ Tph1 and CD69⁺ Tfh1 were more abundant in NMO than in OND (Fig. 4F). Furthermore, the frequency of CD69⁺ Tph1 CSF was substantially higher in CSF than in blood in NMO (Fig. 4G).

DCs were divided into four clusters with X-shift. DC1, 2 and 4 were CD11c⁺ conventional DC subsets, whereas DC3 was CD123⁺ plasmacytoid DC (Fig. 4H, Supplementary Fig. 4D,E). DC4, characterized by high CXCR3 and CCR6 (Fig. 4I), was the most dominant subset in the CNS across the patient groups (Fig. 4J, K). No difference in the composition of DC subsets was observed among the CSF groups of NMO, MS, and OND (Fig. 4J).

Taken together, the acute phase of CSF of NMO contained two B-helper T cell subsets, Tph1 and Tfh1 cells. High expression of PD-1 and CD69 on these subsets suggests their CNS residency.

CD21^{lo} B cell subsets differentiate into ASCs in vitro

Next, we examined B cell subsets in the blood of non-acute NMO patients. We obtained from 25 patients with NMO with diverse clinical courses (Fig. 5A, Supplementary Table 1, 2). There was a relative increase of SWM, DN, and ASCs among blood B cells in NMO than age- and sex-matched controls (Fig. 5B). Importantly, as observed in the CSF, the frequency of CD21^{lo} populations in SWM, DN, and naïve B cells was higher in NMO patients (Fig. 5B).

Our observations that the NMO CSF and blood samples contained a series of B cell subsets with enriched ASCs and their precursors (Fig. 2, 3, 5B) suggest that B cells in NMO display a propensity to differentiate into ASCs. To test this hypothesis, PBMCs obtained from the same 25 patients with NMO as described above (Fig. 5A) were cultured for 5 days with soluble CD40L and IL-21 (to mimic the stimulation from CD4⁺ helper T cells), and analyzed for B cell proliferation and ASC differentiation (Fig. 5A). We found that NMO blood samples yielded higher frequency of ASCs (Fig. 5C) and CD20⁺CD138⁺ plasma cells than control samples (Fig. 5D, Supplementary Fig. 5A). B cell proliferation as well as IgG production were also significantly higher in the culture of NMO blood samples (Fig. 5D).

To gain insights regarding the origin of B cell subsets that differentiated to ASCs in the culture, we examined the correlations in the frequency of B cell subsets before culture (day 0) and ASCs at day 5. We found a strong correlation between day 5 ASCs and day 0 CD21^{lo} SWM cells ($r=0.69$, $p<0.0001$, $n=40$), CD21^{lo} DN ($r=0.58$, $p<0.0001$), and CD21⁺ SWM cells ($r=0.64$, $p<0.0001$) (Fig. 5E), while a strong negative correlation with day 0 CD21⁺ naive cells ($r= -0.65$, $p<0.0001$, $n=40$) (Supplementary Fig. 5B). These results suggest that CD21^{lo} DN B cells and SWM (both CD21^{lo} and CD21⁺) can differentiate into ASCs in the culture with IL-21 and sCD40L. It is important to note that these were the B cell subsets increased in the NMO CSF.

B cells also act as antigen-presenting cells. Specifically, previous studies indicate that CD21^{lo} memory B cells display high antigen-presenting capacity.^{41,42} Indeed, in NMO, CD21^{lo} SWM and DN displayed a higher proportion of CD86⁺ HLA-DR^{hi} cells than their CD21^{hi} counterparts. Approximately 10% of CD21^{lo} NAV were CD86⁺HLA-DR^{hi} cells, whereas few CD21^{hi} NAVs expressed CD86 (Supplementary Fig. 5C, D). Additionally, CD21^{lo} SWMB cells expressed CD40 more strongly than CD21^{lo} DN (Supplementary Fig. 5E).

C5a stimulation promotes ASC differentiation of CD21^{lo} B cells

Treatment with eculizumab, a humanized monoclonal antibody against the complement C5, has been effective in treatment-refractory NMO.⁸ The mode of action of eculizumab is generally considered to prevent C5 cleavage into proinflammatory C5a and C5b, a subcomponent of the membrane attack complex.

Previous reports showed that C5aR1 is expressed in human tonsil B cells and is involved in chemotaxis.⁴³ A recent report also demonstrated that C3aR and C5aR signaling are necessary for effective GC B cell activation and affinity maturation in mice.⁴⁴ Therefore, we wondered whether C5a might contribute to the B cell biology also in NMO. We found that C5aR1 expression was significantly higher on B cells in NMO blood samples than those in the control (Fig. 6A, B). C5aR1⁺ B cells in NMO co-expressed C5aR2, although no significant differences were observed in C5aR2 expression between NMO and the control. Among B cells, SWM and DN expressed higher C5aR1 than naïve B cells in NMO (Fig. 6B). In particular, CD21^{lo} populations in these subsets expressed high C5aR1 (Fig. 6B). By analyzing the CSF and blood samples from untreated first-episode patient groups, we found that C5aR1 expression on B cells and ASCs was higher in NMO as compared to MS (Fig. 6C, Supplementary Fig.6A). C5aR1 expression was particularly high on the dominant B cell subsets in the NMO CSF, including Mem1 (CD21^{lo} DN), Mem4 (CD21^{lo}CD69⁺ SWM and DN), Nav1 (CD21^{lo} NAV), and ASC2 (CD69⁺ ASC), thus ASC and their precursors (Fig. 6C).

To examine whether C5aR1 signaling affects ASC differentiation of B cells in NMO, we cultured PBMCs obtained from NMO in the presence of recombinant C5a along with CD40L and IL-21. We found that C5a supplementation increased the frequency of ASCs at day 5 (Fig. 6D). Furthermore, C5a supplementation increased B cell proliferation as well as the frequency of CD21^{lo} SWM at day 5. IgG production and the differentiation of CD138⁺ plasma were also enhanced by C5a. (Supplementary Fig.6B). By contrast, no effect was observed by C5a supplementation in the control PBMCs (Fig. 6D).

Various cells, including monocytes, DCs, and B cells, express C5aR1.⁴⁵ To examine whether C5a stimulation can directly promote B cell differentiation, we performed the culture with B cells isolated from NMO blood samples (n=6). We found that C5a supplementation increased the generation of ASCs, CD21^{lo} SWM cells, and B cell proliferation (Fig. 6E). Furthermore, the frequency of CD21^{lo} SWM (r=0.80, p<0.0001, n=40) and CD21^{lo} DNs (r=0.62, p<0.0001, n=40) at day 0 strongly correlated with ASC day 5 in the culture supplemented with C5a. Importantly, the frequency of C5aR1⁺ B cells (r=0.68, p<0.0001, n=40) at day 0 significantly correlated with the frequency of ASC at day 5 (Fig. 6F).

To examine the impact of the treatment with eculizumab (C5 blocker) on B cells *in vivo*, we analyzed the phenotype of B cells in the blood obtained from NMO patients before and 2 months after the treatment (n=5). No other maintenance therapy was changed during this period. We found that eculizumab treatment decreased C5aR1 expression on B cells in all five cases (Fig. 6G). Furthermore, when PBMCs were cultured with C5a, the effect of C5a on the increase of ASC differentiation was canceled after the treatment with eculizumab (Fig. 6G).

Collectively, these data suggest that C5a stimulation promotes the differentiation of B cells into ASCs in NMO.

CD21^{lo} B cell subsets can differentiate into “CSF type ASC” under CD40L stimulation

Next, we examined whether CD21^{lo} B cell subsets display a higher propensity to differentiate into ASCs than CD21^{hi} B cell subsets. Because CD21^{lo} B subsets are infrequent and vulnerable to mechanical stress such as sorting, sorted B cell subsets were cultured with CD40L-L cells⁴⁶ for seven days in the presence of IL-21 and C5a (Supplementary Fig.7A).

Although the frequency of ASCs at post-culture did not differ between CD21^{lo} SWM and CD21⁺ SWM, ASCs derived from CD21^{lo} SWM were more prone to express CD138 and CXCR3 (n=4, Fig. 7A, B), being consistent with the characteristics of ASCs in the CSF of our data and previous reports.¹³ A comparison of CD21^{lo} DN and CD21⁺ DN exhibited similar trends (n=4, Fig. 7B). CD21^{lo} NAV also differentiated more efficiently into CD21^{lo} B cells and expressed high CXCR3 compared to CD21⁺NAV (n=5, Supplementary Fig.7B).

Last, we examined whether CD21^{lo} SWM contained AQP4-IgG-producing cells. We sorted CD21^{lo} SWM and CD21⁺ SWM from blood samples of initial NMO attacks (n=2) and cultured as described above. We analyzed AQP4-IgG in supernatants in a semi-quantitative cell-based assay. Strikingly, AQP4-IgG was detected in the supernatant of CD21^{lo} SWM in both cases, with one showing higher intensities than in CD21⁺ SWM (Fig. 7D, Supplementary Fig. 7C).

CD21^{lo} B cell subsets correlate with the annual relapse rate

Finally, we evaluated whether alterations in B cell subsets correlate with clinical parameters of NMO, including age, disease duration, total number of attacks, annualized relapse rate (ARR), and Expanded Disability Status Scale (EDSS) (Supplementary Fig.8). Among these parameters, ARR positively correlated with the frequency of CD21^{lo} SWM ($r=0.56$, $p<0.005$, $n=25$), CD21^{lo} DN ($r=0.40$, $p<0.05$) and C5aR1⁺ B cells ($r=0.43$, $p<0.05$) in the blood (Fig. 8A). ARR also correlated with the frequency of ASC at day 5 of the PBMC culture with and without C5a (Fig. 8B). Thus, B cell alterations in NMO are most strongly associated with the relapse rate. This observation further suggests that an increased generation of CD21^{lo} B cell subsets along with enhanced C5a signals contribute to the pathogenesis of NMO.

Discussion

In this study, we performed a comprehensive analysis of immune cells in paired CSF and blood samples and identified ten immune cell clusters. Among the identified clusters, only B cells and ASCs were higher in frequency among the CSF of patients with untreated acute-phase NMO than in the CSF samples from ONDs. This is largely in line with the current view on NMO pathogenesis that B cells play major pathogenic roles. Importantly, our study further identified multiple aspects regarding the alterations in B cell biology in NMO.

First, we found that the dominant B cell subsets in the CSF differed between the acute-phase NMO and MS. Among B cell populations, CD21^{lo} “activated” naïve B cells (Nav1 in Fig. 2), CD21^{lo} DN cells (Mem1), and CD21^{lo} SWM cells (Mem4) were more dominant in the CSF of NMO than in the CSF of MS. Among ASCs, ASCs lacking the expression of CXCR3 (ASC1) were dominant in the CSF of NMO as compared to MS. These differences suggest that the mechanism associated with B cell alterations differs between NMO and MS.

Second, B cells in the CSF of NMO were enriched with the CD21^{lo} populations, a subset expanded in various types of autoimmune diseases.^{38,47} Notably, a majority of CD21^{lo} B cells in the CSF of NMO expressed the tissue-resident marker CD69. As CD21^{lo} B cells do not upregulate CD69 upon BCR stimulation in previous reports,⁴⁷ and our data also show that they express low levels of CD69 upon CD40L stimulation, we surmise that CD69 expression on CD21^{lo} B cells in the CSF of NMO

1 does not reflect their activation status *in situ*, but rather indicates their residency in the CNS. The
2 presence of CD69⁺ tissue-resident B cells has been demonstrated in the lung^{48,49} and in the gut⁵⁰
3 in mice and humans. Moreover, we found an increase of CD21^{lo} B cell subsets also in the CSF of
4 treatment-refractory NMO patients. CD21^{lo} B cells present in inflamed tissues of human
5 autoimmune diseases are widely considered to represent precursors of ASCs developed via the
6 extrafollicular pathway.^{38,47} Consistently, our *in vitro* culture experiments with supplementation
7 of IL-21 and CD40L demonstrated that CD21^{lo} DN cells and CD21^{lo} SWMB cells in NMO patients
8 displayed a propensity to differentiate into CXCR3⁺CD138⁺ ASCs as found in CSF. Collectively,
9 NMO displays a series of CD21^{lo} B cell subsets ranging from “activated” naïve, DN, and SWM in
10 the CNS as CNS-resident B cells. These B cells might contribute to autoantibody production *in*
11 *situ*. Alternatively, these CD21^{lo} B cells might play important pathogenic roles as antigen-
12 presenting cells to activate autoreactive T cells locally, given their high expression of co-
13 stimulatory molecules, at least in the blood.^{35,41,47}

14 Regarding the generation of CD21^{lo} B cell subsets, recent studies with samples obtained from
15 patients with monogenic defects in IL-21R, IFN- γ R1, and CD40/CD40L demonstrated lower
16 frequencies of CD21^{lo} B cells,⁵¹ indicating the importance of IL-21, IFN- γ , and CD40/CD40L for
17 their differentiation. Furthermore, an *in-vitro* culture study showed that stimulation with R848 (a
18 ligand for TLR7 and 8) and CD40L was important to induce AQP4-IgG production in the culture
19 of PBMCs from NMO patients.⁵² Given the key roles of TLR7 and CD40 signals for the generation
20 of CD21^{lo} B cells,⁵³⁻⁵⁵ this observation further supports the central pathogenic role of CD21^{lo} B cell
21 subsets in NMO.

22 Third, our study suggests that IFN- γ is highly integrated into the NMO pathogenesis. A majority
23 of B cells in the CSF of NMO expressed CXCR3, a chemokine receptor induced on B cells by
24 IFN- γ stimulation.⁵⁶ Nonetheless, CXCR3 expression was not restricted to B cells in the CSF of
25 NMO and was observed across CSF samples from other diseases. Therefore, IFN- γ -inducible
26 chemokines CXCL9, CXCL10, and CXCL11 and their common receptor CXCR3 likely play a
27 central role in the migration of B cells from the periphery to the CNS. In NMO, CXCL10 levels
28 in CSF are elevated,⁵⁷ and therefore, this axis is likely active to recruit B cells into the CNS.
29 Furthermore, we found that NMO patients in the acute phase showed an increased frequency of
30 IFN- γ -producing CXCR3⁺ B helper T cell subsets, including Tph1 and Tfh1 cells in the CSF. This

is consistent with previous reports showing an increase of IL-21 and IFN- γ in the CSF of acute NMO,^{57,58} and an increase of Tph-like cells in the peripheral blood of NMO.^{59,60} We further showed that Tph1 and Tfh1 cells in the CSF of NMO highly expressed CD69 and PD-1, suggesting their CNS residency. Recently, B-helper CD4⁺ T cells in the peripheral blood of stable NMO were shown to display an exhausted phenotype with high expression of inhibitory molecules including PD-1, TIGIT, and CTLA4.⁶¹ Therefore, whether PD-1^{hi} Tph1 cells in the CSF show an exhausted phenotype or not might be of interest. Altogether, our study suggests that the CXCR3-CXCL10 axis plays a major role in recruiting CD21^{lo} B cells as well as Tph1 and Tfh1 cells to the CNS in NMO, and these recruited B and T cells become CNS-resident. Such T and B cells might contribute to the generation of AQP4-IgG *in situ*. This aligns with previous reports showing the presence of AQP4-IgG⁺ B cells with unique BCR repertoires in the CSF.^{14,16} A recent study on the analysis of B cell lineages in the CSF of patients with autoimmune encephalitis (AE) demonstrated that autoreactive B cell clones were abundantly present in the CSF, particularly enriched within ASCs. Interestingly, there were few increases of the mutation of BCR genes in autoreactive B cells in the CNS of AE, accompanied by the lack of CD4⁺ T cell infiltration to the CNS.⁶² Thus, how and where autoantibodies are generated seems to differ among inflammatory CNS diseases, and T and B cell interactions in the CNS might be a feature of NMO. Furthermore, our hypothesis is also supported by the observation that ASCs lacking the expression of CXCR3 were also enriched in the CSF of NMO. CXCR3⁻ ASCs might represent an ASC subset generated in the CNS of NMO patients.

Fourth, our study showed that CD21^{lo} B cells in the CSF and blood of NMO patients displayed high C5aR1 and C5aR2. These receptors seem functional, as supplementation of C5a promoted B cells to proliferate, produce IgG, and differentiate into ASCs. Given that C5a levels are elevated in CSF and plasma in NMO,^{63,64} C5a signals in B cells might further promote their differentiation into ASCs in the periphery and the CNS, and eculizumab might inhibit this mechanism.

Last, we provided evidence that B cell alterations in NMO are most strongly associated with the relapse rate. Importantly, CD21^{lo} SWM in acute NMO contained AQP4-IgG-producing cells. The strong correlation between CD21^{lo} SWM and CD21^{lo} DN B cells with the relapse rate further supports their pathogenic roles.

1 A limitation of our study was the small sample size in the untreated acute phase of NMO compared
2 to MS and OND, making statistical significance challenging to achieve and potentially affecting
3 the generalizability of our findings. Another limitation was that CSF samples from post-treatment
4 NMO patients were predominantly from refractory cases, as we did not collect CSF for research
5 purposes only. This selection bias may have influenced the results.

6 Despite these limitations, our findings provide valuable insights into the types of B cell alterations
7 in NMO. With the emergence of high-efficacy treatments for NMO, it is critical to gain a deeper
8 understanding of pathogenesis and stratify patients for optimal treatment. Our study provides
9 evidence that CD21^{lo} B cell subsets contribute to the pathogenesis and represent the development
10 of novel therapeutic approaches.

11 12 **Data availability**

13 Anonymized data are available on request from any qualified investigator.
14

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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Figure legends

Figure 1 Comprehensive immune cell analysis of CSF & blood samples from acute-phase NMO and MS. (A) The global experimental design. Blood and CSF collected from patients were separately stained with CD45-PerCP (blood) and CD45-APC/Fire810 (CSF), and then mixed for the staining of other markers in the flow panel. FCS data were obtained with spectral flow cytometry, and immune cells within the blood and CSF samples were analyzed based on CD45 staining. The flow data were concatenated and analyzed for dimensional reduction and immune cell clusters. Created in BioRender. Ryusei, N. (2025) <https://BioRender.com/l67r603>. **(B)** Ten immune cell clusters identified with CSF samples of acute-phase NMO (n=4, 26,546 cells), acute-phase MS (n=8, 38,307 cells), and OND cases (n=22, 18,765 cells); and blood samples of acute-phase NMO (n=4, 117,659 cells), and acute-phase MS cases (n=7, 173,948 cells). CD20⁺ B cells (CD20⁺B, Pop0), antibody secreting cells (ASC, Pop1), CD8⁺ T cells (CD8⁺T, Pop2), NK cells (NK, Pop3), dendritic cells (DC, Pop4), CD4⁺ T cells and double negative T cells (CD4⁺&DN T, Pop5), monocytes (Mono, Pop6), intermediate monocyte (Intermediate-Mono, Pop7), neutrophils (Neut, Pop8), and basophils (Baso, Pop9). **(C)** Marker expression of each cluster. The MFI of each

parameter calculated by relative intensities is shown in a heat-map format. **(D)** Composition of immune cell clusters in each sample group. The frequency of cell clusters within CD45⁺ cells is presented in a pie chart. **(E)** Comparison of the frequency of each cluster in the CSF samples between disease groups. Kruskal-Wallis test followed by Dunn's test. **(F)** Comparison of %ASC within B-lineage cells between CSF and blood samples. Paired-t test. **(G)** Comparison of the frequency of each immune cell subset in the CSF between disease groups (acute-phase NMO, n=8; acute phase MS, n=10; ONDs, n=27). The frequency based on conventional gating. Kruskal-Wallis test followed by Dunn's test (unpaired). Triangles shows the data of relapse cases (on immunotherapy). ****P < 0.0001, ***P < 0.001, **P < 0.01; actual p values are listed when 0.01 < P < 0.5; ns = not significant.

Figure 2 Enrichment of CD21^{lo} Naïve/Memory B cells in CSF of acute NMO. **(A)** Subclusters within CD20⁺ B cells. CD20⁺ B cells were sub-divided into three naïve (Nav1-3) and four memory (Mem1-4) subsets. **(B)** The characteristics of CD20⁺ B subclusters. Mem1-4 are indicated in blue and Nav1-3 are in gray in UMAP (top-left), and plotted on CD27-IgD biaxial (top-middle). Expression of CD27 of Nav1-3 and Mem1-4 is shown in the histogram (top-right). The frequency of memory and naïve B cells per sample group is shown in the bar graph (bottom-left). Nav1-3 are plotted on CD21-CD38 biaxial, and Mem1-4 are plotted on CD21-CXCR3 (bottom-right). Refer to the color codes in Fig. 2A. **(C)** The marker expression of each B cell subcluster. The MFI of each parameter calculated by relative intensities is shown in a heat-map format. **(D)** CD69 expression by the indicated B cell subsets. **(E)** The frequency of subclusters within CD20⁺ B cells is presented in a pie chart. **(F)** Comparison of the frequency of B cell subsets between CSF and blood samples. Paired t-test. **(G)** Comparison of the frequency of B cell subsets between NMO CSF and MS CSF. Mann-Whitney U test (unpaired). Refer to the color codes in Fig. 1H. **(H)** Subclusters within ASCs. ASCs were sub-divided into 4 subsets (ASC1-4). **(I)** ASC1-4 are plotted on INTβ7-INTβ1 and CD69-CXCR3 biaxial. Refer to the color codes in Fig. 2H. **(J)** Marker expression of each ASC subcluster. **(K)** The frequency of ASC subclusters within ASC is presented in a pie chart. **(L)** Comparison of the frequency of ASC2 within ASC between CSF and blood samples. Paired t-test. **(M)** Comparison of the frequency of ASC subsets between NMO CSF and MS CSF. Mann-Whitney U test (unpaired). **(N)** Comparison of the frequency of B-cell

subsets based on conventional gating in the CSF between NMO and MS, Mann–Whitney U test (unpaired). (O) Comparison of the frequency of the CD69⁺CXCR3⁺CD21^{lo} SWM and CD69⁺CXCR3⁺ASC based on conventional gating between CSF and blood, paired-t test (NMO cases, n=6). Triangles shows the data of relapse cases (on immunotherapy). **P < 0.01; actual p values are listed when 0.01 < P < 0.1; ns = not significant.

Figure 3 Enrichment of CD21^{lo} memory B cells in CSF of non-acute phase NMO. (A) Comparison of the frequency of CD3⁺CD19⁺ cells in live cells (left) and ASCs in CD3⁺CD19⁺ cells (right) between the non-acute phase of NMO CSF (n=4) and OND CSF (n=6). Mann–Whitney U test (unpaired). (B) Comparison of the frequency of ASCs in CD3⁺CD19⁺ cells between CSF and blood in non-acute phase (left) and in acute phase (right) of NMO (n=3, n=6, respectively). The frequency of ASC and CD3⁺CD19⁺ cells is based on conventional gating. Paired T-test. (C) Comparison of the frequency of the indicated subsets in B cells between CSF and blood in non-acute phase of NMO (top). Comparison of the frequency of CD21^{lo} subsets in each B cell subset between CSF and blood in non-acute phase of NMO (bottom). **P < 0.01; actual p values are listed when 0.01 < P < 0.1; ns = not significant.

Figure 4 Enrichment of Tph1 and Tfh1 in CSF of acute NMO. (A) Subclusters within CD4⁺ memory T cells. Initially, the CD4⁺&DN T cluster (Pop5 in Fig. 1A) was sub-clustered into six populations with FlowSOM (top-left). The frequency of subpopulations in each sample group is shown in the bar graph (bottom-left). Next, memory CD4⁺ T cells were analyzed for subclusters and 12 subclusters (C1-12) were defined (top-right). The marker expression of each T cell subcluster is provided in Fig. S3A. (B) Indicated subclusters are plotted on the indicated biaxial. Refer to the color codes in Fig. 3A. (C) The frequency of subclusters within memory CD4⁺ T cells is presented in a pie chart. (D) Comparison of the frequency of the indicated subclusters within memory CD4⁺ T cells between CSF and blood samples. Paired t-test. (E) Comparison of the frequency of the indicated subclusters within memory CD4⁺ T cells among CSF sample groups. Kruskal-Wallis test followed by Dunn's test. (F) Comparison of the frequency of CD4⁺ T-cell subsets in the CSF based on conventional gating between disease groups, Kruskal-Wallis test followed by Dunn's test (unpaired). (G) Comparison of the frequency of the CD69⁺ Tph1 based

on conventional gating between CSF and blood, paired-t test (NMO cases, n=6). **(H)** Subclusters within DCs. 4 subclusters (DC1-4) were defined (bottom). **(I)** DC1-4 are plotted on CCR6-CXCR3 biaxial. Refer to the color codes in Fig. 3F. **(J)** The frequency of DC subpopulations in each sample group is shown in the bar graph (bottom-left). **(K)** Comparison of the frequency of DC4 within DCs between CSF and blood samples. Paired t-test. Triangles shows the data of relapse cases (on immunotherapy). ***P < 0.001, **P < 0.01; actual p values are listed when 0.01 < P < 0.5; ns = not significant.

Figure 5 CD21^{lo} B cell subsets differentiate into ASCs in vitro. **(A)** The experimental design for Fig 5 and 6. PBMCs obtained from patients with NMO (n=25) and control subjects (n=15) were stored in LN₂. Thawed PBMCs were analyzed for B cell subsets by FCM. A fraction of PBMCs was cultured for five days with sCD40L and IL-21 ± C5a. Cultured cells were analyzed for B cell subsets by FCM and IgG measurement in the supernatant. Created in BioRender. Ryusei, N. (2025) <https://BioRender.com/o07p680>. **(B)** The frequency of B cell subsets before culture. Comparison of the frequency of SWM, DN, and NAV in B cells and the frequency of ASCs in CD3⁺CD19⁺ cells between NMO and control (top). Comparison of the frequency of CD21^{lo} subsets in each B cell subset and the frequency of CD3⁺CD19⁺ cells in live cells between NMO and control (bottom). Mann–Whitney U test (unpaired). **(C)** Representative flow data of B cell subsets after five days of culture. **(D)** Comparison of the frequency of ASCs, CD138⁺ PCs, and CFSE^{lo} proliferating cells in CD3⁺CD14⁺CD19⁺ cells, and IgG concentration in the supernatants between NMO and control at day 5 of culture. Mann–Whitney U test (unpaired). **(E)** Correlation between the frequency of B cell subsets before culture and ASCs at day 5. Pearson's correlation coefficient. ****P < 0.0001; ***P < 0.001; **P < 0.01; actual p values are listed when 0.01 < P < 0.05 ; ns = not significant.

Figure 6 C5a stimulation promotes ASC differentiation of CD21^{lo} B cells. **(A)** Representative data on the expression of C5aR1 and C5aR2 on B cells in the blood of NMO patients. Fluorescence minus one (FMO) data for C5aR1 (panel without C5aR1-PE) and C5aR2 are provided. **(B)** Comparison of C5aR1 and C5aR2 expression on total B cells in the blood of NMO and controls (top). Mann–Whitney U test (unpaired). The expression of C5aR1 on the indicated B cell subset

in the blood of NMO is shown at the bottom. Wilcoxon signed rank test (paired). (C) MFI of C5aR1 on B cells and ASCs in the blood of acute phase NMO and MS is shown in the heatmap. MFI of C5aR1 on each B cell and ASC clusters in the blood of acute phase NMO is shown on the right. (D) PBMCs were cultured for 5 days under stimulation with CD40L, IL-21, and C5a (Fig. 5A). Comparison of the frequency of ASCs and CFSE^{lo} proliferating cells in CD3⁺CD14⁺CD19⁺ cells, and CD21^{lo} cells in SWM with or without C5a stimulation. Paired t-test. (E) B cells isolated from NMO blood samples were cultured for five days under stimulation with CD40L, IL-21, and C5a (n=6). Comparison of the frequency of ASCs and CFSE^{lo} proliferating cells in CD3⁺CD14⁺CD19⁺ cells, and CD21^{lo} SWM cells in B cells with or without C5a stimulation. Paired t-test. (F) Correlation between the frequency of B cell subsets and C5aR1⁺ cells in B cells before culture and ASCs at day 5. Pearson's correlation coefficient. (G) PBMC were obtained from patients with NMO before and after the treatment with eculizumab (n=5). C5aR1 and C5aR2 expression on B cells are shown on the left. PBMCs were cultured as in Fig. 6D and analyzed for the frequency of ASCs in B cells at day 5. ****P < 0.0001; ***P < 0.001; **P < 0.01; actual p values are listed when 0.01 < P < 0.05; ns = not significant.

Figure 7 CD21^{lo} B cell subsets can differentiate into “CSF type ASC” under CD40L stimulation. (A) Representative panel of FCM analysis of B cell subsets cultured on CD40L-expressing L cells. (B) Comparison of the cultured B-cell subset derived from CD21^{lo} SWM and CD21⁺ SWM. Paired-t test. (n=4, NMO cases) (C) Comparison of the cultured B-cell subset derived from CD21^{lo} DN and CD21⁺ DN. Paired-t test (n=4, NMO cases). (D) AQP4-IgG was detected in the culture supernatant using a commercially available cell-based assay. Peripheral blood-derived CD21^{lo} SWM (left) and CD21⁺ SWM (second from left) from a case of first-episode acute NMO were cultured, and concentrated supernatants were 1x-4x-16-diluted and reacted with HEK293T with AQP4 forced expression and imaged with fluorescence microscopy. As a control, the same culture supernatant was reacted with HEK293T cells in MOCK (right). All photographs were courtesy of Cosmic Co. Blue dots show the data from untreated cases in Fig. B,C. ****P < 0.0001; ***P < 0.001; **P < 0.01; Actual p values are listed when P < 0.1; ns = not significant.

Figure 8 CD21^{lo} B cell subsets correlate with the annual relapse rate. (A) Correlation between the indicated B cell subsets and annualized relapse rate (ARR). Pearson's correlation coefficient. (B) Correlation between the frequency of ASCs in CD3⁺CD14⁺CD19⁺ cells at day 5 (Fig. 6) of culture and ARR. Actual p values are listed when $P < 0.1$; ns = not significant.

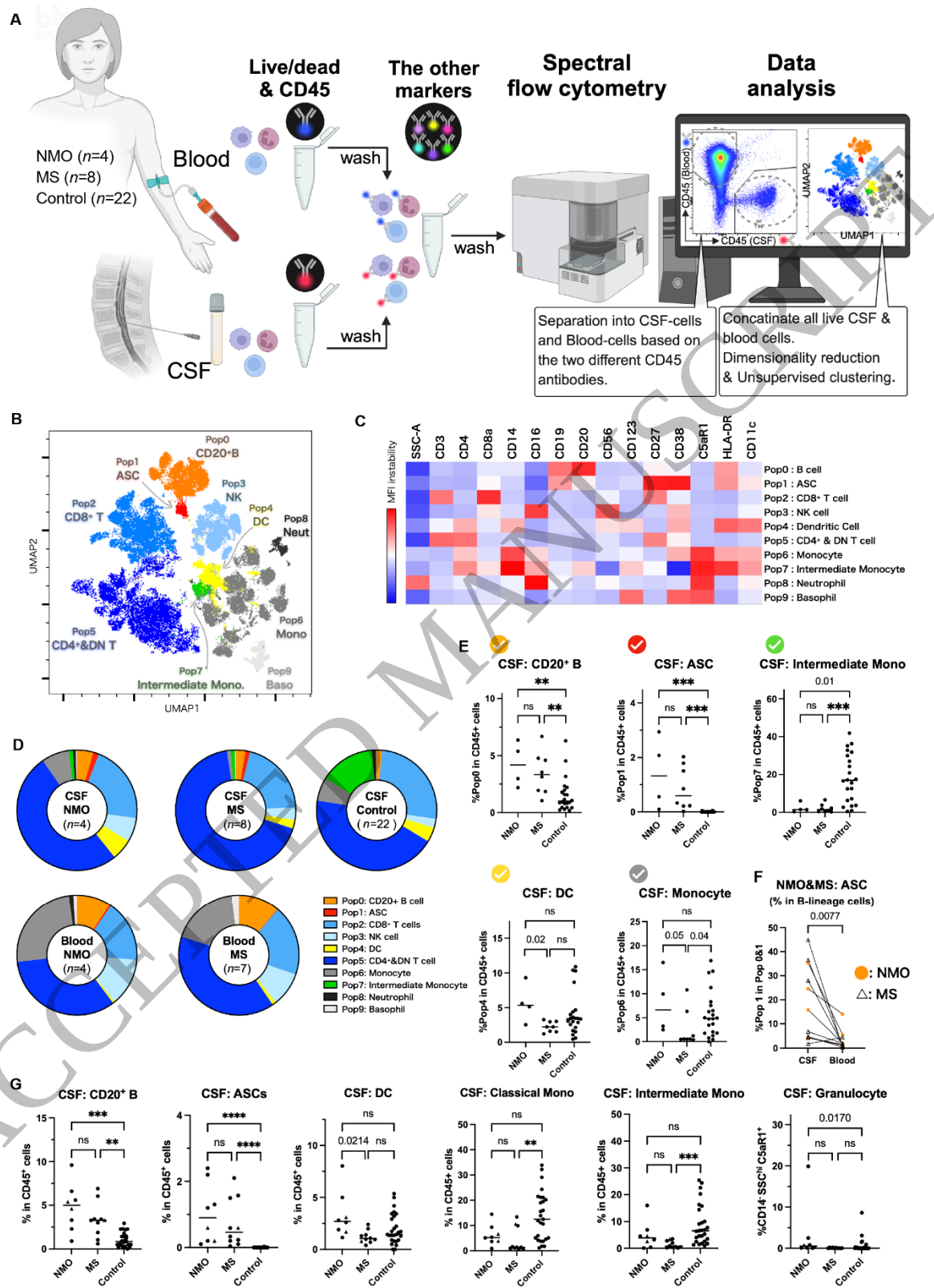


Figure 1
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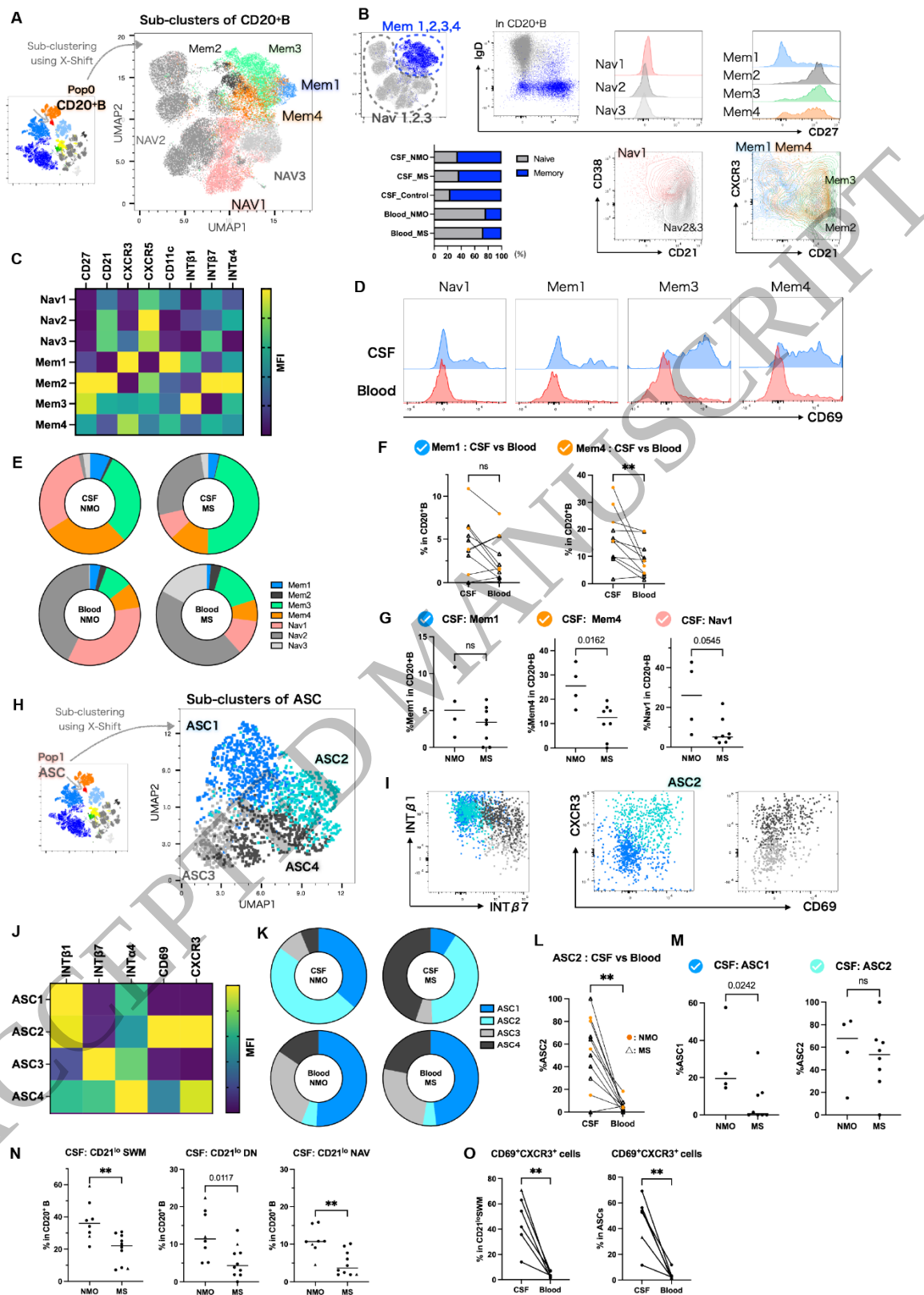


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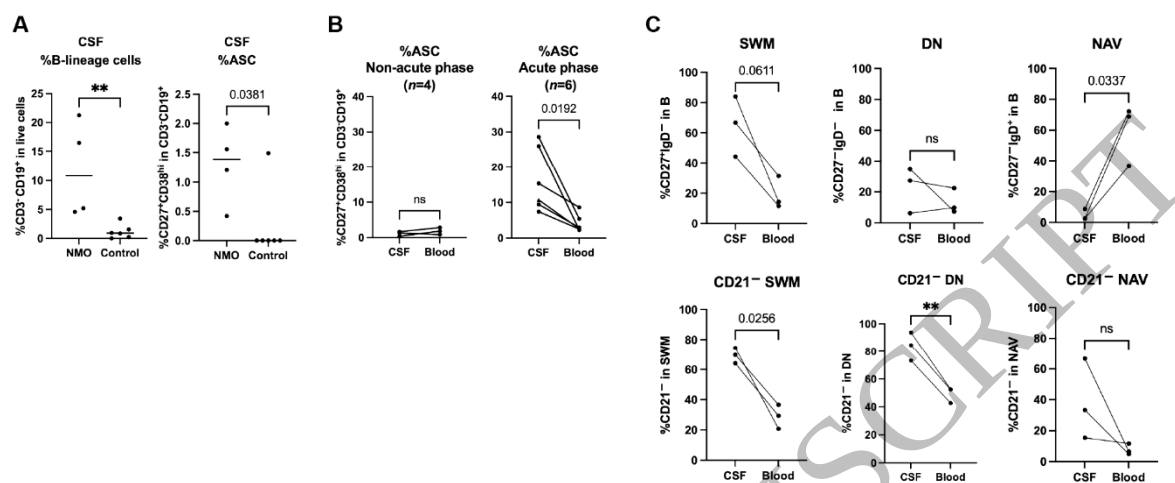


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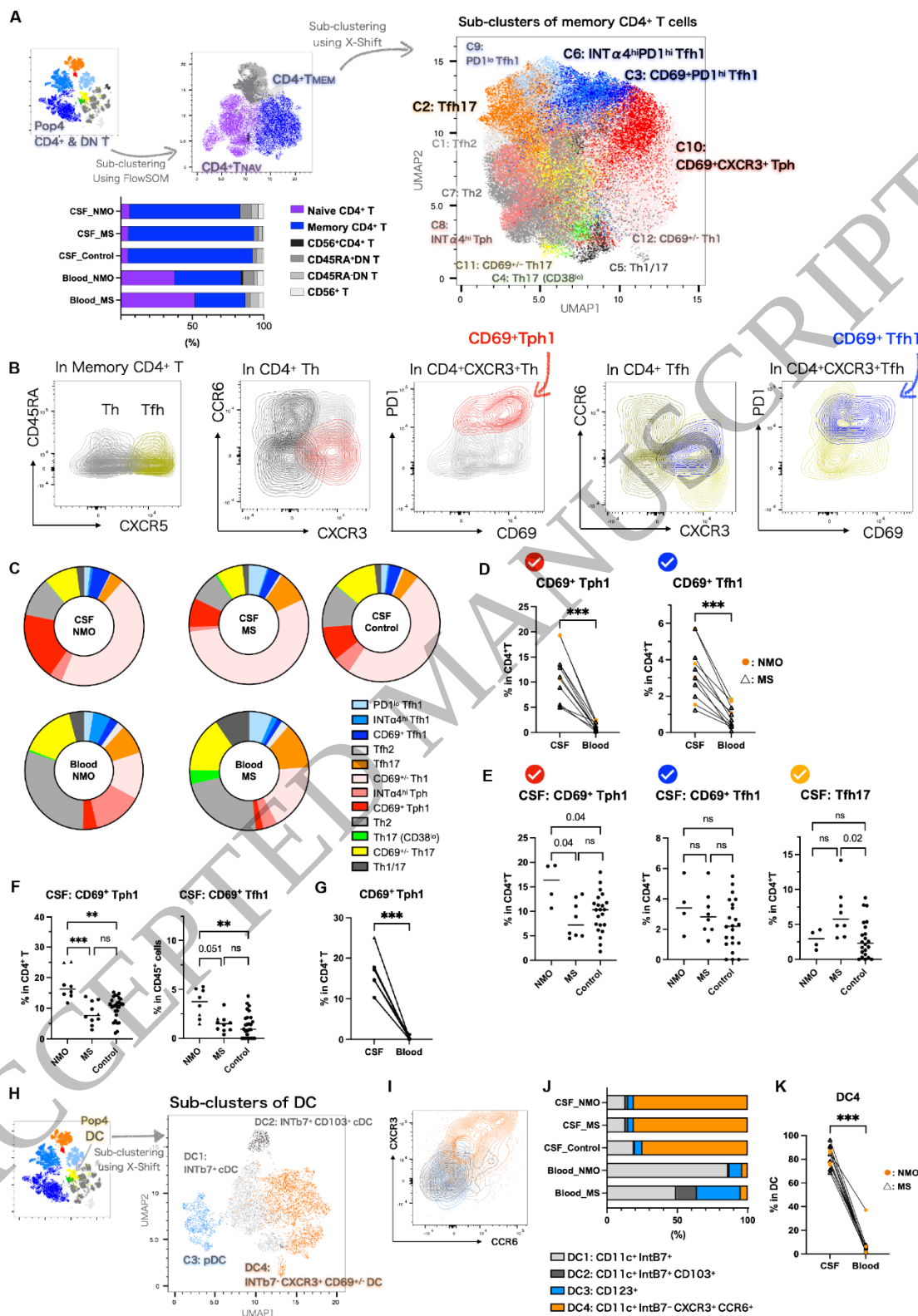


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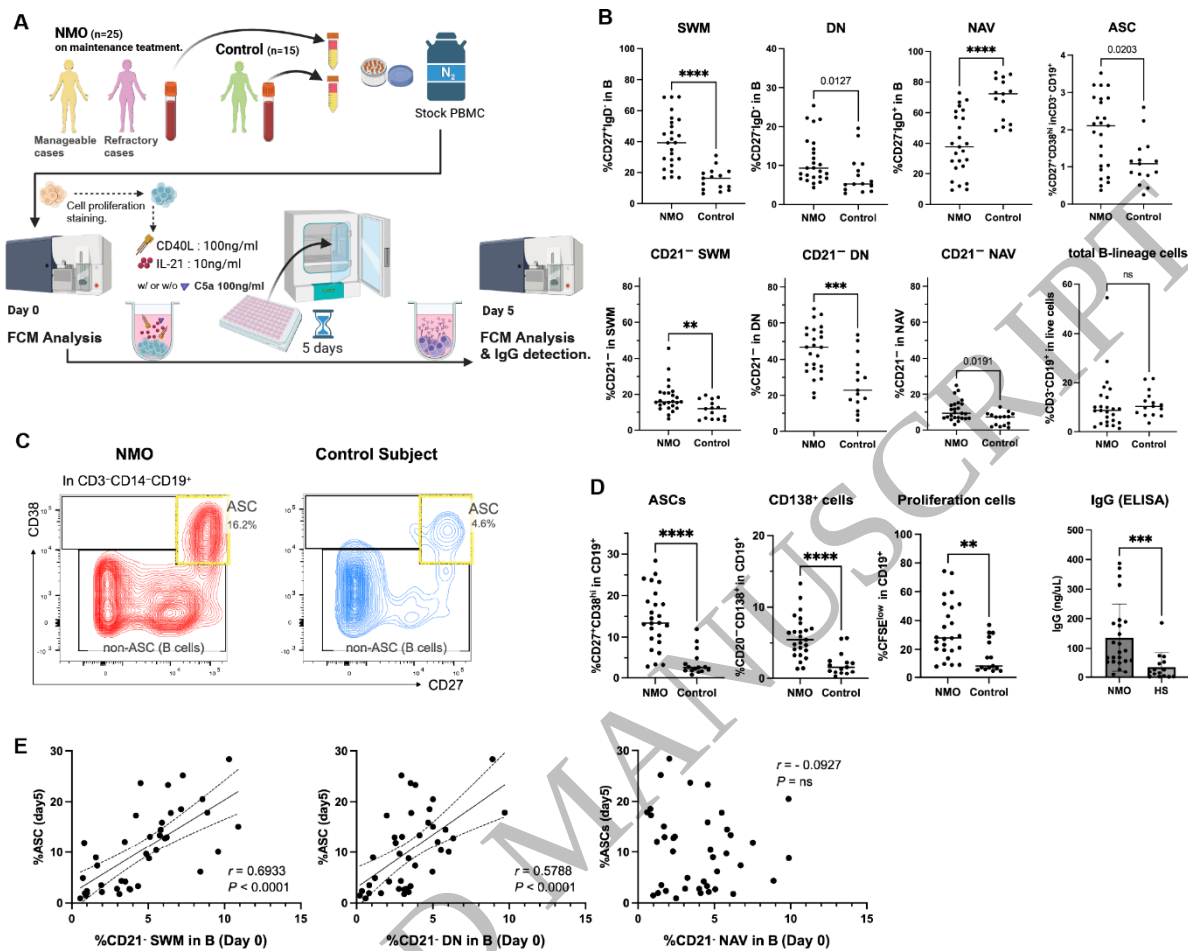


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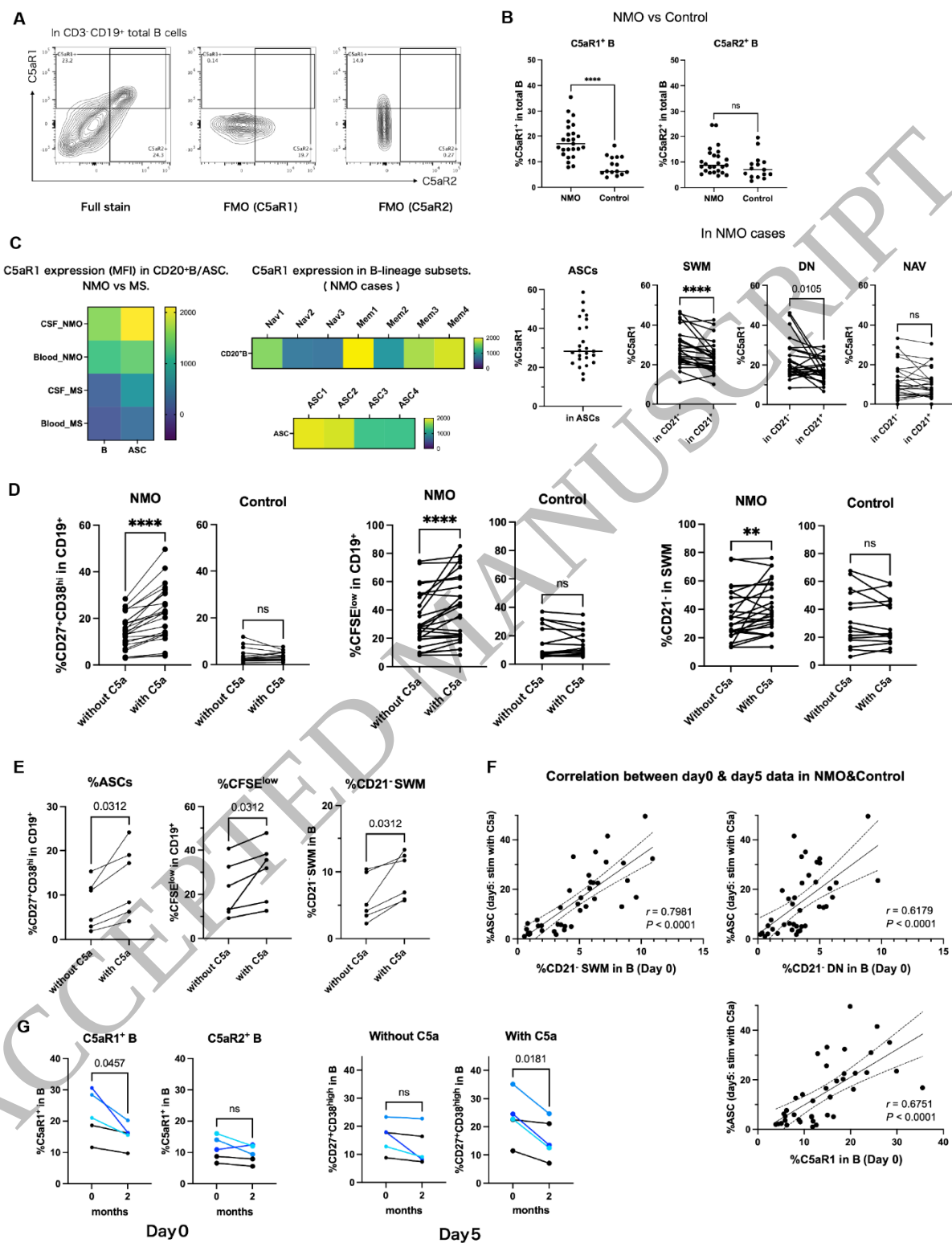


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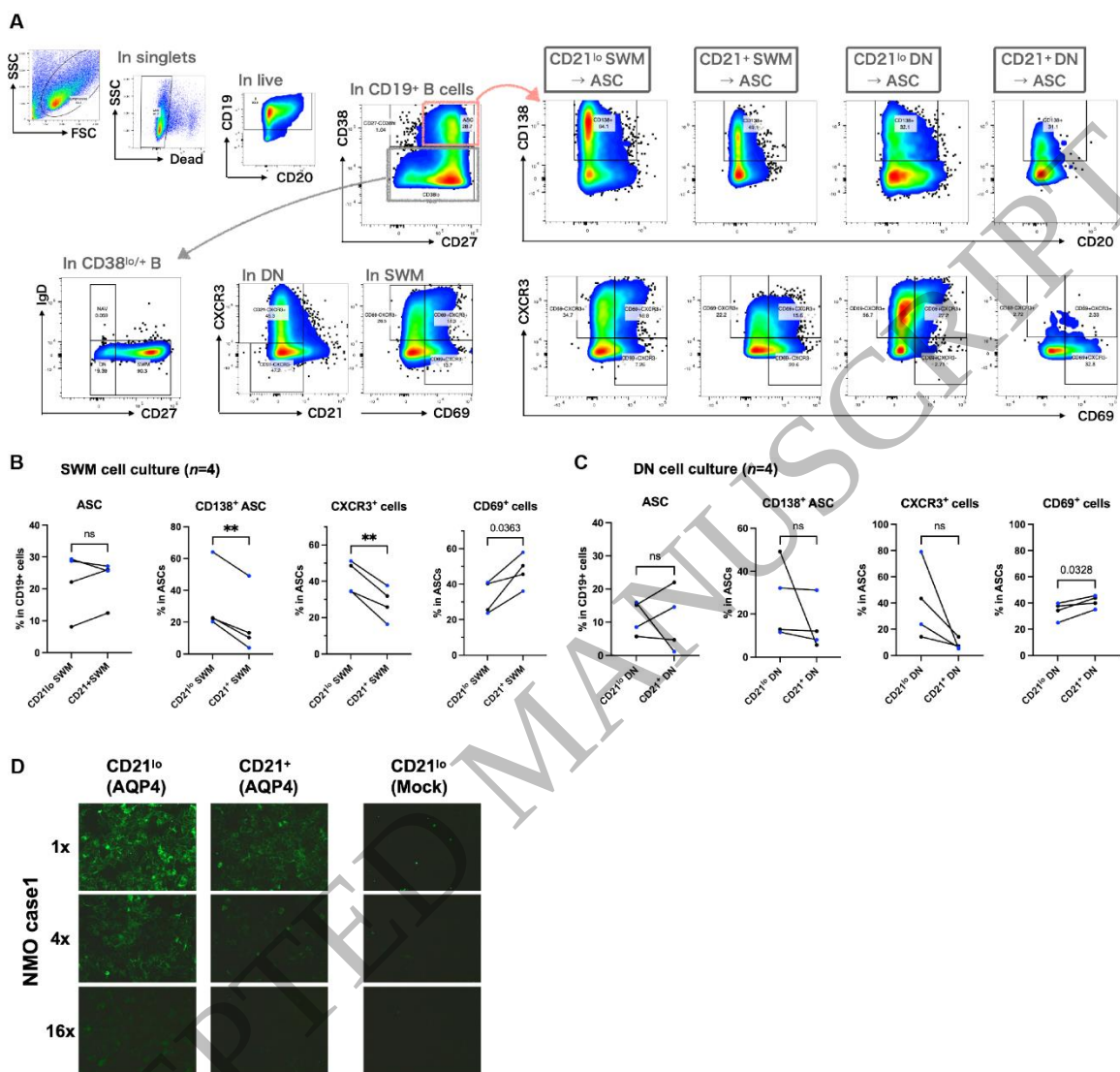


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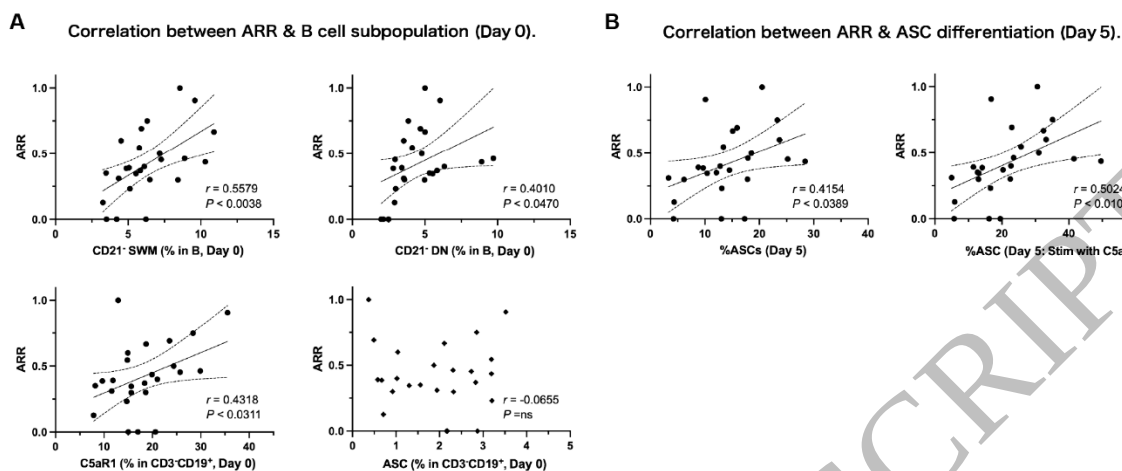


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