Th1 - CD11c$^+$ B Cell Axis Associated with Response to Plasmapheresis in Multiple Sclerosis

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Objective: Although plasmapheresis is a treatment option for patients with autoimmune neurological diseases, treatment response varies greatly among patients. The main objective of this study was to find out if biological/immune traits correlate with a beneficial response.
Methods: We thoroughly analyzed immune phenotypes in paired blood samples from a cohort of 31 patients with multiple sclerosis before and after plasmapheresis, in parallel with clinical evaluation of treatment response.
Results: The frequency of IFN-γ^+ Th1 cells was persistently higher in those who obtained benefit from plasmapheresis (responders) than nonresponders. The Th1 cell frequency before plasmapheresis provided a high predictive value for beneficial response, achieving area under the curve (AUC) of 0.902. Plasmapheresis treatment decreased inflammation-related gene expressions in Th1 cells. Meanwhile, *IFNG* expression in Th1 cells positively correlated with the frequency of CD11c$^+$ B cells, of which a pathogenic role has been suggested in several autoimmune diseases. In line with this, in vitro experiments showed that CD11c$^+$ B cells would increase in response to exogenous IFN-γ compared to IL-4, and secrete high amounts of IgG. B cell receptor analysis indicated that clonal expansion of CD11c$^+$ B cells takes place in patients with multiple sclerosis. Interestingly, CD11c$^+$ B cells, which showed unique gene expression profile, decreased after plasmapheresis treatment along with all the immunoglobulin subsets in the circulation.
Interpretation: Taken together, we postulate that Th1 cell - CD11c$^+$ B cell axis is involved in treatment response to plasmapheresis, giving us clues to better understanding of complicated pathogenesis of autoimmune diseases, and getting closer to a personalized therapy.

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Plasmapheresis (PP) is a procedure which removes immunoglobulins and other humoral factors from the plasma. It has been shown to effectively reduce inflammation, thereby controlling symptoms and signs of various autoimmune neurological diseases, including multiple sclerosis, neuromyelitis optica spectrum disorders, Guillain-Barre syndrome, chronic inflammatory demyelinating polyneuropathy, and myasthenia gravis.[1] With regard to multiple sclerosis, sham-controlled trials that were conducted in the last century showed that plasma exchange (PLEX) significantly improved the disease severity score.[2,3] More recently, immunoadsorption plasmapheresis (IAPP), which selectively removes immunoglobulin but spares most of other constituents including albumin, showed a similar or even better outcome regarding the rate for beneficial treatment response.[4–8] Nevertheless, it should be

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noted that one to two thirds of the patients did not obtain significant benefits from PP in these studies. To overcome the problem of low response rate, it appears to be a rational approach to identify a key immunological pathway involved in the responsiveness to PP.

Prior studies showed that pathology of multiple sclerosis lesions comprises four distinct patterns, presumably corresponding to heterogeneous inflammatory processes.[9,10] Among the 4 patterns, pattern II is characterized by prominent antibody deposition along with T cell infiltration.[9,10] It appears that PP is particularly beneficial for patients with pattern II lesions,[7,11] supporting the prevailing hypothesis that the removal of pathogenic immunoglobulin is a key therapeutic mechanism. However, it is still a challenge to predict response to PP in individual cases.

Here, we show that patients who benefit from IAPP are characterized by a higher frequency of T helper (Th) 1 cells in the peripheral blood before the treatment than nonresponders, which appears to have a significant value for prediction of PP responses. The expression levels of inflammation-related genes in Th1 cells, including *IFNG*, decreased after IAPP treatment. Notably, *IFNG* expression in Th1 cells positively correlated with the frequency of $CD11c^+$ B cells, which are transcriptionally and clonally distinct from other B cell populations, and highly potent in producing antibodies presumably involved in the pathogenesis. These results allow us to postulate that the effect of PP could be attributed not only to removal of antibodies but also to phenotypic change of Th1 cells, which would reduce $CD11c^+$ B cells with potential pathogenicity. Collectively, this study sheds light on the heterogeneity in the immunopathogenesis of multiple sclerosis, and provides a new mechanistic insight into the IAPP treatment, which appears to be more complex than removing disease-relevant antibodies.

Methods

Subjects

Demographics of the patients with multiple sclerosis and healthy control subjects are described in the Table. All the patients were treated at National Center Hospital of Neurology and Psychiatry, Japan, from 2017 to 2019. McDonald criteria 2010 was used to make the diagnosis of multiple sclerosis.[12] We have been aware that there is a group of patients who can be treated successfully with plasmapheresis even a while after the last relapse or even when they are already in a progressive state. Therefore, we recruited such patients as well as some patients soon after a relapse. Patients in relapse were defined as those within 1 month after the last relapse in this study. All the patients had a history of insufficient response to treatment with high-dose steroids. All the patients were treated with IAPP or double-filtration plasmapheresis (DFPP) for 4 to 7 times in one treatment course. PlasautoΣ (Asahi Kasei Medical, Tokyo, Japan) was used for both treatments. Plasmaflo OP-05 W (Asahi Kasei Medical) was used to separate plasma from cellular components. Thereafter, IAPP was performed with IMMUSORBA TR-350 (Asahi Kasei Medical), a tryptophan immobilized polyvinyl alcohol gel column, and DFPP was performed with Cascadeflo EC-20 W (Asahi Kasei Medical). Treated plasma volume was 1.5 L per session of both procedures. Then, 600–750 ml of 5% albumin was administered per session of DFPP. The blood samples were collected within 1 week before the first and after the fourth PP. The blood sample was obtained only before the treatment for one patient who was treated with DFPP. A neurologist, who was blinded to any laboratory results obtained by flow cytometer, evaluated the treatment response. The responders were defined as patients with objective clinical improvement with any change of Expanded Disability Status Scale (EDSS), including functional system scoring after the treatment, regardless of subjective evaluation. The EDSS was evaluated within 1 week before and about 1 month after PP. In the analysis of IFN-γ^+, IL-$17A^+$, and IFN-γ^-IL-$17A^-Foxp3^+$ T cells in Figure 1, data are shown with 2 distinct dots for each of 5 patients who underwent the IAPP course twice at different time points. The absolute Th1 cell number in the peripheral blood was calculated based on the number of lymphocytes that was tested within 3 days prior to flow cytometry assay (see Fig 1D). The Ethics Committee of National Center of Neurology and Psychiatry approved the study protocol according to the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Cell Preparation and Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll–Paque PLUS (GE Healthcare Bioscience, Ontario, Canada). They were stained for cell surface antigens, followed by intracellular staining using Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, San Diego CA, USA) or BD Cytofix Fixation Buffer/BD Phosflow Perm Buffer III (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The antibodies and isotype controls used in this study were as follows: FITC-anti-Foxp3 (PCH101; eBiosciences), V500-anti-CD3 (UCHT1), V500-anti-CD4 (RPA-T4), APC-Cy7-anti-CD19 (SJ25C1), V500-anti-CD27 (M-T271), APC-H7-anti-CD45RA (HI100), PE-Cy7-anti-CD45RA (L48), AlexaFluor-488-anti-CD56 (B159), PE-anti-CD180 (G28-8), V500-anti-

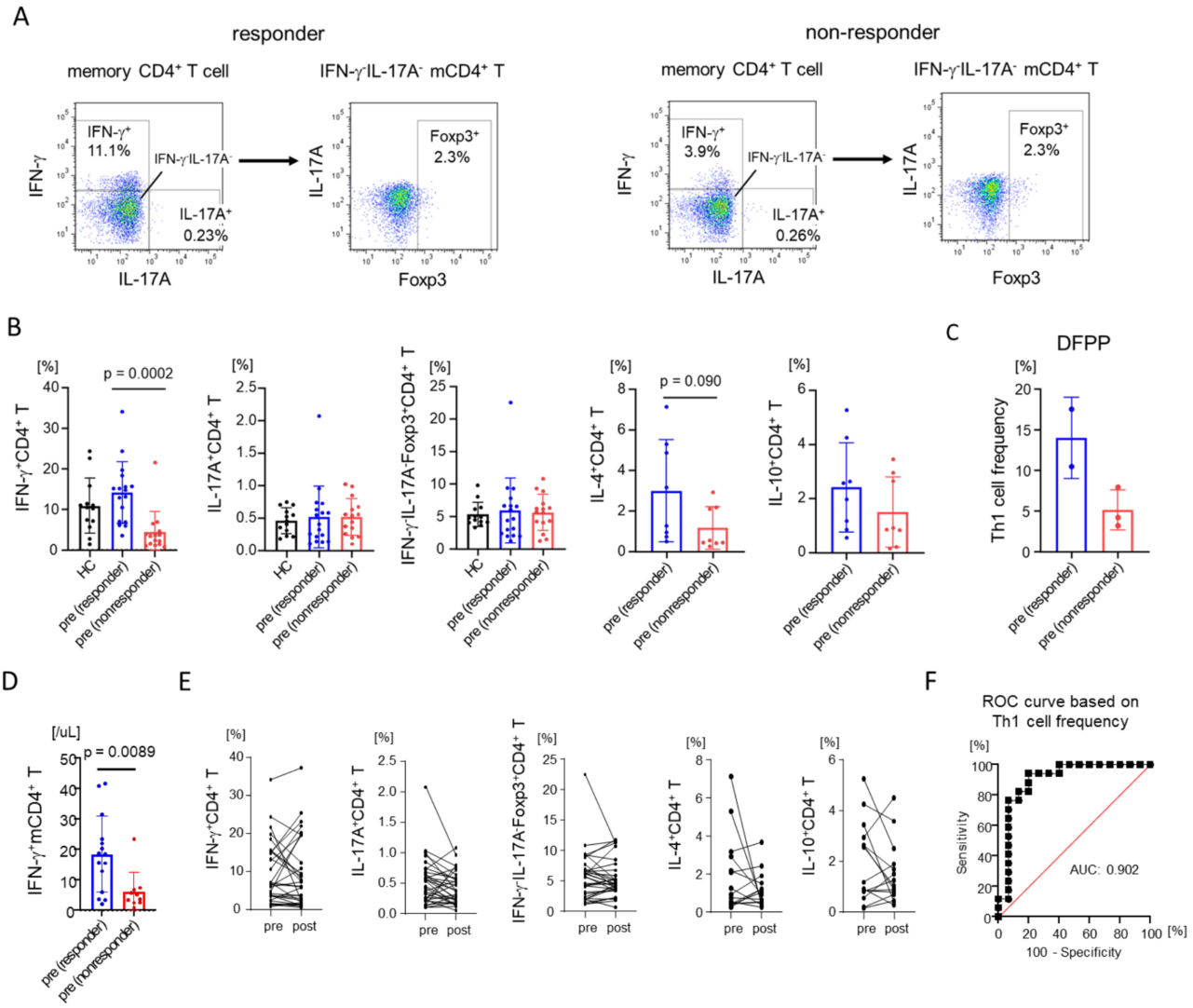

FIGURE 1: IAPP responder is characterized by Th1 cell predominance. (A) Representative flow cytometry plots showing inflammatory and regulatory CD4^{+} T cells in the peripheral blood of responders and nonresponders to IAPP prior to the treatment with IAPP. (B) The frequencies of inflammatory and regulatory T cells among memory CD4^{+} T cells in the peripheral blood of healthy controls, responders, and nonresponders, before the IAPP treatment. In the 3 panels from the left for assessment of Th1, Th17, and Treg cells (IFN-γ^{+}, IL-17A^{+}, and IFN-γ^{-}IL-17A^{-}Foxp3^{+}), data are shown with 2 distinct dots for each of 5 patients who underwent IAPP course twice. (C) The frequency of Th1 cells among memory CD4^{+} T cells was analyzed in the peripheral blood of responder and nonresponder patients to DFPP, before the treatment. (D) The absolute Th1 cell number in the peripheral blood before the IAPP treatment. Data are shown with 2 distinct dots for each of 3 patients who underwent the IAPP course twice. (E) No significant alterations in the frequency of each T cell subset after IAPP. (F) Receiver operating characteristic (ROC) curve based on the frequency of Th1 cells (IFN-γ^{+}CD45RA^{-}CD4^{+} T cells) for prediction of responders before the IAPP treatment. Th1 cell frequency of 7.02% had the highest positive likelihood ratio for prediction of responders. An unpaired was used for statistical analysis in B, C, and D, and a paired *t* test was used in E. Error bars represent the mean ± SD. AUC = area under the ROC curve; DFPP = double-filtration plasmapheresis; IAPP = immunoadsorption plasmapheresis. [Color figure can be viewed at www.annalsofneurology.org]

IgD (IA6-2), V500-mIgG1k (X40), V500-mIgG2a (G155-178; BD Biosciences), PerCP-Cy5.5-anti-CCR6 (G034E3), AlexaFluor-700-anti-CD3 (UCHT1), PE-anti-CD3 (UCHT1), PerCP-Cy5.5-anti-CD3 (UCHT1), PE-Dazzle-594-anti-CD4 (RPA-T4), APC-Cy7-anti-CD8a (RPA-T8), PE-Cy7-anti-CD11c (3.9), APC-anti-CD14 (M5E2), PerCP-Cy5.5-anti-CD16 (3G8), PE-Cy7-anti-CD24 (ML5), PE-Cy7-anti-CD25 (BC96), PB-anti-CD27 (O323), PerCP-Cy5.5-anti-CD38 (HIT2), BV421-anti-CD127 (A019D5), PB-anti-HLA-DR (L243), PE-anti-IFN-γ (B27), PE-anti-IFN-γRα (GIR-94), PerCP-Cy5.5-anti-IgD (IA6-2), BV421-anti-IL-4 (MP4-25D2), BV421-anti-IL-10 (JES3-9D7), APC-anti-IL-17A (BL168; BioLegend, San Diego, CA, USA), PE-anti-CD11c (BU15), PB-anti-CD21 (BL13), FITC-anti-CD38 (T16; Beckman Coulter, West Sacramento, CA, USA),

FITC-anti-CXCR3 (49801), and APC-anti-CXCR5 (51505; R&D Systems, Minneapolis, MN, USA). All of these antibodies were well described and could be used without any apparent problems.

Dead cells were analyzed with Zombie Violet Fixable Viability Kit (BioLegend). To stain intracellular cytokines, cells were stimulated with 50 ng/ml of phorbol-myristate-acetate (Sigma-Aldrich, St. Louis, MO, USA), 500 ng/ml of ionomycin (Sigma-Aldrich), and 2 μM monensin (Sigma-Aldrich) for 1 hour before staining. IFN-γ^+mCD4$^+$ T cells were sorted for the analysis of gene expression using IFN-γ Secretion Assay Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) after stimulation for 1 hours without monensin. Cells were analyzed and sorted using FACS Canto II (BD Biosciences) or FACS Aria II (BD Biosciences). The data were analyzed with FlowJo software (BD Biosciences). CD4$^+$ T cells and naive B cell were prepared using a CD4$^+$ T Cell isolation kit, a naive B cell isolation kit, and autoMACS Pro Separator (Miltenyi Biotec) according to the manufacturer's instructions.

Cell Culture

To evaluate the effect of environmental factors, CD4$^+$ T cells (1×10^5) from healthy controls were cultured in the presence of 125 or 250 μg/ml of fibrinogen (Merck Millipore, Darmstadt, Germany), 0.5 ng/ml of SDF-1α (Peprotech, Rocky Hill, NJ, USA), 0.2 ng/ml of GRO-α (Peprotech), or 2 ng/ml of PDGF-BB (Peprotech), for 2 days. AIM-V medium was used. To stain intracellular cytokines, cells were stimulated with 50 ng/ml of phorbol-myristate-acetate, 500 ng/ml of ionomycin, and 2 μM monensin for 4 hours just before staining. To analyze gene expression and cytokine secretion of cultured cells, they were similarly stimulated without monensin.

To evaluate the effect of IFN-γ on the frequency of CD11c$^+$ B cells, naive B cells from healthy controls were cultured for 6 days using RPMI medium (GIBCO, Cape Cod, MA, USA) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Santa Ana, CA, USA), IFN-γ (20 ng/ml), IL-4 (20 ng/ml), IL-2 (50 IU/ml), IL-21 (10 ng/ml; all from Peprotech), BAFF (10 ng/ml; BioLegend), R848 (1 μg/ml; Invivogen, Toulouse, France), and anti-human IgG + IgM F(ab')2 fragment (10 μg/ml; Jackson ImmunoResearch Lab, West Grove, PA, USA).

To analyze the secreted immunoglobulin by B cells, CD11c$^+$, CD11$^-$CD27$^+$, and CD11c$^-$CD27$^-$ B cells were sorted and cultured for 7 days using AIM-V in the presence of IL-2 (50 IU/ml), IL-21 (100 ng/ml), BAFF (10 ng/ml), R848 (1.25 μg/ml), and sCD40L (100 ng/ml). The amount of each immunoglobulin in the supernatant was measured using Antibody Isotyping 7-Plex Human ProcartaPlex Panel (Invitrogen, Waltham, MA, USA). The amount below the lower detection threshold was converted into 0. The amount above the upper detection limit was excluded. The mean value was calculated from 1 to 5 wells for each B cell subset per each patient. The average amount of the 3 subsets was normalized to 1 for each patient.

Analysis of mRNA Expression

To analyze gene expression of Th1 cells, sorted cells were lysed, reverse transcribed, and pre-amplified for 18 cycles using Single Cell-to-CT Kit (Thermo Fisher Scientific, Waltham, MA, USA) and pooled primers as described below (Fluidigm, South San Francisco, CA, USA). The pre-amplified samples were then subjected to polymerase chain reaction (PCR) using TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and Biomark HD System (Fluidigm) according to the manufacturer's protocol. Target gene expression was normalized to the expression of *ACTB*. The primers for PCR in the analysis of Th1 cells were as follows; *IFNG* (forward primer [FP]: ACTGCCAGGACCCATATGTAA, reverse primer [RP]: GTTCCATTATCCGCTACATCTGAA), *CSF2* (FP: TGATGGCCAGCCACTACAA and RP: CAAAGGGGATGACAAGCAGAAA), *STAT1* (FP: ATGCTGGCACCAGAACGAA and RP: GCTGGCACAATTGGGTTTCAA), *STAT3* (FP: GGAAATAATGGTGAAGGTGCTGAAC and RP: CCGAGGTCAACTCCATGTCAAA), *STAT4* (FP: CAGTGCTGGAGGTAAAGGAA and RP: AGAGGCAGATCTGTGTTTCAA), *TBX21* (FP: GGGCGTCCAACAATGTGAC and RP: CCGTCGTTCACCTCAACGATA), *GATA3* (FP: CACGGTGCAGAGGTACCC and RP: AGGGTAGGGATCCATGAAGCA), *RORC* (FP: CAAGACTCATCGCCAAAGCA and RP: TTTCCACATGCTGGCTACAC), *FOXP3* (FP: TGTGGGGTAGCCATGGAAA and RP: GGGTCGCATGTTGTGGAA), *IFNGR1* (FP: AAGCCAGGGTTGGACAAAA and RP: GATATCCAGTTTAGGTGGTCCAA), and *ACTB* (FP: CCAACCGCGAGAAGATGAC and RP: TAGCACAGCCTGGATAGCAA; Fluidigm).

To analyze gene expression of cultured CD4$^+$ T cells, total RNA was prepared from the cultured cells using RNeasy Kit and RNase-Free DNase Set (QIAGEN, Venlo, The Netherlands), and reverse transcribed to complementary DNA using PrimeScript RT Master Mix (Takara Bio, Kusatsu, Japan). PCR was performed using SYBR Premix Ex Taq (Takara Bio), and LightCycler 96 (Roche, Basel, Switzerland). The expression level of each mRNA was determined with normalization to *ACTB*. The primers for PCR in the analysis of CD4$^+$ cells were as follows: *ACTB* (FP: CCAACCGCGAGAAGATGAC and RP: TAGCACAGCCTGGATAGCAA), *IFNG* (FP:

ACTGCCAGGACCCATATGTAA and RP: GTTCCATTATCCGCTACATCTGAA), *STAT1* (FP: ATGCTGGCACCAGAACGAA and RP: GCTGGCACAATTGGGTTTCAA), *STAT4* (FP: CAGTGCTGGAGGTAAAGGAA and RP: AGAGGCAGATCTGTGTTTCAA), *TBX21* (FP: GGGCGTCCAACAATGTGAC and RP: CCGTCGTTCACCTCAACGATA; Fasmac, Atsugi, Japan).

Detection of Fibrinogen, Immunoglobulin, and Other Cytokines

The concentration of fibrinogen in the plasma was determined with AssayMax Human Fibrinogen ELISA Kit (Assaypro, St. Charles, MO, USA). The concentration of immunoglobulin in the serum was determined using Antibody Isotyping 7-Plex Human ProcartaPlex Panel (Invitrogen) according to the manufacturer's instruction. The other cytokines in the serum were measured using Bio-Plex Pro Human Cytokine Screening Panel (48-Plex; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. The cytokines included in the analysis are those for which more than 70% of the tested samples showed values within their standard range.

Next Generation Sequencing of B Cell Receptor Genes and B Cell Receptor Repertoire Analysis

B cell subsets were sorted, lysed in ISOGEN-LS (Nippon Gene, Tokyo, Japan), and further processed for B cell receptor (BCR) sequencing in Repertoire Genesis Inc. (Ibaraki, Japan). The obtained sequencing data were analyzed with a repertoire analysis software, Repertoire Genesis, which was originally developed by Repertoire Genesis Inc. The details are described in previous papers.[13,14] Only the in-frame CDR3 reads that can be translated into amino acid sequences were included in the analysis. Unique reads were defined as distinct ones that have the same IgHV, IgHD, IgHJ, and IgHC and the CDR3 sequences with the same resultant amino acid sequences.

Analysis of Gene Expression Profile of B Cell Subsets

About 15,000 B cells per sample in average were sorted for nCounter analysis with GX Human Immunology V2 kit (NanoString Technologies, Seattle, WA, USA). Cell lysate was prepared with RLT buffer (QIAGEN), and used as input material, according to the manufacturer's protocol. The data were analyzed with advanced analysis function in nSolver software. The nCounter of each RNA was normalized using the geometric mean of 11 housekeeping genes, *EEF1G*, *RPL19*, *TBP*, *SDHA*, *POLR2A*, *PPIA*, *OAZ1*, *G6PD*, *ABCF1*, *TUBB*, and *POLR1B*, which were selected by nCounter algorithm. The genes, which were detected above the low count threshold level for less than 25% of the samples, were excluded from the analysis. Next, the normalized data were used for further analysis with iDEP software (version 0.90).[15]

Statistical Analysis

Data were analyzed with Prism software (GraphPad Software, La Jolla, CA, USA) unless otherwise indicated. An unpaired or a paired t test was used to compare data from 2 groups, using Welch's correction as appropriate. Two-way analysis of variance ()ANOVA with Holm-Sidak or Dunnett's comparison test was used to compare data from more than 2 groups, as appropriate. Pearson's analysis was used to evaluate correlations. Differences were considered significant when the p value was <0.05.

Data Availability

The gene expression profile data of B cell subsets with nCounter system were deposited to the Gene Expression Omnibus database in National Center for Biotechnology Information (NCBI). The accession number is GSE158326.

Results

Th1 Cell Frequency in the Peripheral Blood Is Higher in PP Responders

To portray the immunological characteristics that correlate with treatment effect of PP, immune cells in the peripheral blood of patients with multiple sclerosis were analyzed before and after the treatment with IAPP. When evaluated by an objective clinical improvement based on EDSS, including functional system scoring after the treatment, 53% of the patients were judged as being IAPP responders (see the Table). The response rate was not different between patients within and more than 1 month after a relapse (75% vs 50%, $p = 0.61$) nor between relapsing–remitting and secondary-progressive multiple sclerosis (50% vs 67%, $p = 0.71$). The median EDSS changed from 4.25 (interquartile range [IQR] $= 2.5$) to 3.75 (IQR $= 2.63$) in responders to IAPP, and did not change in nonresponders (see the Table). The clinical improvement was sustained for 4.5 ± 1.6 months (mean $\pm$ SD) for the responders. The most striking immunological difference was observed in IFN-γ^+CD4$^+$ Th1 cell frequency before treatment, which was significantly higher in IAPP responders than in nonresponders (see Fig 1A, B). The same trend of a higher Th1 frequency in PP responders was observed in another cohort of patients who received DFPP (see Fig 1C). The median EDSS changed from 7 (IQR $= 1.0$) to 6.75 (IQR $= 0.75$) in responders to DFPP, and did not change in nonresponders (see the Table). The absolute Th1 cell number was also higher in

TABLE. Demographics of all the Participants

		HC (n = 13)	Responder to IAPP (n = 16)	Nonresponder to IAPP (n = 11)	Responder to DFPP (n = 2)	Nonresponder to DFPP (n = 3)	Repertoire analysis (n = 2)	B cell gene expression analysis (n = 3)
Age (mean [SD])		42.1 [8.8]	46.1 [9.9]	42.6 [9.3]	53 [0]	44 [5.3]	51.5 [7.8]	43.7 [13.0]
M: F		6: 7	5: 11	1: 10	0: 2	0: 3	1: 1	1: 2
Disease duration (years) (mean [SD])			13.3 [10.1]	12.3 [6.3]	12.5 [2.1]	20.7 [12.2]	7 [2.8]	9.7 [0.6]
EDSS (median [range]) (before PP)			4.25 [3–7.5]	4.5 [2–8.5]	7 [6–8]	7 [6–7.5]	3.5 [3.5–3.5]	3.5 [2–4.5]
ARR (mean [SD])			0.9 [1.1]	1.2 [1.1]	1.0 [1.4]	1.0 [1.0]	0 [0]	0.7 [1.2]
Disease status at treatment			relapse in 3 cases	relapse in 1 case	no relapse case	no relapse case	no relapse case	no relapse case
RRMS: SPMS			10: 6	8: 3	2: 0	0: 3	1: 1	1: 2
OCB (positive)			7/16	4/10	0/1	2/3	0/2	1/3
Maintenance treatment	GA		4	4	0	0	0	0
	IFN-b		1	0	0	0	1	1
	Fingolimod		1	0	0	0	0	0
	steroid		10	10	2	2	1	2
	IS		8	7	2	3	1	1

Demographics of the patients with multiple sclerosis and healthy control subjects are described. No patients presented with clinical characteristics suggestive of neuromyelitis optica spectrum disorder or myelin oligodendrocyte glycoprotein (MOG) antibody-associated disease. Anti-aquaporin 4 antibody and anti-MOG antibody were negative in all the 29 and 7 patients who were tested, respectively. Five patients, who were treated twice with IAPP at different time points, are shown here based on the information at the first time of the treatment. The blood sample was obtained only before the treatment for one patient who was treated with DFPP. Intravenous methylprednisolone treatment was performed after IAPP in 4 among the total of 17 treatment courses of responders, and in 4 among the total of 15 courses of nonresponders. Five responders and one nonresponder were treated with intravenous methylprednisolone before IAPP. One responder was treated with intravenous high-dose immunoglobulin after IAPP. Intravenous methylprednisolone treatment was performed after DFPP in 2 responders and 2 nonresponders. One responder and one nonresponder were treated with intravenous methylprednisolone before DFPP. One responder was also treated with intravenous high-dose immunoglobulin after DFPP. One responder and one nonresponder to IAPP were included in the analysis of B cell repertoire analysis (Fig 6E, F). Two responders and one nonresponder were included in B cell gene expression analysis (Fig 7).

ARR = annualized relapse rate; DFPP = double-filtration plasmapheresis; EDSS = Expanded Disability Status Scale; GA = glatiramer acetate; HC = healthy control; IAPP = immunoadsorption plasmapheresis; IFN-β = interferon-β; IS = immunosuppressant; OCB = oligoclonal bands; PP = plasmapheresis; RRMS = relapsing–remitting multiple sclerosis; SPMS = secondary-progressive multiple sclerosis.

responders than nonresponders before IAPP (see Fig 1D). In contrast, the frequency of IL-17A$^+$CD4$^+$ Th17 cells, another inflammatory Th cells, and IFN-γ$^-$IL-17A$^-$Foxp3$^+$CD4$^+$ regulatory T (Treg) cells did not differ between responders and nonresponders (see Fig 1A, B). IL-4$^+$CD4$^+$ Th2 cells, and IL-10$^+$CD4$^+$ T cells showed a tendency to be higher in responders without significance (see Fig 1B). Although the frequencies of these cells were not altered after IAPP treatment (see Fig 1E), the Th1 cell frequency appeared to have a predictive value for identifying responders before starting the treatment (see Fig 1F). Th1 cell frequency of 7.02% had the highest positive likelihood ratio for prediction of responders with a sensitivity of 76.5% and a specificity of 93.3%.

Further analysis showed that the frequency of follicular helper T cells, which are specialized for mainly providing B cell help in the germinal centers, did not correlate with treatment response (Fig 2A, B). There was no difference between responders and nonresponders in the frequencies of B cell lineage groups, NKT cells, NK cells, HLADR$^+$ NK cells, and several monocyte types (see Fig 2C–F). The treatment with IAPP did not alter the frequencies of these subsets (data not shown). As these thorough analyses indicate that a high frequency of Th1 cells has a significant predictive value for responders (see Fig 1B, F), we explored for the underlying mechanism for this intriguing observation.

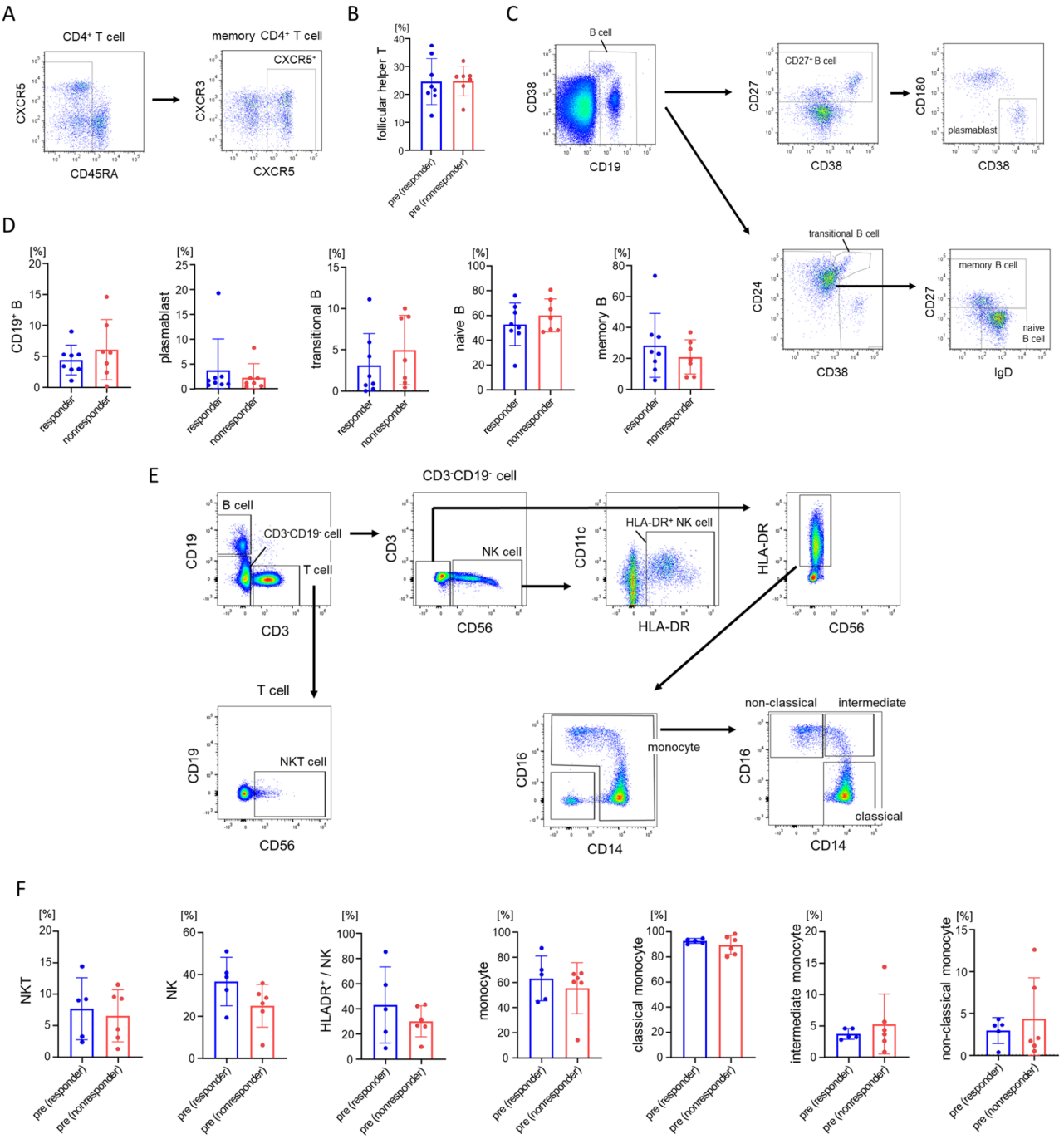

FIGURE 2: The frequencies of various immune cell subsets are not different between responders and non-responders before the treatment with IAPP. (A) Representative flow cytometry plots showing CXCR5^{+}CD45RA^{-}CD4^{+} follicular helper T cells. (B) The frequency of follicular helper T cells among memory CD4^{+} T cells was analyzed in the peripheral blood of responder and nonresponder patients to IAPP, before the treatment. The frequency was compared before and after the treatment. (C) Representative flow cytometry plots showing B cell subsets. (D) The frequency of B cells among mononuclear cells, and plasmablasts, transitional B cells, memory B cells, and naive B cells among CD19^{+} B cells were analyzed in the peripheral blood of responder and nonresponder patients before IAPP treatment. (E) Representative flow cytometry plots showing NKT cells, NK cells, HLA-DR^{+} NK cells, and classical/intermediate/non-classical monocytes. (F) The frequency of NKT cells among CD3^{+} T cells, NK cells among CD3^{-}CD19^{-} non-T non-B cells, HLA-DR^{+} cells among NK cells, monocytes among CD3^{-}CD19^{-}CD56^{-} non-T non-B non-NK cells, and classical/intermediate/non-classical monocytes among all monocytes, were analyzed in the peripheral blood of responder and nonresponder patients to IAPP, before the treatment. Error bars represent the mean ± SD. IAPP = immunoadsorption plasmapheresis. [Color figure can be viewed at www.annalsofneurology.org]

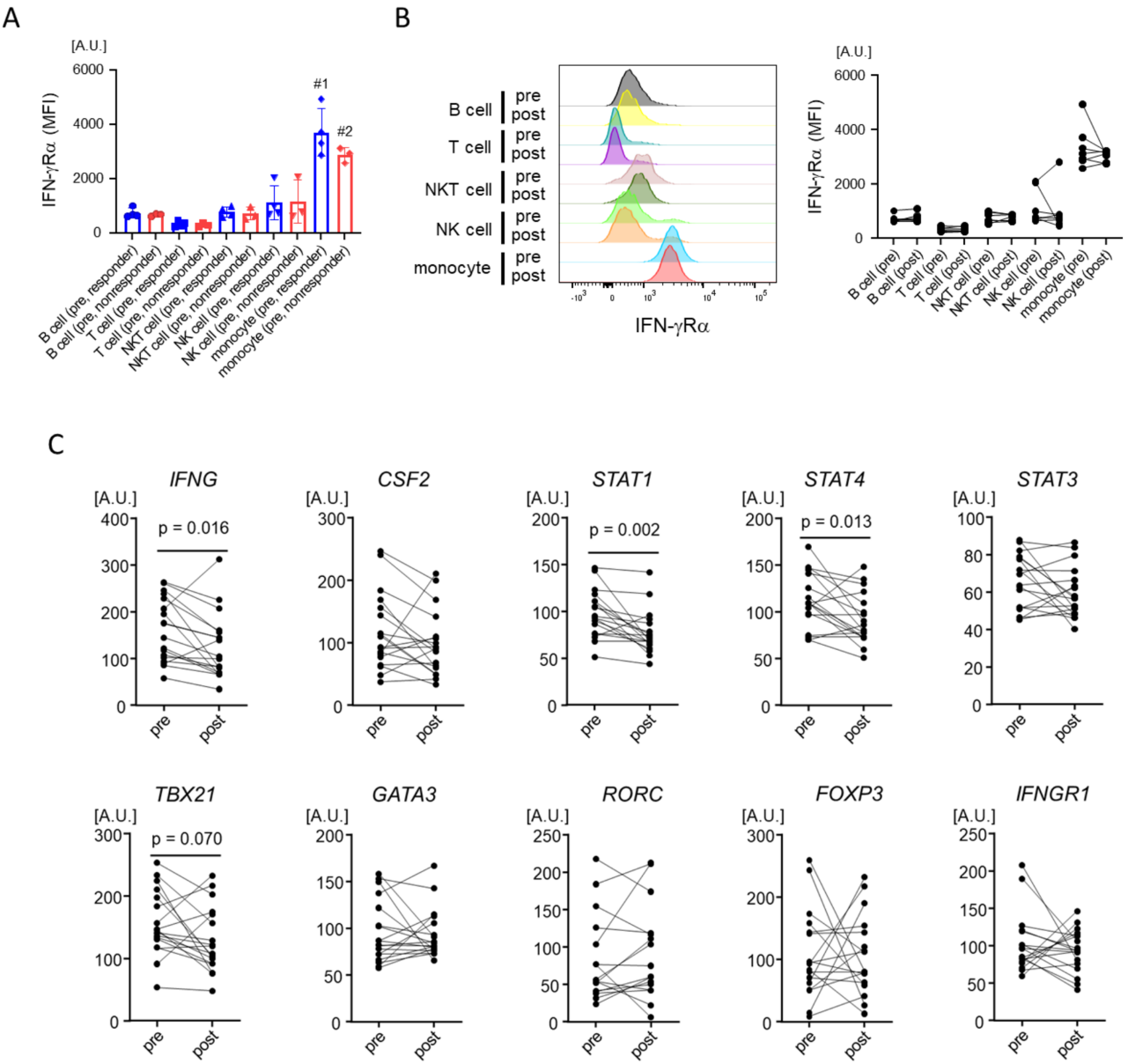

FIGURE 3: Inflammatory gene expression of Th1 cells decreases after PP treatment. (A) The expression of IFN-γRα on various immune cell subsets of IAPP responders and nonresponders before IAPP. The *p* value: #1 < 0.0001 versus B cell (R), <0.0001 versus T cell (R), <0.0001 versus NKT cell (R), <0.0001 versus NK cell (R), #2 0.001 versus B cell (NR), <0.0004 versus T cell (NR), <0.001 versus NKT cell (NR), and <0.004 versus NK cell (NR). (B) Representative histogram showing the expression of IFN-γRα on the immune cell subsets before and after IAPP (left panel). No significant alterations were observed after the treatment (right panel). (C) Expression of genes related to T cell functions in IFN-γ^{+}mCD4^{+} T cells before and after IAPP. The expression of Th1-characteristic genes (*IFNG*, *STAT1*, and *STAT4*) decreased after the treatment, whereas other genes were not significantly altered. Gene expression was normalized to the expression of *ACTB*. Two-way analysis of variance (ANOVA) with Holm-Sidak correction was used for statistical analysis in A. An unpaired *t* test was used for statistical analysis in C. Error bars represent the mean ± SD. NR = nonresponders; IAPP = immunoadsorption plasmapheresis; PP = plasmapheresis; R = responders. [Color figure can be viewed at www.annalsofneurology.org]

Expressions of Inflammation-Related Genes in Th1 Cells Are Reduced After the Treatment With IAPP

At first, expression of IFN-γ receptor α (IFN-γRα) was evaluated to search for the mechanism underlying the correlation between the IFN-γ^{+} Th1 cell frequency and treatment response. We confirmed that the expression of IFN-γRα was significantly higher on monocytes than other immune cell subsets irrespective of treatment response (Fig 3A). However, it was not differentially expressed on any cell subsets between IAPP responders and nonresponders before the treatment (see Fig 3A). Moreover, the expression was not altered after IAPP (Fig 3B), implicating that IFN-γRα

is not involved in mediating the treatment response to IAPP.

Next, the gene expression profile of Th1 cells was investigated in order to detect a possible cell-intrinsic change by the treatment. We found that expressions of Th1-characteristic genes, *IFNG*, *STAT1*, and *STAT4*, were significantly decreased by the treatment (Fig 3C). In addition, the expression of *TBX21*, encoding T-bet, a central transcription factor of Th1 cells, tended to be decreased after IAPP (see Fig 3C). In contrast, no alteration related to IAPP treatment was detected in the expressions of some signature genes for Th2, Th17, and Treg cells (see Fig 3C). These gene profile data suggested that the IAPP treatment would reduce expression of inflammation-related genes in Th1 cells, which might be correlated with the link between IAPP responders and a higher Th1 cell frequency (see Fig 1B, F).

We further searched for a soluble factor that might be associated with the beneficial treatment effect of IAPP. Although none of 30 factors that we evaluated showed a predictive value, fibrinogen and GRO-α decreased, and SDF-1α and PDGF-BB increased after IAPP treatment (Fig 4A, B, data not shown). However, none of these factors had significant effect on the Th1-related genes at least in our experimental setting using primary CD4$^+$ T cells (Fig 4C, D).

The Frequency of CD11c$^+$ B Cells is Positively Correlated With Th1 Cell Frequency, and Reduced After IAPP Treatment

It has recently been reported that CD11c$^+$ B cells are increased in the peripheral blood of patients with systemic lupus erythematosus (SLE), and the cells secrete a high amount of autoantibodies.[16] CD11c$^+$ B cells are characterized by a higher expression of *TBX21*,[16] which could be induced by IFN-γ.[17] Although it is generally thought that the primary mechanism of PP is to remove disease-relevant antibodies, we hypothesized that PP might influence the number or functions of this specific B cell population to mediate treatment effect. When the samples were divided based on the Th1 cell frequency, the frequency of CD11c$^+$ B cell was significantly higher in the Th1-high group (Fig 5A). Individual analysis showed that the CD11c$^+$ B cell frequency positively correlated with the Th1 cell frequency, and the expression of *IFNG* in Th1 cells (Fig 5B). CD21$^{-/low}$ B cells have been shown to be autoreactive and increased in rheumatoid arthritis, Sjogren syndrome, and idiopathic thrombocytopenic purpura.[18–20] CD11c$^+$ B cells had significantly lower expression of CD21 than the CD11c$^-$ counterpart, further supporting autoreactivity of CD11c$^+$ B cell population (Fig 5C). The expression of CXCR5 was also decreased in

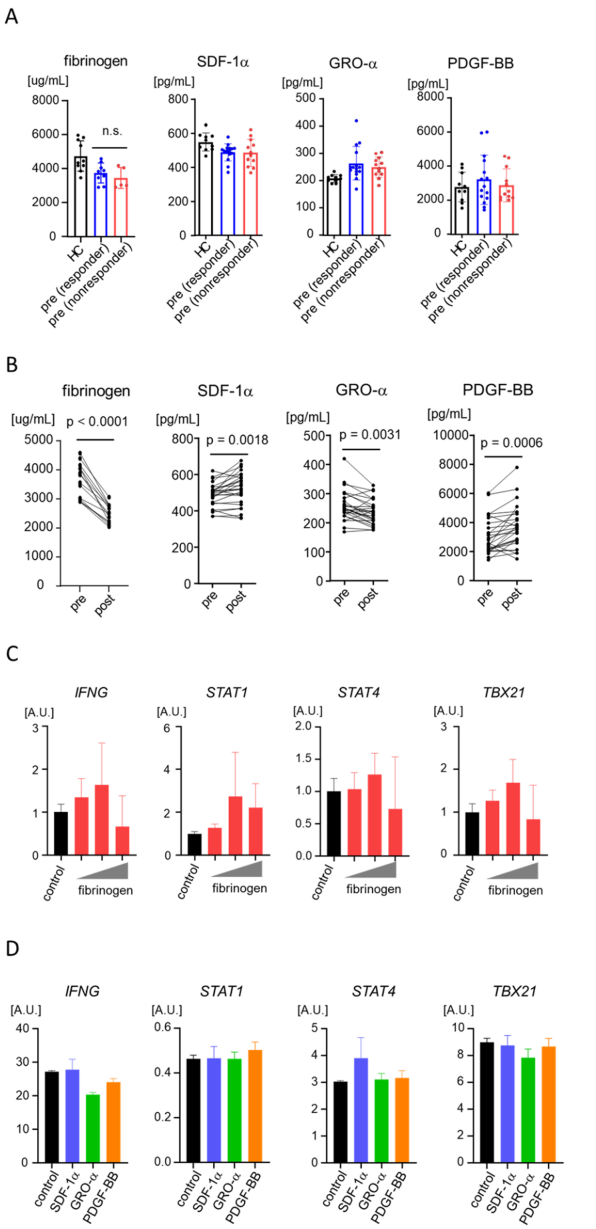

FIGURE 4: Fibrinogen decreases after treatment with IAPP. (A) The concentration of fibrinogen was measured in the plasma, and the concentrations of SDF-1α, GRO-α, and PDGF-BB were measured in the serum, from healthy controls and patients with multiple sclerosis before IAPP treatment. (B) Fibrinogen and GRO-α were decreased, and SDF-1α and PDGF-BB were increased after IAPP treatment. (C) CD4$^+$ T cells were cultured with 0, 125, 250, and 4000 μg/ml of fibrinogen, for 2 days. The expressions of *IFNG*, *STAT1*, *STAT4*, and *TBX21*, which are characteristic genes of Th1 cells, were measured in cultured CD4$^+$ T cells in the presence of fibrinogen. Three independent experiments were combined with the values normalized to controls. There were 3 or 4 in each group for each experiment. (D) CD4$^+$ T cells were cultured in the presence of SDF-1α, GRO-α, or PDGF-BB, for 2 days. The expressions of *IFNG*, *STAT1*, *STAT4*, and *TBX21* are shown. There were 3 in each group. An unpaired *t* test was used in A, and a paired *t* test was used in B for statistical analysis. Error bars represent the mean ± SD in A and C, and ± SEM in D. IAPP = immunoadsorption plasmapheresis. [Color figure can be viewed at www.annalsofneurology.org]

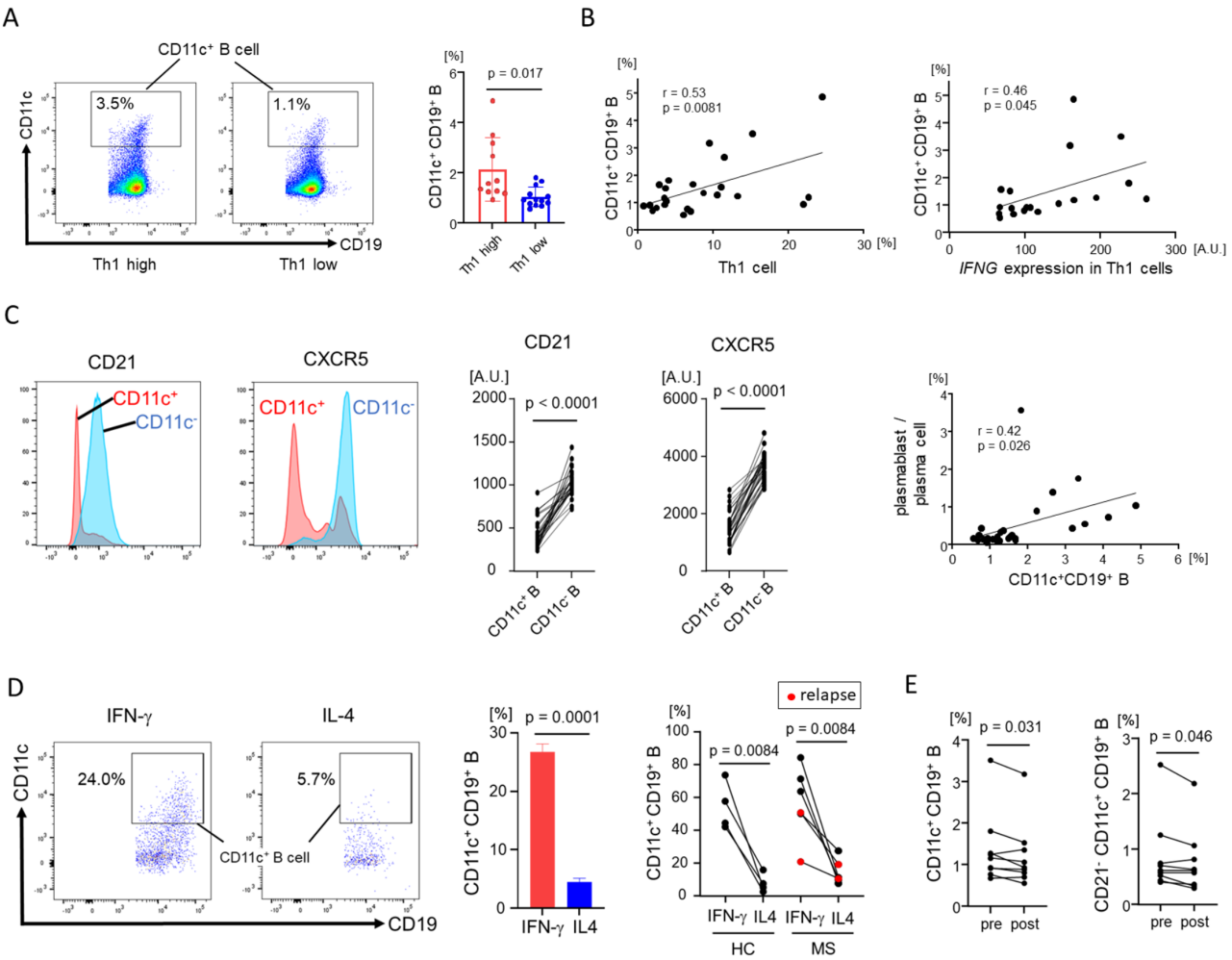

FIGURE 5: CD11c$^+$ B cells are correlated with Th1 cell frequency. (A) Representative flow cytometry plots showing the frequency of CD11c$^+$ B cells among non-plasma CD19$^+$ B cells in the peripheral blood. They were increased in subjects with high Th1 cell frequency. The cutoff value for high Th1 frequency was set to 7.02%, which had the highest positive likelihood ratio for prediction of responders based on ROC curve shown in Fig 1E. (B) The frequency of CD11c$^+$ B cells positively correlated with Th1 cell frequency, gene expression of *IFNG* in Th1 cells, and plasmablast/plasma cell frequency in the peripheral blood. (C) Representative histogram showing the expression of CD21 and CXCR5 on CD11c$^+$ and CD11c$^-$ B cells. Their expression levels were compared based on the MFI. (D) Naive B cells from a healthy subject were cultured for 6 days in the presence of IFN-γ or IL-4, in addition to IL-2, IL-21, BAFF, R848, and anti-IgG/IgM F(ab')2 fragment. The frequency of CD11c$^+$ B cells was higher in the presence of IFN-γ compared to IL-4 (left and middle). The same effect was observed with B cells from patients with multiple sclerosis (right). (E) The frequency of CD11c$^+$ and CD21$^-$ CD11c$^+$ B cells decreased after treatment with IAPP. An unpaired or a paired *t* test was used appropriately for statistical analysis in A, C, D, and E. Pearson's analysis was used to evaluate correlations in B. Error bars represent the mean ± SD in A and SEM in D. ROC = receiver operating characteristic. [Color figure can be viewed at www.annalsofneurology.org]

CD11c$^+$ B cells (see Fig 5C), which is in line with the previous studies showing a relatively lower migratory potential of this subset to germinal centers.[16,18,21] As expected from the correlation of Th1 cells and CD11c$^+$ B cells (see Fig 5A, B), exogenous IFN-γ significantly increased the frequency of CD11c$^+$ population among cultured naive B cells, compared to IL-4, a Th2-characteristic cytokine (Fig 5D). This effect was similarly observed among healthy controls and patients with multiple sclerosis (see Fig 5D). Importantly, the frequencies of CD11c$^+$ B cells and CD21$^-$CD11c$^+$ B cells decreased in the peripheral blood after IAPP treatment (Fig 5E). This observation can be in harmony with the results that *IFNG* expression by Th1 cells decreased after IAPP (see Fig 3C), and positively correlated with CD11c$^+$ B cell frequency (see Fig 5B).

CD11c$^+$ B Cells Potently Produce Antibodies With Unique BCR Repertoire

After observing the possible involvement of CD11c$^+$ B cells in response to PP in multiple sclerosis, we next

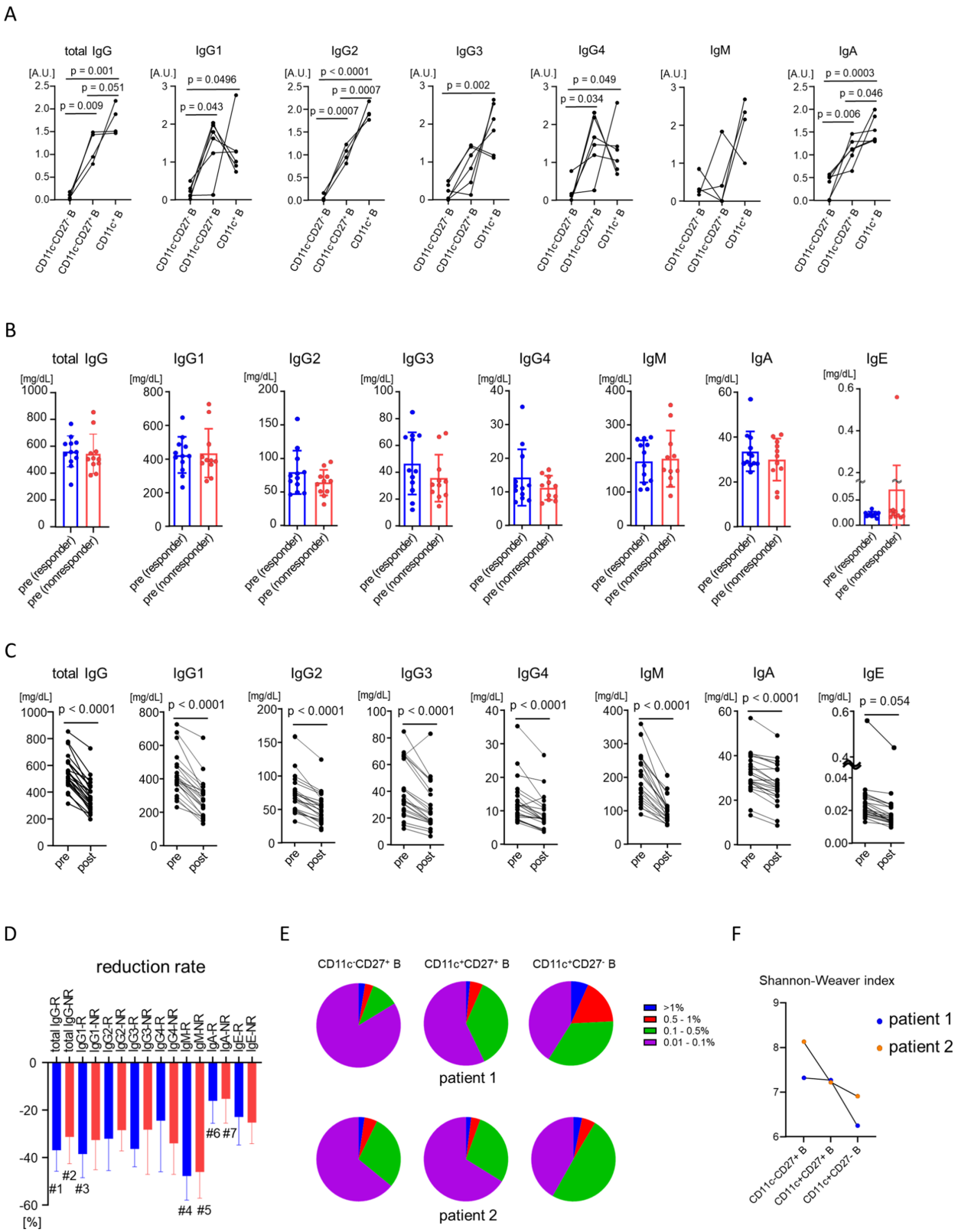

FIGURE 6: CD11c^{+} B cells secrete a high amount of immunoglobulin characterized by BCR repertoire bias. (A) CD11c^{+}, CD11^{-}CD27^{+}, and CD11c^{-}CD27^{-} B cells were sorted and cultured for 7 days in the presence of IL-2, IL-21, BAFF, R848, and sCD40L. The amount of each immunoglobulin subset in the supernatant was measured. The average amounts of the 3 subsets were normalized to 1 for each sample. There were 6 patients with multiple sclerosis. (B) The concentration of each immunoglobulin subset in the serum. No differences were observed between responders and nonresponders before treatment

(Figure legend continues on next page.)

evaluated antibody secretion by this population in comparison to other B cell subsets. All IgG subclasses and IgA were much more robustly produced by the $CD11c^+$ population than by $CD11c^-CD27^-$ naive B cells (Fig 6A). Moreover, with regard to secretion of IgG2 and IgA, $CD11c^+$ B cells were even more efficient than $CD11c^-CD27^+$ memory B cells (see Fig 6A). In addition, the frequency of plasmablasts/plasma cells positively correlated with $CD11c^+$ B cell frequency (see Fig 5B). Although the concentration of immunoglobulin in the serum did not predict treatment response (Fig 6B), all the immunoglobulin subsets were decreased by IAPP treatment, as expected (Fig 6C). No difference was found in the reduction rate between responders and nonresponders (Fig 6D). On the other hand, the reduction rate analysis (see Fig 6D) showed that IgG and IgM are more efficiently removed by IAPP treatment compared with IgA and IgE. The concentration of immunoglobulins may be different in the cerebrospinal fluid (CSF) between PP responders and nonresponders, because $CD11c^+$ B cells are more abundant there than in the peripheral blood,[22] which needs further investigation.

Next, the BCR repertoire of IgG was analyzed in B cell populations that are classified based on the expression of CD11c and CD27. When the proportion of highly prevalent combinations of IgH chains (IgHV, IgHD, and IgHJ) was evaluated, it was notable that $CD11c^+CD27^-$ B cells contained a larger number of expanded unique combinations, compared with $CD11c^+CD27^+$ or $CD11c^-CD27^+$B cells (Fig 6E). Supportive for this, the calculated index of clonal diversity appeared to be lower in $CD11c^+CD27^-$ B cells, when the sequence of CDR3 was incorporated into the analysis (Fig 6F). Taken together, we postulate that the $CD11c^+CD27^-$ B cell population is relatively more enriched for clonally expanded B cells, which are presumed to contain disease-relevant ones. Analysis of shared BCR repertoires detected no specific clonal similarity among these 3 B cell populations (data not shown).

$CD11c^+$ B Cells Are Transcriptionally Distinct from Other B Cell Subsets

To further evaluate the characteristics of $CD11c^+$ B cells, we analyzed the gene expression profile of different B cell populations based on the expression of CD11c and CD27. First, these populations were clearly differentiated by principal component analysis, and most variable gene sets (Fig 7A, C). As expected, $CD11c^+$ B cells had higher expression of *ITGAX*, encoding CD11c (Fig 7B–D). Consistently with the data that $CD11c^+$ B cells are prevalent in the presence of IFN-γ (see Fig 5D), *TBX21*, a gene induced by IFN-γ, was highly expressed by $CD11c^+$ B cells (see Fig 7B–D). In addition, *IFNGR1* expression was relatively higher in $CD11c^+$ B cells compared to $CD11c^-CD27^+$ memory B cells, indicating their enhanced responsiveness to IFN-γ (see Fig 7D). As expected from lower expression of CD21 (see Fig 5C), the corresponding gene, *CR2*, was relatively lower in $CD11c^+$ populations (see Fig 7C, D). Higher expression of *CD80* and *CD86* suggested that $CD11c^+$ B cells could exhibit higher co-stimulatory functions or maintain activated status (see Fig 7C, E).

TLR7 signaling, which is activated by single-stranded RNA, is critically involved in autoantibody-mediated diseases.[23,24] The pivotal role of TLR7 ligands in autoantibody production has been described mainly in SLE,[23,24] where autoantibodies respond to RNA- and DNA-containing autoantigens. However, the role of TLR signaling has been shown also in other autoantibody-mediated disorders, such as neuromyelitis optica.[25] *TLR7* was highly expressed in $CD11c^+CD27^-$ B cells (see Fig 7C, F). This might partially contribute to their higher expression of *TBX21* (see Fig 7B–D), as it was shown that IFN-γ and TLR7 stimulation synergistically induced T-bet expression in B cells.[26] Additionally, *TLR2* and *TLR4* expressions were relatively higher in $CD11c^+$ B cells (see Fig 7C, F). Stimulation of these TLRs on B cells is known to induce the proliferation and differentiation of the B cells into plasma cells.[27] These alterations were accompanied by a

with IAPP. (C) The concentration of each immunoglobulin subset in the serum was measured before and after treatment with IAPP. (D) The reduction rate of each immunoglobulin subset by treatment with IAPP. It was not different between responders and nonresponders. The *p* values (among total IgG, IgM, IgA, and IgE for R and NR, respectively): #1 0.0001 vs IgM-R, <0.0001 vs IgA-R, and <0.0001 vs IgE-R, #2 < 0.0001 vs IgM-NR, <0.0001 vs IgA-NR, 0.02 vs IgE-NR, #4 < 0.0001 vs IgA-R, <0.0001 vs IgE-R, #5 < 0.0001 vs IgA-NR, <0.0001 vs IgE-NR, #6 0.006 vs IgE-R, and #7 0.0007 vs IgE-NR. The *p* values (among IgG subclasses, R and NR, respectively): #3 0.04 vs IgG4-R. (E) The BCR repertoire was analyzed for 3 B cell subsets. All the reads of BCR in IgG subclass were sorted based on the combination of IgHV, IgHD, and IgHJ chains. Each specific BCR combination was further classified according to their frequency among all the BCR reads, and shown as circle graphs. The analysis included the BCR combinations that account for at least 0.01% of all the reads. (F) The Shannon-Weaver index H′ of BCR repertoire in IgG subclass based on the combination of IgHV, IgHD, and IgHJ chains, IgHC region, and CDR3 sequence. Two-way analysis of variance (ANOVA) with Holm-Sidak correction was used for statistical analysis in A and D. A paired *t* test was used in C. Error bars represent the mean ± SD in B and D. BCR = B cell receptor; IAPP = immunoadsorption plasmapheresis; NR = non-responders; R = responders. [Color figure can be viewed at www.annalsofneurology.org]

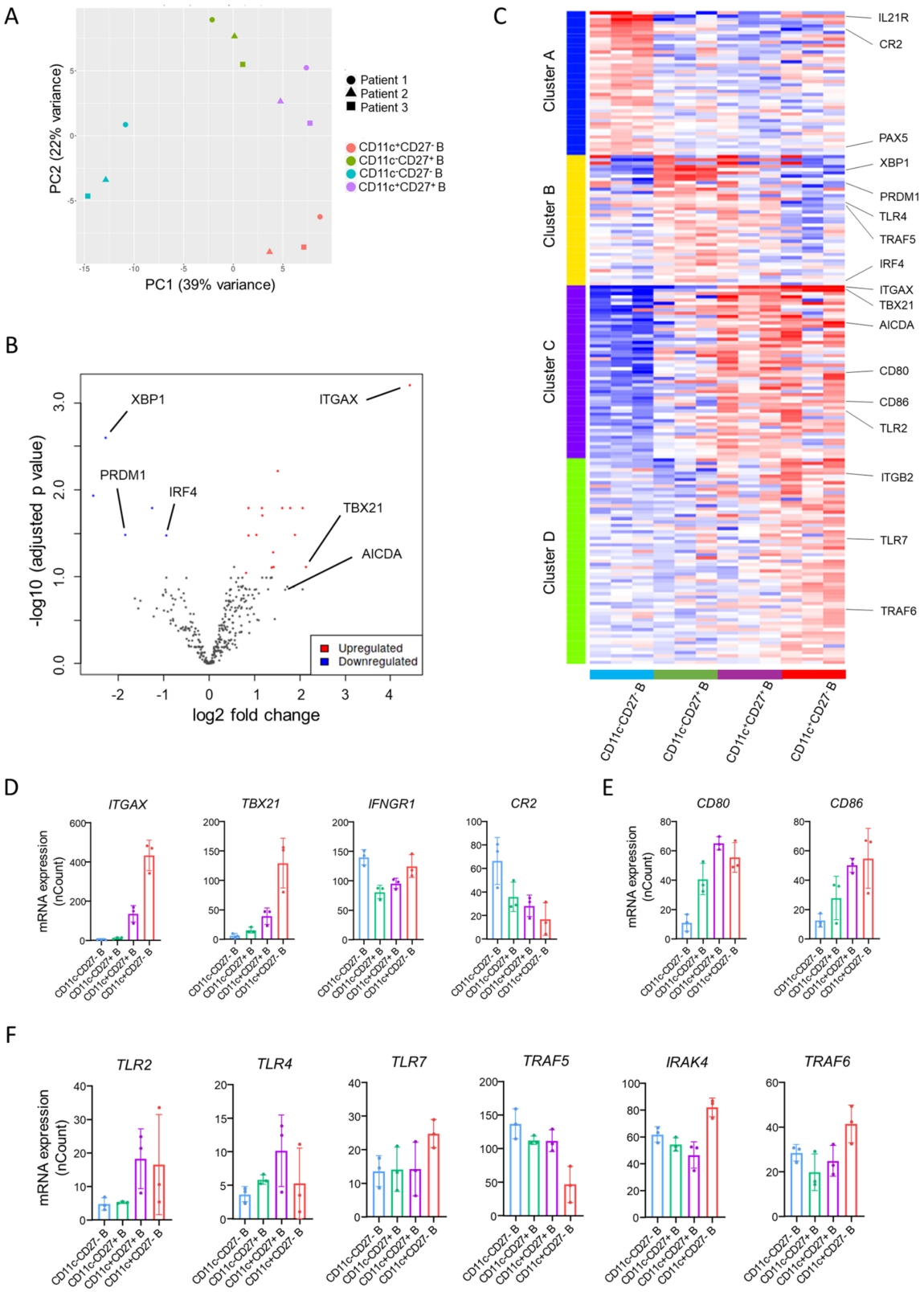

FIGURE 7: CD11c$^+$ B cells are transcriptionally distinct from other B cell subsets. (A) Principal component analysis based on gene expression data of the 4 B cell populations (CD11c$^{+/-}$/CD27$^{+/-}$) from 3 patients with multiple sclerosis. The expression data were obtained with nCounter system. (B) A Volcano plot showing differential gene expression of CD11c$^+$ B cells, including CD11c$^+$CD27$^-$ and CD11c$^+$CD27$^+$ cells, compared to CD11c$^-$CD27$^+$ B cells. (C) K-means clustering of 200 most variable genes detected by nCounter system. (D) The expression of genes that are involved in IFN-γ signaling and autoimmunity. (E) The expression of activation-related genes. (F) The expressions of TLR-related genes were detected by nCounter system. Error bars represent the mean ± SD.

reduced expression of *TRAF5* in $CD11c^+CD27^-$ B cells (see Fig 7C, F). This is consistent with the fact that *TRAF5* is a negative regulator of TLR7 signaling.[28] Moreover, higher expressions of *IRAK4* and *TRAF6* support the increased responsiveness of the $CD11c^+CD27^-$ B cells to the TLR stimulation, because these molecules would mediate downstream signaling of most TLRs, including TLR2, TLR4, and TLR7 (see Fig 7C, F).[29] Collective data in our study, consistent with a previous study for SLE,[21] might suggest that $CD11c^+$ B cells, especially $CD11c^+CD27^-$ B cells, stimulated by TLR signaling contribute to the development of autoantibody-mediated pathology in multiple sclerosis. In fact, disease exacerbation in multiple sclerosis is frequently observed during or after systemic infection, including potently TLR2- and TLR4-stimulating bacterial, and TLR7-stimulating viral infection.[30,31] Moreover, exacerbations associated with systemic infection are known to result in more sustained neurological deficits than other exacerbations.[32]

In summary, this study has revealed that a subgroup of patients with multiple sclerosis who have relatively high frequencies of Th1 cells in the peripheral blood can be successfully treated with PP. In such patients, there was a correlative increase of $CD11c^+$ B cells that might produce pathogenic autoantibody under the control by Th1 cells. The beneficial effects of PP are associated with decreased inflammatory characteristics of Th1 cells, and a decreased frequency of $CD11c^+$ B cells, a potential source of autoantibodies. We therefore assume that removal of autoreactive immunoglobulins is not a sole mechanism of PP treatment for multiple sclerosis. The responders to PP could be predicted with the frequency of Th1 cells, which deserves further investigation to develop precision medicine in multiple sclerosis.

Discussion

Although PP may relieve disabling symptoms in a broad range of autoimmune neurological diseases, no beneficial effects of PP were observed in up to two thirds of patients with multiple sclerosis,[2,3,7,8] implying that such patients only take risks of potential complications, such as hemorrhage, infection, and deep venous thrombosis.[3,8] It was speculated that autoreactive immunoglobulins in the circulation are the main therapeutic target of PP.[7,11] However, immune cells and several plasma constituents, including cytokines, were shown to be also affected by PP, and the precise therapeutic mechanism has not yet been fully understood.[33,34] This hinders reliable prediction of treatment response. Here, we propose a novel candidate predictor, Th1 cell frequency, which is supported by detailed analysis of immunological mechanism of PP (see Fig 1B, F). Most importantly, our study suggests that treatment response to PP is clearly different depending on immunological characteristics regarding Th1 cell - $CD11c^+$ B cell axis in individual patients. This might be consistent with a previous finding that a subgroup of patients with distinct traits of pathology appears to benefit from PP.[7,11] PP was also beneficial for some patients with progressive disease, even more than 1 month after the last exacerbation (see the Table). Although previous studies failed to show the therapeutic efficacy of PP in progressive disease, it can be speculated that heterogeneity of immunological traits made it difficult to detect the benefit.[2] $CD11c^+$ B cells are known to secrete a high amount of autoantibodies in patients with SLE.[16] In addition, the frequency of $CD11c^+$ B cells in the peripheral blood positively correlated with the titer of autoantibodies in the serum of the patients.[16] Another study showed that $CD11c^+CD11b^+$ B cells were increased in lupus-prone mice, and depletion of these cells in vivo resulted in a reduced amount of autoantibodies.[35] We have revealed a positive correlation between the frequencies of $CD11c^+$ B cells and Th1 cells (see Fig 5A, B). This is of particular interest considering that PP is effective in patients with a high Th1 cell frequency (see Fig 1B, F). We observed a higher frequency of $CD11c^+$ cells after in vitro culture in the presence of IFN-γ compared to IL-4 (see Fig 5D). This is consistent with results from a previous report that IFN-γ and TLR7 stimulation synergistically promoted expression of CD11b and CD11c in B cells.[26] It was noted that $CD11c^+CD21^-$ B cells were increased in the peripheral blood of a subpopulation of patients with multiple sclerosis.[22] Moreover, their frequency was further elevated in the CSF than in the blood, implying their involvement in the pathogenesis.[22] Importantly, we found that both $CD11c^+$ and $CD11c^+CD21^-$ B cells were reduced after PP (see Fig 5E). This might be attributable to a decreased expression of *IFNG* in Th1 cells after the treatment, because the expression positively correlated with the frequency of $CD11c^+$ B cells (see Fig 5B).

$CD11c^+$ B cells were also characterized by higher expression of *TBX21*, encoding T-bet (see Fig 7D). B cell-specific deletion of T-bet protects mice from murine lupus manifestations, including autoantibody production, renal damage, and rapid mortality,[36,37] whereas IFN-γ is one of the key factors that induces T-bet expression in B cells.[26,38,39] B cell-specific *Ifngr* deletion abolished class-switched autoantibody production and systemic autoimmunity in a B cell-driven autoimmunity model.[38] IFN-γ was also reported to promote development of plasmablasts, and to enhance migratory capacity of B cells to the central nervous system (CNS).[39] Consistently, we found that the frequency of $CD11c^+$ B cell positively

correlates with the frequency of plasmablasts/plasma cells in the peripheral blood (see Fig 5B). These data further support the pathogenic role of CD11c$^+$ B cells.

Our study showed that PP could remove fibrinogen (see Fig 4), which supposedly has a role in sustaining inflammatory pathology of multiple sclerosis, given its presence mainly at the active and chronic active lesions in the CNS of multiple sclerosis.[40,41] In addition, the amount of fibrinogen in the plasma and the CSF correlates with the presence of active lesions and high cortical lesion load, respectively.[42,43] Plasma-derived fibrinogen induces perivascular microglial clustering, and thereby mediates recruitment of T cells and macrophages, which may cause axonal damage in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis.[44,45] Although the pathological contribution of fibrinogen is mostly attributable to its interaction with CD11b, fibrinogen could bind CD11c and TLR4 as well.[45–48] It is an open question whether fibrinogen directly affect CD11c$^+$ B cells.

Our study suggested that successful treatment response to plasmapheresis is not observed when Th1-CD11c$^+$ B cell axis is not working, whereas Th1 cell frequency is similar between responders and healthy controls. Although autoreactive B cells are enriched in the CD11c$^+$ population, this population could be heterogeneous. This might be the case for Th1 cells in the context of Th1-CD11c$^+$ B cell axis, too. Further studies are needed to clarify possible functional difference of Th1-CD11c$^+$ B cell axis among responders, nonresponders, and healthy controls.

Although we focused on the antibody-related function of B cells, a number of recent studies suggested that other antibody-independent functions are highly relevant in the pathogenesis of multiple sclerosis, such as antigen presentation, co-stimulation of adjacent immune cells, and secretion of inflammatory cytokines.[49,50] Our study indicated Th1 cell - CD11c$^+$ B cell axis is involved in responsiveness to PP, presumably mediated by the decrease of CD11c$^+$ B cells and removal of pathogenic immunoglobulins. We also showed that this B cell population is a potent producer of antibodies. However, these findings do not necessarily exclude other possible pathogenic roles of CD11c$^+$ B cells, which may also be related with response to PP. The target antigens of antibodies secreted from CD11c$^+$ B cells and their potential pathogenicity should be determined to address this issue. Besides, IL-4$^+$CD4$^+$ Th2 cells and IL-10$^+$CD4$^+$ T cells tended to be higher in responders (see Fig 1B). It is of interest to investigate the possible involvement of these populations in this context.

This study has some limitations, which could not be fully addressed. The treatment plan was determined by attending neurologists without randomization, although the neurologists were blinded to any laboratory results obtained by flow cytometer. All the patients had a history of insufficient response to treatment with high-dose steroids. These factors might have resulted in selection bias. Additional treatment to PP might have affected the results, although there was no significant difference regarding additional treatment between responders and nonresponders (see the Table). There were no secondary outcome parameters, such as magnetic resonance imaging (MRI) lesions included in this study. We could not identify the exact mechanism underlying PP-induced transcriptional change of Th1 cells, where further studies are warranted.

As CD11c$^+$ B cells appear to be involved in the pathogenesis of SLE,[16] it is of great interest to explore whether our hypothesis about Th1 cell - CD11c$^+$ B cell axis could also be relevant for prediction of PP responses in other autoimmune neurological diseases. Besides, we and other research groups previously showed that gut microbiota is altered in patients with multiple sclerosis.[51,52] We found that it is also significantly different between typical and atypical multiple sclerosis cases.[53] Specific types of dysbiosis might be involved in the responsiveness to PP, which deserves further investigations in the future.

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Potential Conflicts of Interest

Y.L. had a joint research with Asahi Kasei Medical Co. Ltd., which manufactures the medical equipment that is used for participants in this study. T.Y. received grant support for research from Asahi Kasei Medical Co. Ltd. K.K., Y.L., and T.Y. have a pending patent in Japan for methods to collect data for prediction of response to plasmapheresis. All the other authors report no potential conflict of interest.

Author Contributions

K.K., Y.L., H.Y., W.S., D.T., M.M., Y.D., T.O., R.T., T.K., and T.Y. contributed to conception and design of the study. K.K., Y.L., H.Y., and T.Y. contributed to acquisition and analysis of data. K.K., Y.L., and T.Y. drafted a significant portion of the manuscript and figures.

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