RESEARCH ARTICLE OPEN ACCESS

Different Patterns of Autoantibody Secretion by Peripheral Blood Mononuclear Cells in Autoimmune Nodopathies

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Neurol Neuroimmunol Neuroinflamm 2024;11:e200295. doi:10.1212/NXI.0000000000200295

Abstract

Background and Objectives

Autoimmune nodopathies with antibodies against the paranodal proteins show a distinct phenotype of a severe sensorimotor neuropathy. In some patients, complete remission can be achieved after treatment with rituximab whereas others show a chronic course. For optimal planning of treatment, predicting the course of disease and therapeutic response is crucial.

Methods

We stimulated peripheral blood mononuclear cells in vitro to find out whether secretion of specific autoantibodies may be a predictor of the course of disease and response to rituximab.

Results

Three patterns could be identified: In most patients with anti-Neurofascin-155-, anti-Contactin-1-, and anti-Caspr1-IgG4 autoantibodies, in vitro production of autoantibodies was detected, indicating autoantigen-specific memory B cells and short-lived plasma cells/plasmablasts as the major source of autoantibodies. These patients generally showed a good response to rituximab. In a subgroup of patients with anti-Neurofascin-155-IgG4 autoantibodies and insufficient response to rituximab, no in vitro autoantibody production was found despite high serum titers, indicating autoantibody secretion by long-lived plasma cells outside the peripheral blood. In the patients with anti-pan-Neurofascin autoantibodies—all with a monophasic course of disease—no in vitro autoantibody production could be measured, suggesting a lack of autoantigen-specific memory B cells. In some of them, autoantibody production by unstimulated cells was detectable, presumably corresponding to high amounts of autoantigen-specific plasmablasts—well in line with a severe but monophasic course of disease.

Discussion

Our data suggest that different B-cell responses may occur in autoimmune nodopathies and may serve as markers of courses of disease and response to rituximab.

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The Article Processing Charge was funded by the authors.

Glossary

Caspr1 = Contactin-associated protein1; **CNTN1** = Contactin-1; **FACS** = fluorescence-activated cell sorting; **NF** = Neurofascin; **OD** = optical density; **PBMC** = peripheral blood mononuclear cells.

Introduction

Autoimmune nodopathies with IgG4 autoantibodies against Neurofascin (NF), Contactin-1 (CNTN1), and Contactin-associated protein1 (Caspr1) are characterized by distinct clinical features such as acute onset of sensorimotor neuropathy, sensory ataxia, and poor response to treatment with IV immunoglobulins.[1,2] Most patients show good response to rituximab,[3] in some of the patients even leading to complete remission and seronegativity. In others, autoantibodies persist and repeated treatment is necessary. In a minority of patients, rituximab is not efficient and high autoantibody titers persist.[6] Patients with anti-pan-NF autoantibodies directed against the nodal (NF186) and paranodal (NF155) isoform of NF, typically present with a very severe neuropathy but monophasic course of disease.[7-9] It is so far unclear why some patients experience a monophasic course of disease whereas others develop chronic courses with persistent autoantibody production. Prognostic markers predicting the course of disease and/or response to treatment are needed.

In general, autoantibodies are produced by short-lived plasma cells in lymph nodes, plasmablasts in the blood, or long-lived plasma cells in the bone marrow and secondary lymphoid tissues.[10] Furthermore, B1 cells that belong to the innate immune system are a potential source of autoantibodies, especially natural autoantibodies of the IgM class, but also IgG.[11] B-cell responses comprise extrafollicular and germinal center responses: Extrafollicular responses rapidly induce plasmablasts and do not necessarily induce memory B cells, whereas in germinal centers, B cells acquire affinity maturation, and memory B cells and long-lived plasma cells are generated to induce ongoing autoantibody production.[12]

In several other autoantibody-associated diseases, mostly with IgG1 autoantibodies, such as myasthenia gravis with acetylcholine receptor antibodies, anti-NMDA-receptor-, or anti-GAD65-associated disease, autoantibodies may be produced by long-lived plasma cells in the bone marrow and parenchyma that are not depleted by rituximab.[13]

In this study, we aimed to identify predictors of a monophasic or chronic course of disease and response to rituximab by analyzing in vitro autoantibody secretion by peripheral blood mononuclear cells (PBMC).

We therefore investigated (1) autoantibody secretion by stimulated PBMC according to a protocol that has been shown to selectively stimulate memory B cells,[14,15] i.e., investigating the presence of autoantigen-specific memory B cells, (2) autoantibody production by unstimulated PBMC to quantify autoantibody production by circulating B cells that spontaneously secrete autoantibodies (mostly plasmablasts), and (3) a potential discrepancy between high serum autoantibody titers and the lack of autoantibody secretion by PBMC as a potential indicator of long-lived plasma cells in the bone marrow as the source of autoantibodies. Data were compared with response to rituximab to assess the use of PBMC stimulation as a predictor of therapeutic response.

Methods

Study Participants

Fourteen patients with a diagnosis of autoimmune nodopathy based on the detection of anti-paranodal autoantibodies (anti-NF155 n = 5, anti-pan-NF n = 6, anti-CNTN1 n = 2, or anti-Caspr1 n = 1) by ELISA, cell-based assays and binding assays on murine teased fibers and a typical clinical phenotype were included. PBMC and serum were obtained before treatment with rituximab if possible, 4 patients had received rituximab 3 to 14 months before inclusion. Follow-up PBMC were available from 5 patients. Twenty-one healthy controls (median age 45.7 years, range 21–82) and 5 seronegative patients with previous autoimmune nodopathy (1 anti-NF155, 3 anti-CNTN1, 1 anti-Caspr1) were also included.

Isolation of PBMC

PBMC were isolated from EDTA-treated blood by density gradient centrifugation using Lymphoprep separation medium (Serumwerk Bernburg AG, Bernburg, Germany). PBMC were extracted, washed, filtered, counted, and resuspended in freezing medium (90% fetal bovine serum [FBS], 10% dimethyl sulfoxide). The cells were stored at −80°C for 24 hours and further transferred to liquid nitrogen.

Immunophenotyping by Flow Cytometry

Fluorescence-activated cell sorting (FACS) of PBMC was performed on a BD FACSAria III cell sorter (Beckton Dickinson, San Jose, CA) with 4 lasers (488/561/633/405 nm) and appropriate filters. The BD FACS Diva software and a 70 μm nozzle were used for B-cell sorting. 10,000 events were counted for each PBMC suspension.

PBMC were analyzed for the expression of CD19, CD20, CD3, CD38, CD27, and CD43. Stimulated and unstimulated PBMC were washed and resuspended in the staining antibody mixture (99 μL 1x phosphate buffered saline (PBS) supplemented with 1% FBS with 1 μL CD3-FITC, 2 μL CD19-VioGreen, 2 μL CD20-PE, 2 μL CD27-PE-Vio770, 2 μL

CD38-APC, 2 µL CD43-APC-Vio770, Miltenyi Biotech, Bergisch Gladbach, Germany). After incubation for 10 minutes in the dark, the cell suspension was washed and the cell pellet was resuspended in sorting medium (1640 RPMI medium without phenol red, 0.5% bovine serum albumin, 2 mM EDTA) to a concentration of 2×10^6 cells/mL.

Stimulation of PBMC

After thawing and suspension in cell culture medium (RPMI 1640 medium with glutamine, 10% FBS, 1% penicillin/streptomycin), PBMC were washed and filtered. The cells were counted and resuspended in cell culture medium to a concentration of 3×10^6 cells/mL. PBMC were either stimulated by 15 ng/mL interleukin-2 (IL-2, PreproTech, Cranbury) and 2.5 µg/mL toll-like receptor (TLR) 7/8 ligand resiquimod (R848, Enzo Life Sciences GmbH, Lörrach, Germany) or remained unstimulated. 6×10^6 cells per well of stimulated and unstimulated PBMC were seeded on a 12-well plate and incubated for 10 days at 37°C. After 5 days, the cell culture medium was changed. The 5-day and 10-day supernatant was stored at −20°C. After 10 days, stimulated PBMC were frozen as described above.

Enzyme-Linked Immunosorbent Assays (ELISA)

Autoantibodies against CNTN1, NF155, and Caspr1 were measured in the undiluted 5-day and 10-day supernatant by ELISA as previously described.[16,17]

For the detection of anti-tetanus antibodies and human IgG, MaxiSorp 96-well plates (Thermo Fisher Scientific, Waltham) were coated with tetanus toxoid (1:500 in PBS, Merck KGaA, Darmstadt, Germany) or anti-human IgG (1:5,000 in HCO_{3-}, Aligent Technologies, Santa Clara). 5-day and 10-day supernatant (anti-tetanus antibodies: undiluted; human-IgG: 1:100 in PBS) was added, and incubation and washing were followed by the application of horseradish peroxidase (HRP)–conjugated anti-human IgG (1:10,000, Aligent Technologies). Rabbit serum (1:100, Aligent Technologies) and HRP-conjugated anti-rabbit IgG (1:2,000, Aligent Technologies) were used as a control. The optical density (OD) was measured at 450 nm by a Multiscan FC ELISA Reader (Thermo Fisher Scientific). The supernatant was tested in duplets, and the OD of corresponding uncoated wells was subtracted.

The threshold for anti-CNTN1, -NF155, and -Caspr1 was set at 2 SD above the mean OD of controls. The lower threshold for anti-tetanus antibodies and total IgG was set at 2 SD below the mean of controls, for anti-tetanus 3 extreme outliers (probably unvaccinated individuals) were excluded. The concentration of human total-IgG was determined by a human IgG ELISA Kit (Thermo Fisher Scientific) as described in the manual.

Statistical Analysis

Statistical testing was performed using GraphPad Prism version 9.5.1 for Windows (GraphPad Software, San Diego, California, graphpad.com). For the comparison of numerical data, Mann-Whitney *U* test with Bonferroni correction was used. A significance level of <0.05 was applied.

Standard Protocol Approvals, Registrations, and Patient Consents

All participants gave written and oral informed consent to participate, and the study was approved by the Ethics committee of the University of Würzburg (No. 222/20).

Data Availability

Anonymized data not published within this article will be made available by request from any qualified investigator.

Results

Autoantibody Production by PBMC in Patients With Anti-Paranodal Autoantibodies

In 6/8 patients with paranodal IgG4 autoantibodies (2/2 anti-CNTN1, 1/1 anti-Caspr1, 3/5 anti-NF155, excluding anti–pan-NF), autoantibody secretion by stimulated PBMC could be measured, indicating the presence of autoantigen-specific memory B cells in the PBMC of this cohort (Table 1, Figure 1A). To investigate autoantibody production by plasmablasts, the supernatant of unstimulated PBMC was analyzed and was weakly positive in one patient and at the borderline in another anti-NF155–positive patient, but clearly negative in all others (Table 1). Both patients had very high serum titers and were in an early phase of disease. We did not detect any paranodal autoantibodies in the stimulated cell culture supernatant in any of the controls nor in 3 rituximab-naïve patients (2 anti-CNTN1, 1 Caspr1) who had been recruited after complete remission indicating the loss of specific memory B cells after remission (Figure 1A). As a control, anti-tetanus antibodies were positively detected in the supernatant of stimulated PBMC of all controls and patients in remission (except for 3 controls, most probably because of long latency of the last vaccination and one patient in remission after rituximab), reflecting the long-term persistence of anti-tetanus–specific memory B cells after vaccination.

In 2 patients (no. 7, 8) with high anti-NF155 serum titers, no in vitro production by PBMC could be detected (Table 1, Figure 1B). One of them was rituximab-naïve and the other had been treated rituximab 4 months before but had persistent high anti-NF155 titers (1:3,000) despite B-cell depletion confirmed by immunophenotyping. In a further patient (no. 4) with positive anti-NF155 secretion by PBMC shortly after the onset of disease, follow-up PBMC did not secrete any anti-NF155 autoantibodies despite persisting high anti-NF155 serum titers (1:3,000) (Figure 1B). They were obtained 6 months after treatment with rituximab when B cells were still depleted. Thus, autoantibody secretion by plasma cells outside the peripheral blood compartment that are not depleted by rituximab can be suspected, indicating long-lived plasma cells as a potential source of autoantibodies in these 3 patients.

Our data suggest the following subgroups: In most patients, autoantibody production by PBMC was inducible, indicating the existence of autoantigen-specific memory B cells. In a

Table 1 Overview on Serum Titers, Antibody Secretion by PBMC, $CD19^+/CD20^+$ Cells, and Treatment Latency and Response of the Individual Seropositive Patients

Autoantigen	No.	Serum titer	PBMC stim	PBMC unstim	Tetanus (stim PBMC)	Total IgG (μg/mL)	CD19/ CD20 (%)	Last (total) Rtx (mo.)	Response
CNTN1	1	1:15,000 IgG4>2	Pos	Neg	Pos	33.1	3/4	14 (6)	Good
	FU	see Figure 5							
CNTN1	2	1:10,000 IgG4>IgG3	Pos	Pos	Pos	36	9/12	—	Seronegative, mild residual symptoms
	FU	Neg	Neg	Neg	Neg	4.6	2/1	7 (2)	
Caspr	3	1:5,000 IgG4	Pos	Neg	Pos	6.75	5/6	9 (2)	Seronegative, residual symptoms
	FU	Neg	Neg	Neg	Neg	0	4/4	10 (3)	
NF155	4	1:5,000 IgG4	Pos	Weakly pos	Borderline	20.9	5/4	—	Partial
	FU	1:3,000 IgG4	Neg	Neg	Neg	0	2/2	6 (2)	
NF155	5	1:5,000 IgG4	Pos	Neg	Neg	8.6	3/5	—	n/a
NF155	6	1:12,000 IgG4	Pos	Borderline	Pos	102.6	5/3	—	Partial
NF155	7	1:5,000 IgG4	Neg	Neg	Neg	0.01	3/2	4	Unambigious
NF155	8	1:1,000 IgG4	Neg	Neg	Pos	94.1	3/3	—	Temporary mild improvement
	FU	1:4,000 IgG4	Neg	n/a	Neg	45.4	6/4	56 (2)	
Pan NF	9	1:1,000 IgG4, IgG3	Neg	Neg	Neg	32.4	12/8	—	—
PanNF	10	1:500 IgG4	Borderline	Neg	Neg	11.3	4/8	—	—
PanNF	11	1:4,000 IgG3	Borderline	Pos	Neg	0.99	2/1	—	—
PanNF	12	1:2000 IgG4>IgG3	Neg	Pos	Pos	32.4	5/5	—	—
PanNF	13	1:300 IgG3>IgG4	Neg	Neg	Pos	22.4	3/5	—	—
PanNF	14	1:6000 IgG4>IgG1	Neg	n/a	Borderline	6.9	5/1	—	—

Abbreviations: FU = follow-up; No. = number; Rtx = rituximab; stim = stimulated; unstim = unstimulated.

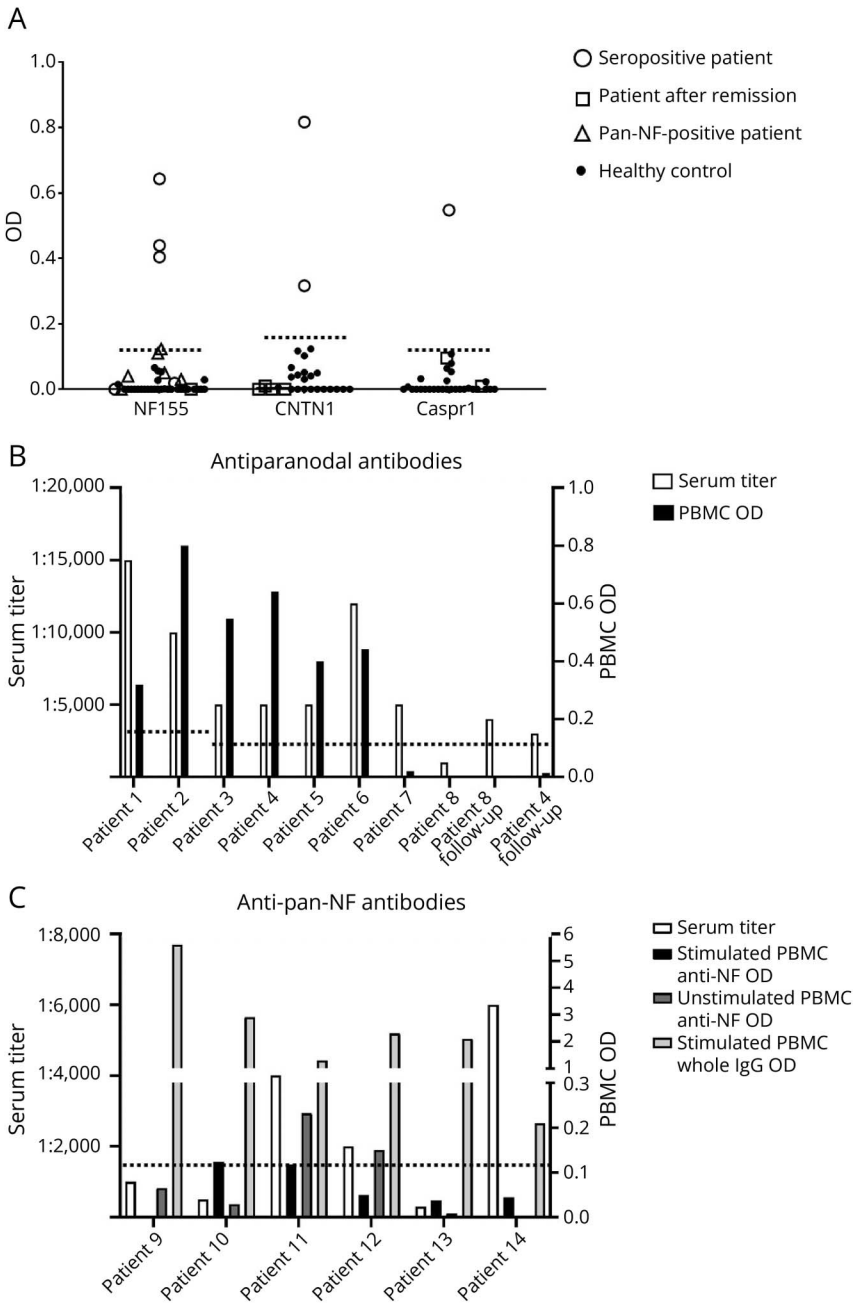
smaller number of patients (all with anti-NF155 autoantibodies), no autoantibody production could be induced by B-cell stimulation, indicating a lack of autoantibody-producing B cells in the peripheral blood.

Association Between In Vitro Autoantibody Secretion and Treatment Response

To evaluate a potential relationship between response to rituximab and in vitro autoantibody production by PBMC, 3 different groups of patients with paranodal autoantibodies (excluding anti–pan-NF) were compared: (1) patients with clinical and seronegativity after treatment with rituximab, (2) patients with a chronic course of disease, but response to rituximab (i.e., increase of the MRC sum score[18] and/or decrease of ODSS[19]), and (3) patients with a chronic course of disease, not clearly responding to rituximab (Figure 2).

Two patients (no. 2 and 3, anti-CNTN1 and anti-Caspr1) achieved complete serologic remission and good clinical improvement after therapy with rituximab (Figure 2, blue lines/dots). In both patients, in vitro autoantibody production by PBMC could be measured during the active phase of disease,

Figure 1 Optical Densities (A: y-Axis; B, C: Right y-Axis) and Serum Titers (B, C: Left y-Axis) in Different Cohorts and Individual Patients (x-Axes)



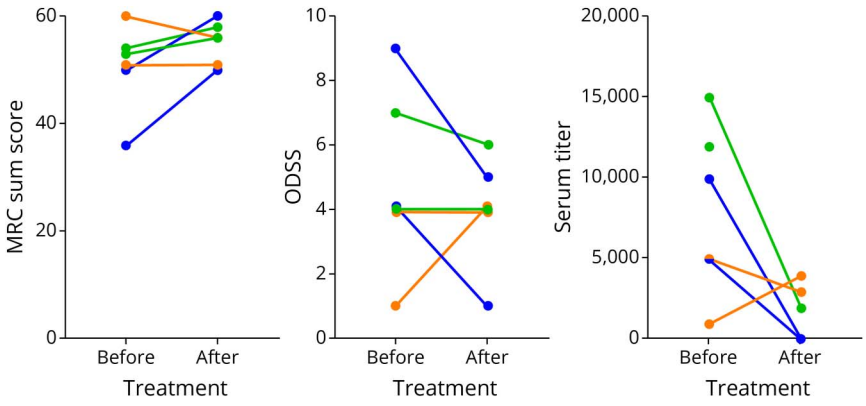
The dashed lines mark the cut-off of ELISA. (A) shows the in vitro secretion of different paranodal autoantibodies: Autoantibody secretion by stimulated PBMC could only be measured in patients with paranodal autoantibodies (i.e., seropositive) (white circles), not in any patient after remission of disease (white squares) or in any control (black circles) (A). Two patients with anti–pan-NF autoantibodies (white triangle) had an OD around the cut-off, all others were negative (A). (B) illustrates serum titers and PBMC OD of individual seropositive patients: Patients 1–6 were positive in the PBMC supernatant, in patients 7 and 8, and the follow-up sample of patient 4 autoantibody secretion by PBMC was clearly below the cut-off despite high serum titers.(C) illustrates serum titers and PBMC OD of individual patients with anti–pan-NF autoantibodies (patients 9–14): In 6 patients with moderate-to-high anti–pan-NF titers, no relevant in vitro autoantibody secretion by PBMC could be measured, but in 2 patients, the supernatant of unstimulated PBMC was just above the cut-off (C). abs = antibodies; Caspr1 = Contactin-associated protein1; CNTN = Contactin; NF = Neurofascin; OD = optical density; PBMC = peripheral blood mononuclear cell; stim = stimulated; unstim = unstimulated.

but not after remission, indicating the existence of autoantigen-specific memory B cells during the active phase of disease and probably their complete and ultimate depletion by rituximab (Figure 3A).

Two patients with a chronic course of disease but good response to rituximab (no. 1: anti-CNTN1, no. 6: anti-NF155) were analyzed, one further patient with a chronic course of disease did not receive rituximab (no. 5). In vitro autoantibody secretion by PBMC was detectable in all of them. Both patients who were treated with rituximab responded with clinical improvement and decrease of autoantibody titers but did not achieve complete remission (Figure 2, green lines/dots). They needed long-term treatment with rituximab to keep autoantibody titers and clinical symptoms at low levels, indicating the recovery of autoantigen-specific memory B cells after B-cell depletion (Figure 3B).

Three patients with anti-NF155 autoantibodies did not sufficiently or only partially respond to rituximab and autoantibody titers remained high despite B-cell depletion (Figure 2, orange lines/dots). In none of these 3 patients (one of them

Figure 2 Clinical Scores (MRC Sum Score[18] and ODSS[19]) and Serum Autoantibody Titers Before and After Treatment With Rituximab in 6 Patients With Anti-Paranodal Abs



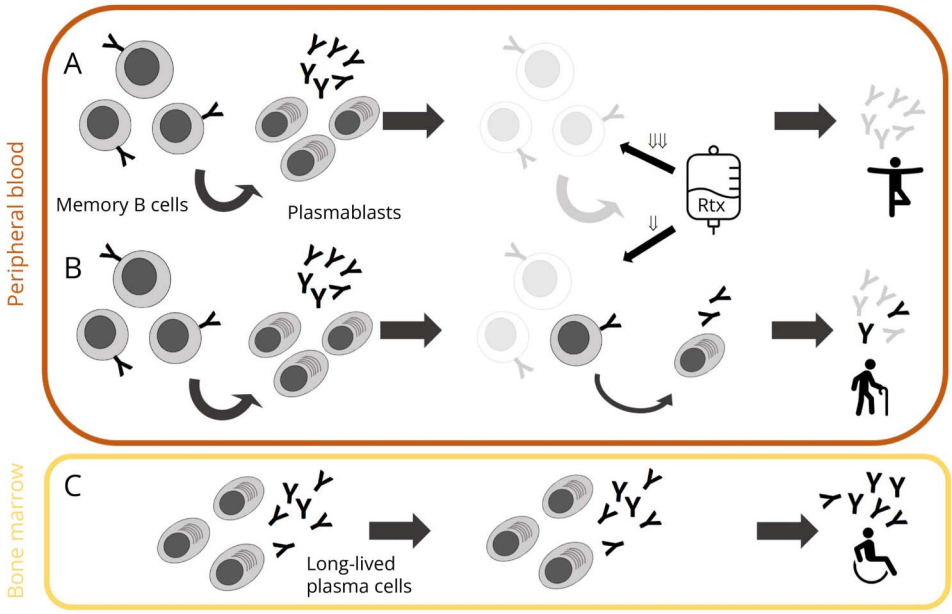
Two patients (blue) with a monophasic course of disease and in whom autoantibody secretion by PBMC could be measured during the acute phase of disease (patient no. 2, 3, corresponding to A in Figure 3) substantially improved after treatment and became seronegative. Two patients (green) with chronic course but response to rituximab and detection of autoantibodies in the supernatant of stimulated PBMC (patient no. 1, 6, corresponding to B in Figure 3) also improved, but to a lesser extent, and the autoantibody titer decreased in one patient (and was not available in the other one). Two patients (orange) who were negative in the supernatant of stimulated PBMC (at onset or follow-up) and without ambiguous response to treatment with rituximab (patient no. 4, 8. corresponding to C in Figure 3) did not improve in the clinical scores and autoantibody titers only mildly decreased or even increased. (From one further patient of this categories, no MRC sum scores and ODSS were available.) MRC = medical research council; ODSS = Overall Disability Sum Score; Rtx = rituximab.

rituximab-naïve), autoantibody secretion by PBMC was detectable despite high serum titers. In one patient (no. 8), PBMC from a second timepoint 4 years later were still tested negative and serum anti-NF155 titers were still high. In one of these patients with a relapsing-remitting course of disease (no. 7), titers finally decreased more than 3 months after treatment, accompanied by clinical improvement, but treatment response could not be clearly differentiated from the natural relapsing-remitting course of disease. In another patient (no. 4), autoantibody secretion by PBMC had been detectable during the subacute onset but not during the course of disease and high serum titers persisted. These results indicate (additional) autoantibody production by cells other than PBMC, most likely long-lived plasma cells in the bone marrow or secondary lymphoid tissues (Figure 3C). The latency between first symptoms and treatment with rituximab was several years in one patient (patient 8), but in the other 2 patients, it was in the range of a few months, like most of the other patients.

Clinical symptoms of all patients are summarized in Table 2.

Thus, we could show that in vitro autoantibody production after B-cell stimulation is associated with monophasic or chronic courses of disease and response to treatment with

Figure 3 Hypothesis on Differential Responses to Rituximab Treatment



In patients who achieve complete remission, autoantigen-specific memory cells are completely depleted by rituximab, resulting in the loss of autoantibody-producing plasmablasts and seronegativity (A). Autoantibody secretion by PBMC can be measured before treatment but not afterward (corresponding patients no. 2, 3). In patients with response to rituximab but chronic course of disease, autoantigen-specific memory B cells are not completely depleted, resulting in the persistence of smaller amounts of autoantibody secreting plasmablasts and response to treatment but no seronegativity (B), thus requiring repeated treatment (corresponding patients no. 1, 5, 6). In some patients, autoantibodies may be produced by long-lived plasma cells in the bone marrow that cannot be reached by rituximab, leading to persistence of high serum titers and no clinical improvement (C, corresponding patients: 4, 7, 8). Rtx = rituximab.

Table 2 Summary of Clinical Data of the Patients

No.	Autoantigen	Serum titer	Duration	Symptoms	Treatment (since diagnosis)	Outcome
1	CNTN1	1:15,000	14 y	Sensorimotor neuropathy, tremor, ataxia	PE, IA, rituximab, azathioprine, cyclophosphamide, methylprednisolone	Improvement, chronic course
2	CNTN1	1:10,000	5 mo	Sensorimotor neuropathy, ataxia	PE, rituximab, IVIG	Seronegative, mild residual symptoms
3	Caspr1	1:5,000	1 mo	Sensorimotor neuropathy	PE, rituximab	Seronegative, residual symptoms
4	NF155	1:5,000	8 mo	Sensorimotor neuropathy, ataxia, mild tremor	PE, rituximab, IVIG, prednisolone	Mild improvement after Rtx, good response to PE
5	NF155	1:5,000	6 y	Sensorimotor neuropathy, ataxia	PE, prednisolone	Temporary improvement, chronic course
6	NF155	1:12,000	2 y	Sensorimotor neuropathy, ataxia	PE, rituximab, methylprednisolone, IVIG	Stable symptoms, chronic course
7	NF155	1:5,000	4.5 y	Sensorimotor neuropathy, ataxia, tremor	IA, rituximab, IVIG, methylprednisolone	Relapsing-remitting, chronic course
8	NF155	1:1,000	7 y (tremor)	Tremor, sensorimotor neuropathy	PE, rituximab, IVIG	Temporary mild improvement, chronic course
9	Pan-NF	1:1,000	11 mo	Tetraplegia, sensory and cranial nerve involvement, respiratory insufficiency	IA, rituximab, IVIG, prednisolone	Seronegative, complete remission
10	Pan-NF	1:500	2 mo	Sensorimotor neuropathy	PE, IVIG	Seronegative, complete remission
11	Pan-NF	1:4,000	3 mo	Tetraplegia, sensory and cranial nerve involvement, respiratory	PE, rituximab, IVIG, methylprednisolone	Death
12	Pan-NF	1:2,000	2 mo		PE, rituximab, IVIG	Seronegative, residual symptoms
13	Pan-NF	1:300	4 mo		PE, rituximab, methylprednisolone, IVIG	Seronegative, residual symptoms
14	Pan-NF	1:6,000	6 mo		PE, IA, rituximab, bortezomib, prednisolone	Seronegative, residual symptoms

Abbreviations: IA = immune adsorption; PE = plasma exchange; Rtx = rituximab.

rituximab. No in vitro autoantibody production at timepoints of high autoantibody titers occurs in chronic courses of disease and is associated with insufficient response to rituximab.

Autoantibody Production by Stimulated PBMC Is Not Found in Patients With Anti–Pan-NF Autoantibodies

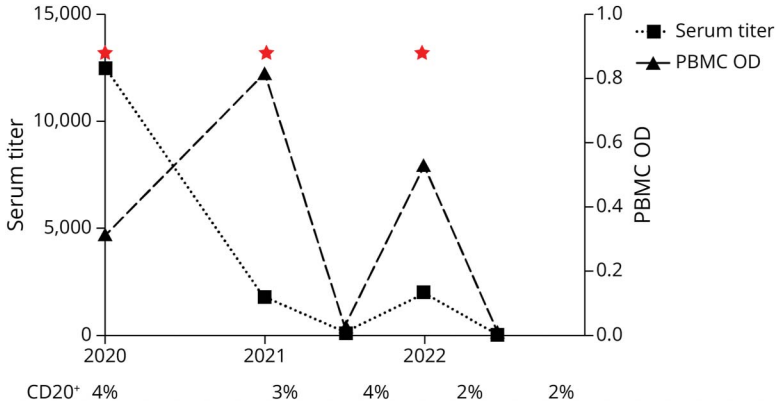
PBMC of 6 patients with anti–pan-NF autoantibodies (no. 9-14, titers 1:500 to 1:6,000, see Table 1, all with a monophasic course of disease, Table 2) were stimulated and the supernatant tested for anti-NF autoantibodies. Autoantibody production was not detectable in any supernatant of stimulated wells, but 2 patients showed values around the cut-off value (Figure 1, A and C). Thus, we could not find any evidence of a relevant amount of anti–pan-NF-specific memory B cells. In 2 patients, the supernatant of unstimulated wells was just above the cut-off value (Figure 1C), possibly indicating autoantibody production by plasmablasts because they secrete autoantibodies without stimulation.

Total IgG in the supernatant of stimulated PBMC, $CD19^+$, $CD20^+$, and $CD3^+$ cells were within similar range in all groups, except for a decrease of total IgG secretion and $CD20^+$ cells in patients after remission who had mostly received rituximab (Figure 4, A, C, and D, $CD3^+$ not shown). The serum autoantibody titers tended to be lower in patients with anti–pan-NF autoantibodies and in samples with negative supernatants of stimulated PBMC, but they largely overlapped arguing against low titers as an explanation for the lack of in vitro autoantibody secretion by PBMC (Table 1, Figure 4B).

Longitudinal Assessment of Autoantibody Production

From one patient (no. 1) with anti-CNTN1 autoantibodies, PBMC from 5 time points within 3 years were available. The patient had a chronic course of disease with onset in 2006 and treatment with rituximab every 6 to 12 months since 2014. Symptoms had improved after treatment with a decrease of autoantibody titers but seropositivity persisted.

Figure 4 PBMC-Secreted Total IgG, Serum Auto-ab Titers of Samples With Positive/Negative Supernatants of PBMC, and Amounts of $CD20^+$ Cells and $CD19^+$ Cells in Stimulated PBMC



Total IgG, $CD20^+$ cells, and $CD19^+$ cells were within similar range in patients and controls, only total IgG and $CD20^+$ cells were decreased in patients after remission, most probably because of treatment (A, C, D). Serum auto-ab titers of samples with negative supernatants of stimulated PBMC tended to be lower (B).*$p < 0.05$ (Mann-Whitney *U* test). abs = antibodies; CD = cluster of differentiation; NF = Neurofascin; PBMC = peripheral blood mononuclear cells.

We could detect in vitro autoantibody secretion at time points with high serum titers (1:15,000, 1:2,000, 1:3,000) but not at time points with low titers (1:100, 1:200) (Figure 5) and generally low IgG in the supernatant, accompanied by B-cell depletion. Even if the number of follow-up samples is too low to determine a statistically significant correlation, the observable association between in vitro autoantibody production and serum titers indicates a major contribution of memory B cells and peripheral blood autoantibody production to perpetuate seropositivity and argues in favor of consequent B-cell depletion with rituximab.

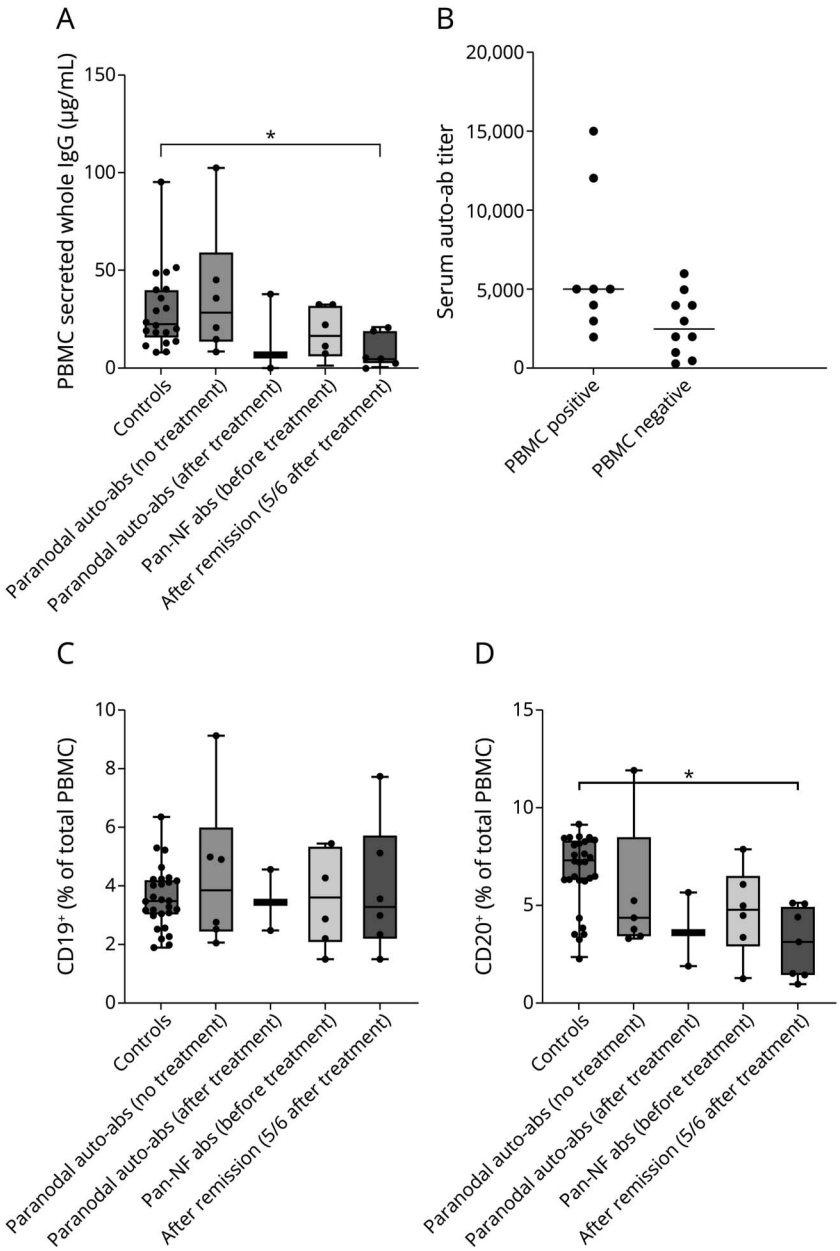
Discussion

By investigating the production of anti-paranodal autoantibodies by PBMC in vitro, we could identify 3 different patterns: In one group, autoantibody production by stimulated PBMC, but not by unstimulated PBMC was detectable. These patients showed good response to rituximab. In the second cohort, no in vitro autoantibody production by PBMC was found, despite high serum titers. This cohort comprised 3 patients with anti-NF155 autoantibodies and insufficient or only partial response to rituximab at that time point. The third cohort comprised 6 patients with anti–pan-NF autoantibodies and monophasic course of disease. No in vitro production of anti-NF was detectable in the stimulated supernatant.

Our data provide evidence of different responses of PBMC to B-cell stimulation, presumably reflecting different sources of anti-paranodal autoantibodies: Autoantibody production by unstimulated PBMC in 2 patients with anti–pan-NF autoantibodies could be explained by large amounts of autoantigen-specific plasmablasts. Indeed, all these patients had a very acute and severe course of disease, thus well in line with an acute and temporary production of autoantibodies. No autoantibody production by stimulated PBMC was detected, revealing the lack of autoantigen-specific memory B cells in this cohort. The lack of memory B cells may explain the severe but usually monophasic course of disease that is typically observed in anti–pan-NF-associated nodopathy.[20] At the level of B-cell responses, this might be explained by an extrafollicular B-cell response that may not induce memory B cells and long-lived plasma cells, thus leading to a monophasic course of disease. A contribution of extrafollicular B-cell responses to autoimmunity has also been discussed in systemic lupus erythematodes and rheumatoid arthritis and has been suggested to be responsible for acute deterioration in these diseases.[21] Future studies using single-cell technology are needed to definitely identify B-cell pathways in autoimmune nodopathies.

From a diagnostic point of view, the absence of autoantibody-specific memory B cells may be a marker for a monophasic course of disease not requiring long-term immunosuppressive treatment: Currently, treatment with rituximab is often recommended in patients with autoimmune nodopathies,[6,22] but our data suggest that in patients with anti–pan-NF autoantibodies, acute secretion of autoantibodies by plasmablasts may play a major role, rather than ongoing generation of new plasmablasts because of repeated autoantigen exposure to memory B cells. Because rituximab mainly depletes CD20-positive B cells, bortezomib or daratumumab that directly affect plasma cells may be more efficient and should be considered as an additional treatment. Indeed, response to daratumumab and bortezomib has recently been reported in anti–NF155- and anti–pan-NF-associated neuropathies,[23,24] and one patient of our cohort was also treated with bortezomib and showed marked improvement. In patients with IgG4 autoantibodies, obexelimab that bispecifically binds to CD19 and FcγRIIb may be a promising option because it inhibits CD19-/CD20-positive B cells, plasmablasts, and plasma cells without inducing B-cell depletion.[25]

Figure 5 Longitudinal Analysis of Autoantibody Secretion by Stimulated PBMC and Serum Autoantibody Titer of a Patient With Anti-CNTN1 Autoantibodies (Patient No. 1)



Red stars mark the time points of rituximab treatments, percentage of $CD20^+$ cells (%) of total PBMC at different time points are given below the graph. The serum titer decreased after treatment with rituximab and was still low 6 months after treatment but then increased. In vitro autoantibody secretion by PBMC could be measured at timepoints of high serum titers and with latencies of 1 year after rituximab, not 6 months after rituximab when serum titers were still low. The association between serum titers and autoantibody secretion by PBMC argues in favor of plasmablasts as the source of autoantibodies in this patient with chronic course of disease. OD = optical density; PBMC = peripheral blood mononuclear cells.

In most of the patients with autoimmune nodopathies and anti-paranodal IgG4, we could stimulate autoantibody production by PBMC in vitro, indicating autoantigen-specific memory B cells that are stimulated by cytokine and TLR stimulation.[26] Thus, when speculating on the potential trigger of relapsing-remitting courses of disease, not only repeated exposure to autoantigens but also systemic inflammatory conditions, e.g., during infections may be considered. In the longitudinal assessment, in vitro autoantibody secretion by PBMC was associated with positive serum titers, arguing in favor of short-lived plasma cells/plasmablasts as the major source of autoantibodies, but at least in patients with persistent seropositivity additional long-lived plasma cells cannot be excluded. Our data are in line with other studies of autoimmune diseases with IgG4 autoantibodies that also reported autoantibody secretion by short-lived plasma cells and plasmablasts.[13] In patients with anti–MuSK-IgG4-positive myasthenia gravis, for example, short-lived plasma cells have been shown to secrete autoantibodies whereas in patients with anti-acetylcholine receptor autoantibodies of the IgG1 subclass, long-lived plasma cells are supposed to be the major source of autoantibodies.[27] Rituximab induces depletion of anti–CD20-positive cells like memory B cells but does not directly affect plasmablasts or long-lived plasma

cells.[13] In patients with myasthenia gravis, this may explain the very good response to rituximab in patients with anti-MuSK autoantibodies compared with patients with anti-acetylcholine receptor autoantibodies.[28] Our data suggest that in vitro stimulation of memory B cells to produce autoantibodies may also be a predictor of treatment response to rituximab in patients with typical IgG4-autoantibody–positive autoimmune nodopathy. No in vitro autoantibody production after B-cell stimulation was detectable in patients after remission, and no relapses were observed in these patients so far, i.e., autoantigen-specific memory B cell can be completely depleted, suggesting a low risk of relapses after complete seronegativity—in contrast to anti–MuSK-IgG4-positive myasthenia gravis were persistence of memory B cells has been described and is considered the cause of relapses.[29] However, these observations need to be confirmed in larger cohorts.

In 3 patients, high autoantibody titers but no in vitro production by stimulated PBMC were detectable indicating long-lived plasma cells as a possible source of autoantibodies. All these patients had a chronic course of disease with only partial or insufficient response to rituximab. In one patient, the latency between onset of disease and treatment was very long, but in the other, it was similar to other patients, so the potential induction of long-lived plasma cells cannot solely be explained by delayed treatment. Bortezomib or daratumumab may also be a good option in these patients because they may deplete long-lived plasma cells.[13,24]

In summary, we provide evidence of different autoantibody sources in patients with autoimmune nodopathies: Memory B cells and short-lived plasma cells/plasmablasts seem to play a role in most patients and explain excellent response to rituximab in this disease. However, in a small subgroup of patients who do not sufficiently respond to rituximab, long-lived plasma cells may be a relevant source of autoantibodies and the lacking detection of autoantigen-specific memory B cells may be a marker for insufficient response to rituximab. Anti–pan-NF autoantibodies may be induced by extrafollicular B-cell responses leading to high amounts of plasmablasts. To definitely identify autoantibody-secreting B-cell subtypes in autoimmune nodopathies and to elucidate B-cell differentiation and activation, PBMC need to be sorted in future studies and T-cell–dependent B-cell activation also needs to be investigated as performed in other autoantibody-associated diseases.[30,31] To definitely rule out a relevant effect of autoantibody titers on the lack of in vitro autoantibody secretion in patients with anti–pan-NF autoantibodies, groups with matched serum titers need to be assessed in larger studies. Autoantibody production by stimulated PBMC in relation to serum titers may be a prognostic marker and a marker for response to rituximab, possibly also in other autoantibody-associated diseases. Treatments targeting plasmablasts and/or long-lived plasma cells may be considered in patients without autoantibody production by stimulated PBMC and may also be effective in patients with anti–pan-NF autoantibodies. At the pathogenic level, immunologic studies elucidating B-cell pathways would be of high interest. On the therapeutic level, clinical trials directly targeting long-lived plasma cells and plasmablasts may be worthwhile in a subcohort of patients.

Acknowledgment

The authors thank Sonja Gommersbach and Barbara Reuter for excellent technical assistance and Edgar Meinl (Munich) for providing Neurofascin protein for ELISA. SR was funded by a grant of the Graduate School of Life Sciences, University of Würzburg. KD is funded by the Interdisciplinary Center for Clinical Research (IZKF), University Hospital Würzburg. KD and CS are part of the Clinical Research Group ResolvePain, funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, KFO5001).

Study Funding

The authors report no targeted funding.

Disclosure

The authors do not declare any competing interests directly related to this study. Potential competing interests outside the submitted work: C. Sommer reports consulting fees from Kedrion, Nevro, Agiax, Takeda, Grifols, Bayer, Roche, Merz, Omega, LFB and honoraria for presentations from Kedrion, TEVA, CSL Behring, Takeda, Pfizer, Grifols, Amicus, Alnylam. She is the IASP past president, Member of the Board of the Peripheral Nerve Society and Deputy Editor of the *European Journal of Neurology*; C. Franke received honoraria for lectures from Bristol Myers Squibb and Boehringer Ingelheim and is a Board Member of the German Society of Neurology (DGN); C. Geis received support from the German Research Council (GE2519/8-2, 9-2) and honoraria for lectures and support for attending meetings from Roche and Alexion and participates on Advisory Boards of Alexion, Roche and Sobi; F. Schöberl received honoraria for lectures from Alnylam and participates on Advisory Boards of Alnylam, Amylyx and Alexion; T. Högen received honoraria for lectures from the University of Augsburg, German Society for Neurophysiologie (DGKN) and Palliative Care Academy Regensburg e.V. and Christophorus Academy for Palliative Care and travel support from the German Society for Neurophysiologie (DGKN); M. Mäurer received honoraria for lectures from CSL Behring, Sanofi and Alexion; K. Doppler received honoraris for lectures from Grifols, Takeda and Roche and is a Board Member of the Inflammatory Neuropathy Consortium of the Peripheral Nerve Society. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* March 18, 2024. Accepted in final form July 9, 2024. Submitted and externally peer reviewed. The handling editor was Associate Editor Marinos C. Dalakas, MD, FAAN.

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Continued

Appendix *(continued)*

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