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IgG4 Valency Modulates the Pathogenicity of Anti–Neurofascin-155 IgG4 in Autoimmune Nodopathy

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Abstract

Background and Objectives

IgG4 autoantibodies to neurofascin-155 (Nfasc155) are associated with a subgroup of patients with chronic inflammatory demyelinating polyneuropathy (CIDP), currently named autoimmune nodopathy. We previously demonstrated that those antibodies alter conduction along myelinated axons by inducing Nfasc155 depletion and paranode destruction. In blood, IgG4 have the potency to exchange their moiety with other unrelated IgG4 through a process called Fab-arm exchange (FAE). This process results in functionally monovalent antibodies and may affect the pathogenicity of autoantibodies. Here, we examined this issue and whether FAE is beneficial or detrimental for Nfasc155 autoimmune nodopathy.

Methods

The bivalency and monospecificity of anti-Nfasc155 were examined by sandwich ELISA in 10 reactive patients, 10 unreactive CIDP patients, and 10 healthy controls. FAE was induced in vitro using reduced glutathione and unreactive IgG4, and the ratio of the κ:λ light chain was monitored. To determine the pathogenic potential of bivalent anti-Nfasc155 IgG4, autoantibodies derived from patients were enzymatically cleaved into monovalent Fab and bivalent $F(ab')_2$ or swapped with unreactive IgG4 and then were injected in neonatal animals.

Results

Monospecific bivalent IgG4 against Nfasc155 were detected in the serum of all reactive patients, indicating that a fraction of IgG4 have not undergone FAE in situ. These IgG4 were, nonetheless, capable of engaging into FAE with unreactive IgG4 in vitro, and this decreased the levels of monospecific antibodies and modulated the ratio of the κ:λ light chain. When injected in animals, monovalent anti-Nfasc155 Fab did not alter the formation of paranodes; by contrast, both native anti-Nfasc155 IgG4 and $F(ab')_2$ fragments strongly impaired paranode formation. The promotion of FAE with unreactive IgG4 also strongly diminished the pathogenic potential of anti-Nfasc155 IgG4 in animals and decreased IgG4 clustering on Schwann cells.

Discussion

Our findings demonstrate that monospecific and bivalent anti-Nfasc155 IgG4 are detected in patients and that those autoantibodies are the pathogenic ones. The transformation of anti-Nfasc155 IgG4 into monovalent Fab or functionally monovalent IgG4 through FAE strongly decreases paranodal alterations. Bivalency thus appears crucial for Nfasc155 clustering and paranode destruction.

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

Anti–neurofascin-155 study group coinvestigators are listed in appendix 2 at links.lww.com/NXI/A736 the end of the article.

Glossary

CASPR1 = contactin-associated protein 1; **CIDP** = chronic inflammatory demyelinating polyneuropathy; **CNTNI** = contactin-1; **FAE** = Fab-arm exchange; **IVIg** = IV immunoglobulin; **MuSK** = muscle-specific kinase; **Nfasc155** = neurofascin-155.

Cell adhesion molecules at the nodes of Ranvier play a crucial role in the organization of the axonal domains and in the rapid propagation of the nerve impulse along myelinated axons.[1] Particularly, the ternary complex formed by the association of neurofascin-155 (Nfasc155), contactin-1 (CNTN1), and contactin-associated protein 1 (CASPR1) is important for the formation of septate-like junctions at paranodal regions for the clustering/stabilization of voltage-gated sodium channels at the nodes of Ranvier and for myelin insulation.[2-5] Antibodies targeting these important cell adhesion molecules have been discovered in a subset of patients with chronic inflammatory demyelinating polyneuropathy (CIDP) and have helped improve the diagnosis and treatment orientation of this disabling neuromuscular disorder.[6-13] Because these patients have distinct clinical features, no overt inflammation or macrophage-mediated demyelination, and a poor response to IV immunoglobulin (IVIg) compared with typical CIDP, this group has recently been named autoimmune nodopathy.[14] Nfasc155 is the major immune target in autoimmune nodopathy (nearly 70% of the cases). The clinical and pathophysiologic distinctiveness of autoimmune nodopathy relies on the antigen specificity (Nfasc155, CNTN1, and CASPR1) and on the fact that these autoantibodies predominantly belong to the IgG4 isotype, which are unable to activate complement and have low affinity for Fcγ receptors.[15] Animal models demonstrated that these antibodies can disrupt the paranodal junctions in the absence of complement activation or macrophage-mediated demyelination.[6,16,17] IgG4 autoantibodies appeared to induce paranodal destruction either through a function-blocking activity or by depleting the target antigens.

A growing number of autoimmune diseases implicate IgG4.[18] IgG4 has the unique characteristic of dynamically exchanging its Fab arms by swapping the heavy chain.[19] This process named Fab-arm exchange (FAE) results in bispecific IgG4 that are monovalent to their target. In myasthenia gravis with antibodies against muscle-specific kinase (MuSK), FAE appears to potentiate the IgG4 pathogenic potential.[20,21] Anti-MuSK IgG4 was shown to block the interaction between MuSK and low-density lipoprotein receptor–related protein 4.[22] Although bivalent anti-MuSK IgG4 can cluster MuSK, the monovalent form obtained after FAE strongly inhibits the aggregation of acetylcholine receptors at the neuromuscular junction and leads to severe muscle weakness in vivo.[20,23] Insofar, MuSK myasthenia gravis is the sole condition where FAE appears to amplify the pathogenic function of IgG4.

In autoimmune nodopathy, the effect of FAE on the pathogenicity of IgG4 has not yet been evaluated. IgG4 to CNTN1 and CASPR1 have a function-blocking activity and disrupt paranodal specialization by inhibiting the interaction between CNTN1/CASPR1 and Nfasc155.[6,16,24] FAE may potentially increase the pathogenic effects of these autoantibodies in a similar manner as for anti-MuSK IgG4. By contrast, anti-Nfasc155 does not have a function-blocking activity.[17] These antibodies induce Nfasc155 clustering on the Schwann cell surface and lead to its depletion, thus abolishing paranode formation and maintenance both in patients and animal models.[6,17,25-27] This finding suggests that anti-Nfasc155 IgG4 can aggregate their target and that a fraction of them may be monospecific in situ. Here, we explored whether IgG4 valency influences anti-Nfasc155 IgG4 pathogenicity and whether FAE is beneficial for paranode maintenance. For that purpose, this study tested the pathogenic potential of monovalent and bivalent anti-Nfasc155 IgG4.

Methods

Patients' IgG

Plasma and sera were obtained from 10 patients with autoimmune nodopathy with anti-Nfasc155 IgG4 antibodies, 10 seronegative patients with CIDP, and 10 healthy donors (Etablissement Français du Sang, Montpellier, France). All subjects provided written informed consent. The study was approved by the Ethics Committee of Montpellier University Hospital (IRB-MTP-2020-01-20200339). IgG1 and IgG4 were purified using the CaptureSelect™ affinity matrix (Thermo Fisher Scientific, Waltham, MA), dialyzed to artificial CSF, concentrated at 10 mg/mL, and filter-sterilized.

Monoclonal Antibody Production

HEK293T cells were transiently transfected with pVITRO1-Trastuzumab-IgG4/κ (#61887; Addgene, Watertown, MA) or pVITRO1-102.1F10-IgG4/λ (#50369; Addgene) for 24 hours; then, the medium was replaced with OptiMEM (Thermo Fisher) for antibody production. IgG was purified with a HiTrap protein A affinity column (Cytiva, Marlborough, MA) on an AKTA Pure (Cytiva). Antibodies were desalted to PBS using a HiTrap Desalting column (Cytiva), filter-sterilized, and stored at −20°C until use.

Antibody Cleavage and FAE

Fab and $F(ab')_2$ fragments were generated using the Pierce Fab preparation kit (Thermo Fisher) or FragIT kit (Genovis, Lund, Sweden) according to the manufacturer's protocols. Fab and $F(ab')_2$ fragments were dialyzed to artificial CSF, concentrated at 10 mg/mL, and filter-sterilized. FAE was performed as previously described.[19,23] IgG4 or IgG1 from Nfasc155-reactive patients were incubated with a 3-fold excess of healthy control IgG4 in the presence or absence of 0.5 mM reduced glutathione (GSH) (Merck-Millipore, Burlington, MA) for 24 hours at 37°C.

Monospecificity ELISA Assay

The extracellular domain of human recombinant Nfasc155 (Met1-Trp1062) with a C-terminal His-tagged was produced in HEK293T cells and purified, then was biotinylated with a 20-fold molar excess of EZ-link sulfo-NHS-biotin (Thermo Fisher) for 30 minutes at 37°C, and desalted to PBS. MaxiSorp microtiter plates were coated with 50 ng of untagged human Nfasc155 overnight at 4°C. Wells were blocked with 0.5% casein sodium 0.05% Tween 20 in PBS for 1 hour at 37°C and then incubated overnight at 4°C with 10 μg of IgG4 in blocking solution for regular tests or with 40 μg of IgG4 for FAE tests (10 μg of patient IgG4 + 30 μg of control IgG4). The day after, wells were incubated with 50 ng of biotinylated Nfasc155 for 1 hour at 37°C, and the reactivity was revealed using peroxidase-conjugated streptavidin (1:2,000; Merck-Millipore) and SIGMAFAST OPD (Merck-Millipore). As positive controls, IgG1 from Nfasc155-reactive patients were used. IgG4 from healthy donors and seronegative patients were used as negative controls. Dilutions of purified anti-Nfasc155 IgG1 were used to obtain a calibration curve, from which the percentage of monospecific IgG4 was calculated.

Quantification of κ/λ Ratios

The amounts of λ and κ light chains in anti-Nfasc155 IgG4 were determined by ELISA against Nfasc155 as indicated above, and the plates were revealed using the peroxidase-conjugated goat anti-human λ or κ light chain (1:2000; Bio-Rad, Hercules, CA). Purified recombinant IgG4/κ and IgG4/λ were used to obtain a calibration curve. For sandwich ELISA, MaxiSorp microtiter plates were coated with 1 μg of donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA) in 50 μL of PBS overnight at 4°C. Wells were blocked as indicated above and then incubated overnight at 4°C with 1 ng of IgG4 from healthy donors or patients. The day after, the reactivity was revealed using the peroxidase-conjugated goat anti-human λ or κ light chain. The quantity of λ or κ light chains was expressed as a percentage of the total amount of λ + κ light chains.

Animals

In vitro nerve incubation experiments and neonatal injection were performed as previously described.[17] New-born Wistar rats received a single intraperitoneal injection of 250 μg of control IgG4, native Nfasc155-reactive IgG4, Fab or $F(ab')_2$ fragments, or 1 mg of swapped antibodies in artificial CSF. Animals were then killed 2 days after the injection. Teased fibers were analyzed from 4 animals for each group.

Immunolabeling

Immunolabeling was performed as previously described.[17] The following primary antibodies were used: rabbit antisera against CASPR1 (1/2000); mouse monoclonal antibodies against Nav channels (K58/35; 1:500; Merck-Millipore); mouse monoclonal antibodies against Myc (11667149001; 1/200; Roche, Basel, Switzerland); and/or goat antibody against CNTN1 (1/2000; R&D Systems, Minneapolis, MN). The following secondary antibodies from Jackson ImmunoResearch were used at a 1:500 dilution: donkey anti-mouse (715-545-151; 715-585-151), donkey anti-goat (705-545-147; 705-585-147), donkey anti-rabbit (711-585-152), and donkey anti-human (709-585-149; 709-545-149). Slides were examined using an ApoTome fluorescence microscope (Carl Zeiss MicroImaging GmbH, Oberkochen, Deutschland), and digital images were manipulated into figures with CorelDraw and Corel Photo-Paint (Corel Corporation, Ottawa, Canada).

Statistics

Statistical significance was assessed by unpaired and paired 2-tailed Student *t* tests, Kolmogorov-Smirnov tests, or one-way ANOVA followed by Bonferroni post hoc tests using GraphPad Prism (GraphPad Software, San Diego, CA). Linear regression and Spearman correlation were performed using GraphPad Prism. p values <0.05 were considered significant.

Standard Protocol Approvals, Registrations, and Patient Consents

All animal experiments were in line with the European community's guiding principles on the care and use of animals (2010/63/EU) and were approved by the local ethical committee and by the "ministére de l'éducation nationale de l'enseignement supérieur et de la recherche" (APAFIS#3847-2016012610089856v5).

Data Availability

Data are available from the corresponding author on request.

Results

A Fraction of IgG4 to Nfasc155 Are Monospecific and Bivalent in Reactive Patients

Our previous finding indicated that IgG4 autoantibodies target and cluster Nfasc155 on the Schwann cell surface. We thus examined whether patients' IgG4 were able to cross-link Nfasc155 in vitro. For that purpose, we selected 10 patients reactive to Nfasc155 from whom a large volume of serum or plasma was available. The clinical history and antibody titers of these patients are indicated in Table 1. IgG1 and IgG4 were purified from these patients, as well as 10 healthy controls and 10 seronegative patients with CIDP. To determine the percentage of monospecific antibodies, IgG4 were tested with a monospecificity ELISA assay based on the ability of IgG4 to link soluble biotinylated Nfasc155 to immobilized untagged Nfasc155 (Figure 1A). IgG4 from seronegative patients with CIDP and healthy controls did not couple Nfasc155. By contrast, IgG4 from Nfasc155-reactive patients significantly cross-linked Nfasc155 with biotinylated Nfasc155 with variable intensities, indicating that patients have different levels of monospecific antibodies (Figure 1B). Dilutions of anti-Nfasc155 IgG1 were used to calculate the percentage of monospecific IgG4 in each patient (Table 1). The potency to cross-link Nfasc155 (Figure 1B) and the percentage of monospecific IgG4 significantly correlated with the titers of anti-Nfasc155 IgG4 in the patients (Spearman $p = 0.018$ and $p = 0.031$, respectively). However, no correlation was found between Nfasc155 cross-linking by monospecific antibodies and the

Table 1 Antibody Titers and Clinical Features of Nfasc155-Reactive Patients

	Titers	Monospecific anti-Nfasc155 IgG4 (%)	Sex	Age	Clinical score (mRS score)	Response to treatment
AN1	2,600	10	M	46	3	IVIg–, Cx partial, Plex+, Ritux+
AN2	19,700	20	M	25	3	IVIg–, Cx+, Plex+
AN3	1,600	19	M	22	3	IVIg–, Cx–, Plex+, Ritux+
AN4	20,500	78	M	53	4	IVIg–, Cx–, Ritux–, Plex+
AN5	6,000	17	M	33	2	IVIg–, Cx+
AN6	3,000	8	M	50	4	IVIg–, Cx+, Plex+, Ritux+
AN7	10,000	21	M	16	3	IVIg–, Cx–, Ritux+
AN8	1,800	7	M	37	3	IVIg partial, Cx partial, Plex+
AN9	6,000	17	M	68	4	IVIg+, Ritux+, Plex+
AN10	6,000	29	M	46	1	IVIg+, Ritux+

Abbreviations: Cx = corticosteroids; IVIg = IV Immunoglobulin; mRS = modified Rankin Scale; Nfasc155 = neurofascin-155; NT = not tested; Plex = plasma exchange; Ritux = rituximab.

clinical severity or the age of the patients (eFigure 1, links.lww.com/NXI/A736). Similarly, no correlation was found between antibody titers and clinical severity.

IgG4 Reactive to Nfasc155 Can Undergo FAE In Vitro

To determine whether anti-Nfasc155 IgG4 can undergo FAE, IgG4 from Nfasc155-positive patients were incubated in vitro with a reducing agent, reduced glutathione (GSH), in the presence or absence of 3-fold excess of IgG4 from a healthy donor. The samples were then tested for their ability to catenate immobilized Nfasc155 with soluble biotinylated Nfasc155. In the absence of GSH, anti-Nfasc155 IgG4 potently interconnected immobilized Nfasc155 and biotinylated Nfasc155 (Figure 1C). The addition of reducing agents potently diminished the ability of IgG4 to cross-connect Nfasc155 in most patients (Figure 1C). This effect was further potentiated by the addition of a 3-fold excess of IgG4 from healthy donors, which led to a 43% reduction of the levels of monospecific antibodies. By contrast, reducing conditions did not affect the monospecificity of anti-Nfasc155 IgG1 (Figure 1D), which is in keeping with previous observations.[19,23]

Ratio of κ and λ Light Chains in Nfasc155-Reactive Patients

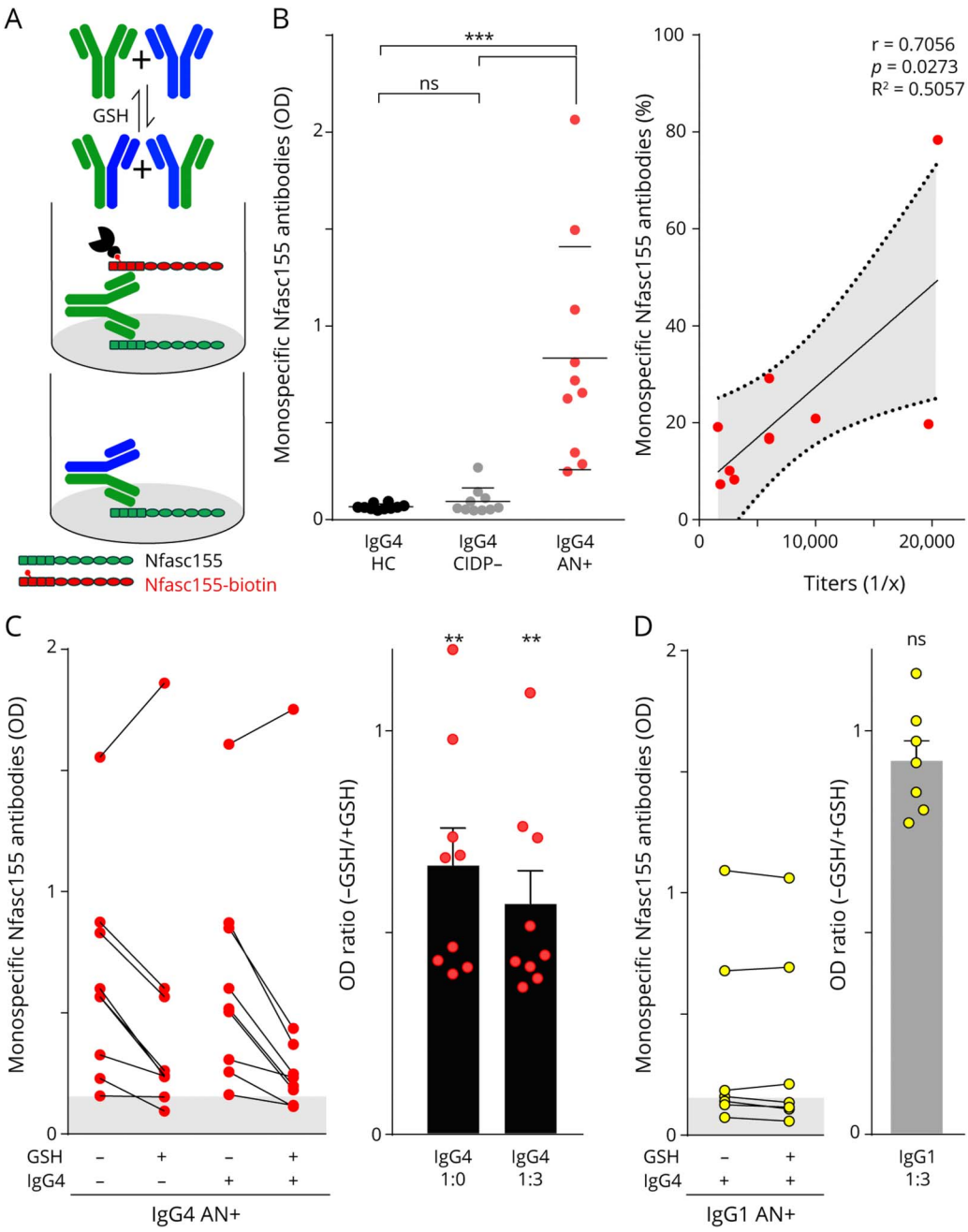
The κ light chain is generally slightly more prevalent than the λ light chain in mature B cells.[28] We exploited this characteristic to confirm our finding and monitored the changes in the κ and λ light chain proportion of anti-Nfasc155 IgG4 after in vitro induction of FAE with monoclonal IgG4/λ or IgG4/κ antibodies. In a first manner, the amount of κ and λ light chains was measured in anti-Nfasc155 IgG4 by ELISA. The amount of κ light chains was found to strongly correlated with that of λ light chains ($p = 0.0016$; Figure 2, A–C), and the mean κ/λ ratio was 1,4 ± 1,0 (eTable 1, links.lww.com/NXI/A736). To determine whether the κ/λ ratios of anti-Nfasc155 IgG4 differ from those of the total IgG4 fraction not only in each patient but also in controls, the κ/λ ratios were quantified by sandwich ELISA in the purified IgG4 fractions from all patients and controls (Figure 2). Although the κ/λ ratios of anti-Nfasc155 IgG4 were slightly more dispersed that those found in the total IgG4 fractions and presented different SDs ($p < 0.05$ with a Bartlett test), the mean values were not significantly different ($p > 0.05$ by one-way ANOVA followed by Bonferroni post hoc tests). Also, no significant differences were found between the κ/λ ratios of Nfasc155-reactive patients and those of healthy donors or seronegative patients. In vitro FAE with a monoclonal IgG4/λ antibody increased mainly the proportion of the λ light chain in Nfasc155-reactive IgG4 and resulted in a significant decrease of the ratio κ:λ (Figure 2C). In vitro FAE with a monoclonal IgG4/κ antibody confirmed this observation and resulted in a significant increase in the proportion of the κ light chain in Nfasc155-reactive IgG4 and in an increase of the ratio κ:λ (Figure 2D).

The correlations between laboratory finding and clinical history indicated that FAE efficiency with recombinant IgG4/λ appeared to slightly follow clinical severity, and anti-Nfasc155 IgG4 from patients with a higher score showed a lower potency to undergo FAE (eFigure 1B, links.lww.com/NXI/A736). However, patients with a high clinical score also showed a higher proportion of λ light chains; it is thus possible that FAE potency with IgG4/λ is underestimated in those patients because antibodies are already predominantly IgG4/λ before FAE (eTable 2).

Bivalency Is Crucial for Pathogenicity, Whereas Monovalency Decreases the Pathogenicity of Nfasc155-Reactive IgG4

To determine whether the valency of IgG4 is important for the pathogenic function, we have performed enzymatic cleavage of

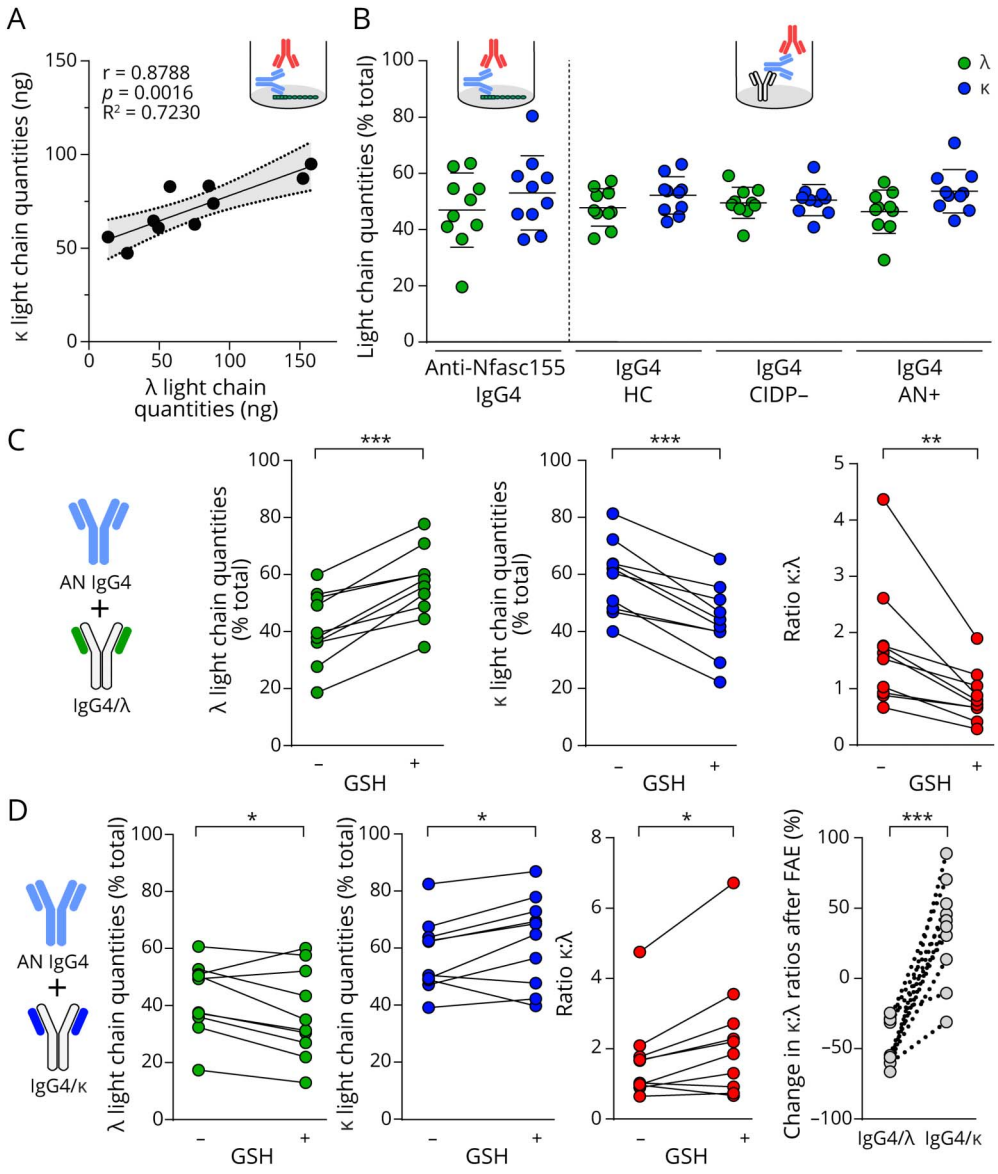
Figure 1 Anti-Nfasc155 IgG4 Cross-Link Nfasc155 and Undergo Fab-Arm Exchange In Vitro



(A) Principle of Fab-arm exchange (FAE) and monospecificity assay. Reduced glutathione (GSH) favors FAE of monospecific IgG4 to generate bispecific IgG4. Monospecific antibodies cross-link untagged Nfasc155 with biotinylated Nfasc155. Bispecific antibodies are unable to cross-link these proteins. (B) Purified IgG4 from healthy controls (HC; n = 10), seronegative CIDP (CIDP−; n = 10) and Nfasc155+ autoimmune nodopathy (AN+; n = 10) were tested by ELISA against Nfasc155 and then incubated with a biotinylated Nfasc155 and revealed with streptavidin-HRP. IgG4 from HCs or seronegative patients did not cross-link Nfasc155. By contrast, IgG4 from Nfasc155+ patients cross-linked Nfasc155 (***$p < 0.001$ by one-way ANOVA followed by Bonferroni post hoc tests). The percentage of monospecific Nfasc155 antibodies correlated with the titers of anti-Nfasc155 IgG4. p Value, Spearman correlation coefficient (r), R square (R^2), and 95% confidence band (dotted lines) are indicated on the graph. (C) To determine whether anti-Nfasc155 IgG4 can undergo FAE, IgG4 from Nfasc155+ patients (n = 9) were incubated with GSH in the absence (1:0) or presence of a 3-fold excess of HC IgG4 (1:3). The addition of GSH decreased IgG4 ability to cross-link Nfasc155. This effect was exacerbated by the addition of 3-fold excess of HC IgG4. The decrease in monospecific antibodies is shown on the right as ratio (**$p < 0.005$ compared with control condition in the absence of GSH or HC IgG4 using paired Student t tests). (D) As controls, IgG1 from Nfasc155+ patients (n = 7) were tested against Nfas155 with the monospecificity ELISA assay. Purified IgG1 cross-linked Nfasc155. GSH and 3-fold IgG4 excess did not affect IgG1-mediated cross-linking ($p > 0.005$ using paired Student t tests). Gray shades delineate positivity limit values. Bars represent mean and SEM, CIDP = chronic inflammatory demyelinating polyneuropathy; ns = nonsignificant; Nfasc155 = neurofascin-155.

purified IgG4 from 2 Nfasc155-reactive patients (AN1 and AN2) with immobilized papain or IdeS to generate monovalent Fab or bivalent $F(ab')_2$ fragments. IgG4 cleavage was confirmed by Western blot, and the ability of Fab and $F(ab')_2$ to bind Nfasc155 and paranodes was demonstrated not only by immunohistology on transfected cells and on teased nerve

Figure 2 Fab-Arm Exchange Modulates the Ratio of κ:λ Light Chain in Nfasc155-Reactive IgG4

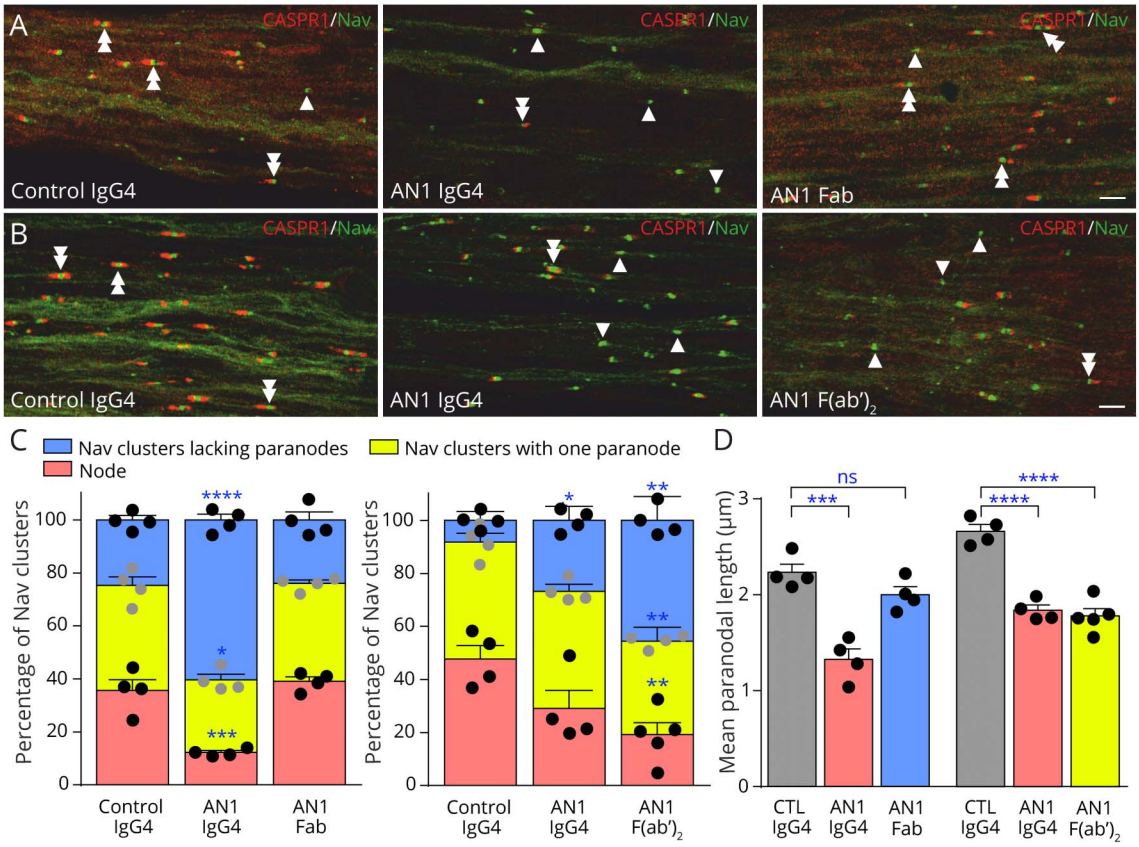


(A and B) The levels of λ and κ light chains of anti-Nfasc155 IgG4 were measured by ELISA against Nfasc155 in 10 reactive patients (A). The levels of λ (green dots) and κ (blue dots) light chains were also quantified by sandwich ELISA in the purified IgG4 fractions from healthy controls (n = 10), unreactive CIDP patients (CIDP−; n = 10), and reactive autoimmune nodopathy patients (AN+; n = 10) and were compared with those found in anti-Nfasc155 IgG4 (B). The levels of κ light chain strongly correlated with those of λ light chain in Nfasc155+ patients. No significant differences in the levels of λ and κ light chains were found between the groups ($p > 0.01$ by one-way ANOVA followed by Bonferroni post hoc tests). *p* Value, Spearman correlation coefficient (r), R square (R^2), and 95% confidence band (dotted lines) are indicated on the graph. (C and D) IgG4 from Nfasc155+ patients (n = 10) were incubated with of a 3-fold excess of monoclonal IgG4/λ (C) or IgG4/κ (D) in the presence or absence of reduced glutathione (GSH). The levels of κ and λ light chains were then measured by ELISA against Nfasc155, and the ratio κ/λ (red dots) was calculated. Fab-arm exchange (FAE) with IgG4/λ significantly increased the levels of λ light chains and decreased the levels of κ light chains in anti-Nfasc155 IgG4 and resulted in a decrease of the κ/λ ratio. Reversely, FAE with IgG4/κ significantly increased the levels of κ light chains and decreased those of λ light chains in anti-Nfasc155 IgG4 and resulted in an increase of the κ/λ ratio (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ compared with control condition in the absence of GSH using paired Student *t* tests). The percentage of increase or decrease of the κ/λ ratio following FAE (gray dots) with IgG4/λ or IgG4/κ was quantified for each patient. Changes in the κ/λ ratio were significantly different following FAE with IgG4/λ or IgG4/κ (***$p < 0.001$ using paired Student *t* tests). CIPD = chronic inflammatory demyelinating polyneuropathy; Nfasc155 = neurofascin-155.

fibers (eFigure 2, links.lww.com/NXI/A736) but also by ELISA. The enzymatic cleavage did not alter the affinity of anti-Nfasc155 Fab and $F(ab')_2$ for Nfasc155.

The pathogenic potential of those fractions was then tested by performing intraperitoneal antibody injection in new-born rat pups (n = 4 animals in each group). At these ages, the blood-nerve barrier is permeable, and IgG4 from Nfasc155-reactive patients can inhibit paranode formation.[17] Animals were then killed 2 days after injection, and the percentages of voltage-gated sodium (Nav) channel clusters with 1, 2, or no CASPR1-positive paranodes were quantified. As controls, animals received a single dose of control IgG4 from healthy donors. The native IgG4 from both Nfasc155-reactive

Figure 3 Bivalent F(ab')^2 From Nfasc155-Reactive IgG4 Abrogate Paranode Formation but Not Monovalent Fab



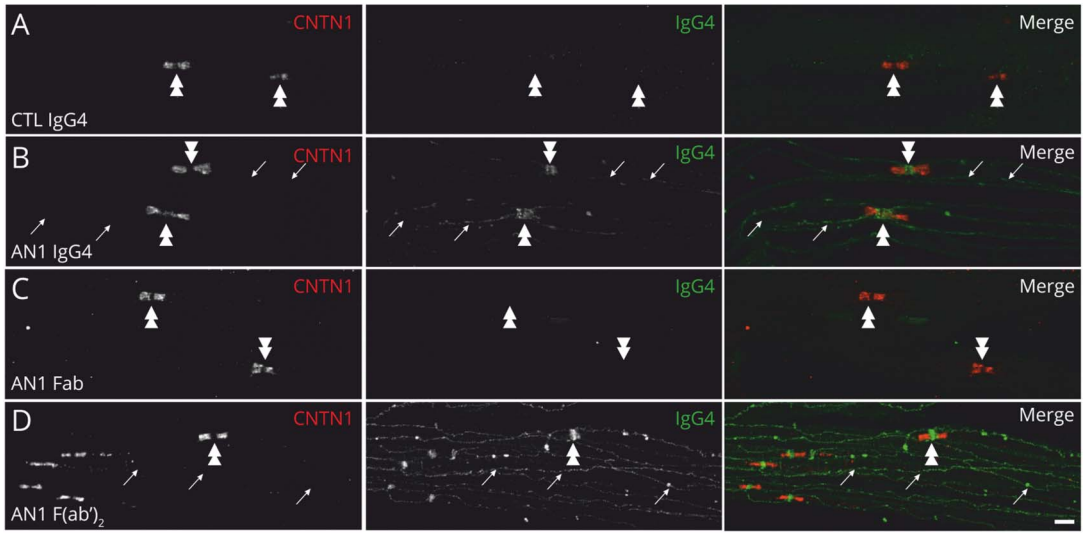
(A and B) New-born rat pups received an intraperitoneal injection of 250 µg of anti-Nfasc155 IgG4, Fab, or F(ab')$_2$ from patient AN1 (n = 4 animals for each condition and age). As controls, animals received 250 µg of IgG4 from healthy donors. Two days after injection, animals were killed, and sciatic nerve fibers were fixed. Teased sciatic nerve fibers were then immunolabeled for voltage-gated sodium channels (Nav; green) to label nodes and heminodes and for CASPR1 (red) to label paranodes. (C and D) The percentage of Nav clusters lacking CASPR1-positive paranodes (arrowheads) or with 1 or 2 flanking CASPR1 positive paranodes (double arrowheads) was quantified, as well as the paranodal length (D) (n = 100–200 nodes or paranodes for each condition). The injection of native IgG4 or F(ab')$_2$ fragments from patient AN1 strongly abrogated the formation of CASPR1-positive paranodes and resulted in a higher percentage of heminodes lacking paranodes (****p < 0.0001, ***p < 0.001, **p < 0.005, and *p < 0.05 by one-way ANOVA followed by Bonferroni post hoc tests). The mean length of paranodes was also shorter after treatment with native IgG4 or F(ab')$_2$ fragments reactive to Nfasc155 (****p < 0.0001 and ***p < 0.001 by one-way ANOVA followed by Bonferroni post hoc tests). By contrast, the injection of the monovalent Fab fragment of IgG4 did not affect the formation or the length of paranodes (G and H). Scale bar: 10 µm. Bars represent mean and SEM CASPR1 = contactin-associated protein 1; ns = nonsignificant; Nfasc155 = neurofascin-155.

patients strongly impaired paranode formation (Figure 3 and eFigure 3, links.lww.com/NXI/A736), and nearly 60% of the Nav channel clusters lacked paranodes by P2 vs 21%–25% in controls (more than 100 nodes were analyzed in 4 animals per groups). The remaining CASPR1-positive paranodes were shorter compared with control animals (Figure 3; the mean of length of more than 100 paranodes was calculated in 4 animals per condition and averaged), whereas the mean node length was not affected (the mean of length of more than 100 nodes was calculated in 4 animals per condition and averaged). F(ab')$_2$ fragments from patients AN1 and AN2 had similar effects and resulted in a significant increase of the percentage of Nav clusters lacking paranodes (Figure 3 and eFigure 3). The remaining paranodes were significantly shorter compared with controls but not to native anti-Nfasc155 IgG4 (Figure 3 and eFigure 3), indicating that both native IgG4 and F(ab')$_2$ fragments were similarly pathogenic. Reversely, the monovalent Fab fragment of IgG4 from both AN1 and AN2 had no effects on the formation of paranodes, and the percentages of Nav clusters with 1, 2, or no CASPR1-positive paranodes were similar to controls (Figure 3 and eFigure 3). Also, monovalent Nfasc155-reactive Fab did not affect paranodal length (Figure 3), indicating that monovalency abolishes the pathogenicity of anti-Nfasc155 IgG4.

Bivalent F(ab')$_2$ IgG4 Antibodies Can Cluster Nfasc155 and Abrogate Paranode Formation

To investigate whether enzymatic cleavage also alters the clustering of Nfasc155 on the Schwann cell surface, sciatic nerves were incubated in vitro for 3 hours in the presence of native IgG4 or Fab and F(ab')$_2$ fragments (Figure 4). An important clustering of IgG4 was found at the vicinity of the nodes of Ranvier and at the mesaxon in myelinated fibers incubated with native Nfasc155-reactive IgG4 or F(ab')$_2$ fragments. By contrast, no clustering was observed on the surface of Schwann cells incubated with Fab fragments or

Figure 4 Bivalent Antibodies Cluster on the Schwann Cell Surface of Myelinated Fibers



(A–D) Rat sciatic nerve were incubated in vitro for 3 hours with control IgG4 (A) or with Nfasc155-reactive IgG4 (B), Fab fragment (C), or F(ab')$_2$ fragment (D) from patient AN1. Fibers were then immunostained for IgG (green) and contactin-1 (CNTN1; red) to label paranodes. By contrast to control IgG4, Nfasc155-reactive IgG4 accumulated at paranode vicinity (double arrowheads) and along the outer mesaxon (arrows). The cleavage into the Fab fragment inhibited IgG4 accumulation at the mesaxon and around paranodes (C). By contrast, the cleavage into the F(ab')$_2$ fragment did not alter the ability to aggregate at the mesaxon and paranode vicinity despite the absence of the Fc region (D). Scale bars: 10 μm. Nfasc155 = neurofascin-155.

healthy control IgG4. Altogether, this further indicated that bivalency is crucial for Nfasc155 clustering and paranodal alterations. This also indicated that the Fc portion of IgG4 is dispensable and is not involved in the pathogenic mechanisms.

FAE Modulates the Pathogenicity of Anti-Nfasc155 IgG4

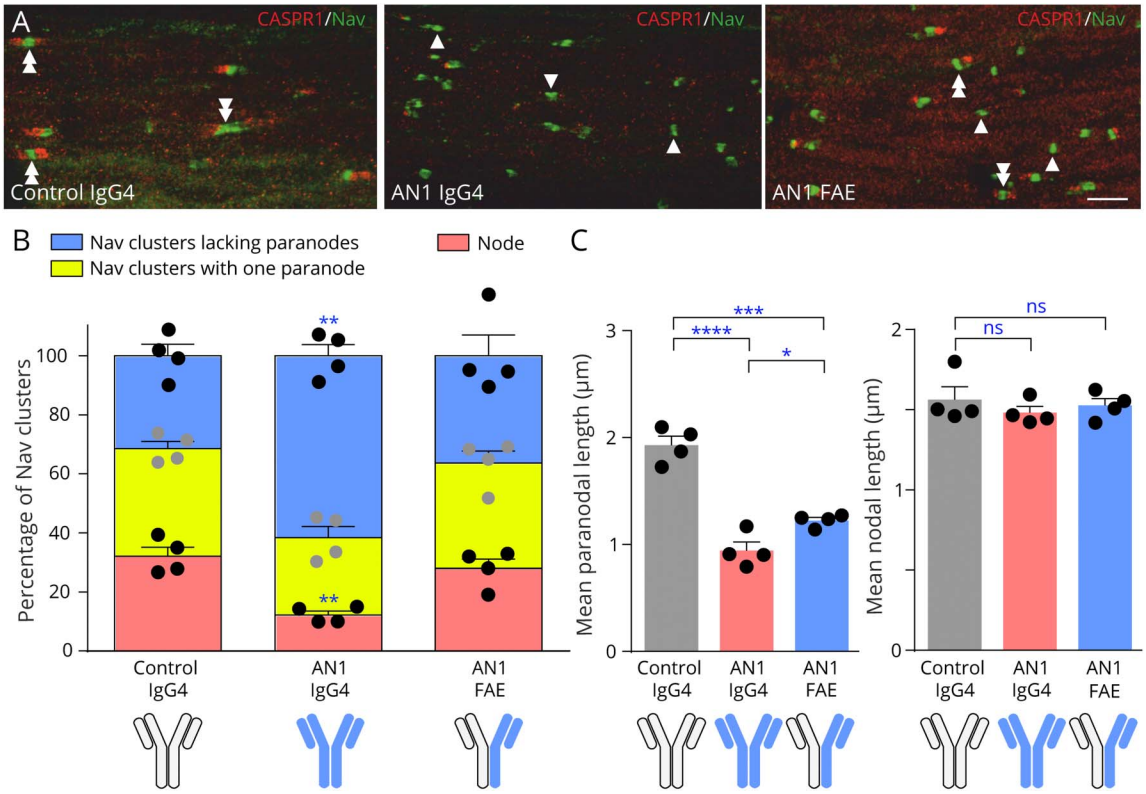
The above finding indicates that in vitro FAE can decrease the monospecificity of anti-Nfasc155 IgG4 and results in functionally monovalent antibodies. We thus tested whether promoting FAE may diminish the pathogenic action of these autoantibodies. For that purpose, IgG4 from 2 Nfasc155-reactive patients AN1 and AN2 were incubated in vitro with a 3-fold excess of healthy control IgG4 and GSH to promote FAE and were then injected into newborn pups. By contrast to native antibodies, the swapped antibodies had no effects on the formation of paranodes in newborn pups, and the percentages of Nav clusters with 1, 2, or no CASPR1-positive paranodes were similar to those of control animals (Figure 5 and eFigure 4, links.lww.com/NXI/A736). However, paranodal length appeared shorter in animals treated with swapped antibodies compared with control animals. The quantification of paranodal length confirmed this observation, and paranodes were found to be significantly shorter in animals treated with swapped IgG4 compared with control animals but still significantly longer than those of animals treated with native Nfasc155-reactive IgG4 (Figure 5 and eFigure 4). This indicated that FAE can potently decrease the pathogenic effect of anti-Nfasc155 IgG4. In our previous study, we found that anti-Nfasc155 IgG4 aggregate near the nodes in injected animals.[17] Here also, native anti-Nfasc155 IgG4 readily clustered along the Schwann and near Nav channels (eFigure 4). This clustering was strongly decreased in animals treated with swapped IgG4 but not abolished (eFigure 4).

Discussion

In this study, we demonstrate that a proportion of monospecific anti-Nfasc155 IgG4 can be detected in patients' serum and can cross-link Nfasc155 both in vitro and in vivo on Schwann cells. The enzymatic cleavage into monovalent Fab or bivalent F(ab')$_2$ enabled us to demonstrate that IgG4 bivalency is crucial for the pathogenic action of anti-Nfasc155 IgG4 and that monovalent antibodies are less pathogenic. Our data demonstrate that these antibodies can undergo FAE in vitro and in situ and that FAE modulates the pathogenic potential of autoreactive IgG4. The presence of monospecific IgG4 antibodies was not due to an inherent inability of anti-Nfasc155 IgG4 to undergo interchain exchange because the simple addition of GSH was sufficient to diminish the amounts of monospecific antibodies. Perhaps anti-Nfasc155 IgG4 have not all undergone FAE in situ with other circulating IgG4. In keeping with this, the κ:λ ratios of anti-Nfasc155 IgG4 did not perfectly match with those of circulating IgG4 in each patient. Nonetheless, patients with Nfasc155 autoimmune nodopathy did not carry exclusively monospecific bivalent, and the percentage of monospecific bivalent anti-Nfasc155 IgG4 ranged between 7% and 78% in patients.

FAE enables the formation of functionally monovalent IgG4 that are anti-inflammatory and participate to immune

Figure 5 Fab-Arm Exchange Decreases the Pathogenicity of Nfasc155-Reactive IgG4



(A) Fab-arm exchange (FAE) was induced in vitro between IgG4 from patient AN1 and healthy donor IgG4. New-born rat pups were injected at birth with control IgG4 (250 µg per animal), IgG4 from patient AN1 (250 µg per animal), or swapped IgG4 from patient AN1 (FAE; 1 mg per animal) and killed after 2 days. Teased fibers were immunostained for voltage-gated sodium channels (Nav; green) and CASPR1 (red) (n = 4 animals for each condition and age). (B and C) The percentage of Nav clusters lacking CASPR1-positive paranodes (arrows) and Nav clusters with 1 or 2 flanking paranodes (double arrowheads) was counted in each group. The injection of native Nfasc155-reactive IgG4 reduced the formation of CASPR1-positive paranodes and significantly decreased the mean length of paranodes. By contrast, swapped reactive Nfasc155 IgG4 did not significantly affect paranode formation. Nonetheless, paranodal length was decreased in animals treated with swapped IgG4 (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.005$, and *$p < 0.05$ by one-way ANOVA followed by Bonferroni post hoc tests) (n = 100–200 nodes or paranodes for each condition). Scale bars: 10 µm. Bars represent mean and SEM. CASPR1 = contactin-associated protein 1; ns = nonsignificant; Nfasc155 = neurofascin-155.

tolerance through the blocking of the autoantigen epitope and by competing with other subclass antibodies.[29] Class switch to IgG4 following chronic exposure to antigens is thus suspected to dampen the immune reaction. Nonetheless, IgG4 antibodies are associated with numerous autoimmune pathologies,[30] and recently, FAE has been shown to promote the pathogenicity of anti-MuSK antibodies in myasthenia gravis through function-blocking activity.[20] In a previous study, we showed that anti-Nfasc155 IgG4 does not have a function-blocking activity.[17] Instead, anti-Nfasc155 antibodies appeared to cross-link Nfasc155, promote Nfasc155 degradation, and thus abolish the association with the CNTN1/CASPR1 complex at the paranode. In neonatal animals, the injection of native anti-Nfasc155 IgG4 thereby inhibited paranode formation and resulted in either shorter or no paranode. Proteolytic cleavage into monovalent Fab abrogated the pathogenic capacity of anti-Nfasc155 IgG4. Promoting FAE in vitro also strongly decreased the effect of anti-Nfasc155 IgG4 but did not abolish paranode shortening. FAE is a dynamic process, and our data demonstrate that a proportion of IgG4 still remain monospecific even in the presence of a three-fold excess of unreactive IgG4. Also, we cannot exclude the fact that a fraction of the swapped antibodies rearranged in vivo into monospecific anti-Nfasc155 IgG4. Thus, the limited effects of swapped anti-Nfasc155 IgG4 are likely mediated by residual monospecific antibodies. To avoid this issue, a mutant form of IgG4 (S228P, F405L, and R409K) and of anti-MuSK IgG4 (S228P) were recently used to create under controlled reducing conditions a stable bispecific antibody.[21] We did not use this technique here because anti-Nfasc155 IgG4 has not been cloned.

Using IgG1 against Nfasc155, we have been able to estimate the percentage of monospecific anti-Nfasc155 IgG4. These levels were generally low with a median of 18% and were proportional to antibody titers, suggesting that monospecific antibodies may either be newly synthesized or may simply persist because of their prevalence. However, these levels did not correlate with clinical severity or response to treatment. Most patients were unresponsive to IVIg but responded positively to either plasmapheresis or rituximab. This further highlighted that low levels of monospecific anti-Nfasc155 IgG4 are sufficient to be

detrimental and that antibody-depleting strategies are the most efficient in those patients.

The absence of clinical correlate with the percentage of monospecific anti-Nfasc155 IgG4 is the main limitation of our study. From this small cohort study, the quantification of monospecific IgG4 does not seem to have clinical value for prognosis or treatment choice. However, a larger cohort study recently demonstrated that anti-Nfasc155 IgG4 titers follow clinical severity scores and are useful to monitor disease status.[31] Anyhow, this study demonstrates that IgG4 valency dictates the pathogenicity of anti-Nfasc155 and that FAE can potently decrease the pathogenic potential of these autoantibodies. Of course, one question comes to mind: why are those patients unresponsive to IVIg[6,12,32-35] if IgG4 effectively decreases autoantibody pathogenicity? IgG4 represent less than 3% of IVIg. Thus, the amount of IgG4 present in a usual dose of IVIg (2 g/kg) may be insufficient to mask patients' pathogenic antibodies. In addition, IVIg are known to act through several modes of action involving the Fc portion of IgG including the activation of immunomodulatory FcγRIIb, the saturation of FcRn, or the inhibition of complement.[36] IgG4 does not activate complement, FcγRIIb, and our data further demonstrated that the Fc domain of anti-Nfasc155 IgG4 is dispensable. Thus, by contrast to other isotypes, IgG4 are not acting through Fc-mediated effector function, and the mode of actions of IVIg appears irrelevant for Nfasc155 autoimmune nodopathy.

The fact that bivalent antibodies are pathogenic points out that IgG1 or IgG3 could also be pathogenic. IgG1 or IgG3 to Nfasc155 are infrequently found in autoimmune nodopathy. Only a few patients here presented with IgG1 to Nfasc155, and lower titers of IgG1 have previously been reported compared with IgG4.[37] Nonetheless, we found here that IgG1 antibodies were able to cross-link Nfasc155 and may thus be pathogenic. Several recent studies described the presence of IgG1 or IgG3 to pan-neurofascin in patients with CIDP or Guillain-Barré syndrome.[9,38,39] In addition to complement activation, these antibodies may alter paranodal regions by favoring Nfasc155 aggregation and degradation. Recently, IgG3 to CNTN1 have also been shown to affect the surface expression of CNTN1 in neurons.[40] The cleavage of anti-CNTN1 IgG into Fab, but not the $F(ab')_2$ fragment, abolished these effects. This further suggests that the monospecificity of IgG1 and IgG3 to CNTN1 may participate in their pathogenic actions. It still remains to be demonstrated whether this is also true for anti-CNTN1 IgG4. In any cases, affinity maturation of IgG4 appears to be required for the development of the pathogenic activity, as demonstrated in MusK myasthenia gravis.[41] Thus, antibody affinity and titers are likely to further influence antibody pathogenicity.

The mechanisms responsible for IgG4 swapping in situ are not yet well defined. GSH was originally suspected to enable FAE in cells because a low-molecular-weight compound extracted from erythrocyte lysate could promote FAE.[19] Here, GSH also promoted the FAE of anti-Nfasc155 IgG4 in vitro. The blood levels of GSH have been reported to be decreased not only in some inflammatory diseases but also during aging.[42] FAE may thus be less efficient in some patients because of GSH deficiency. A correlation was previously reported between the severity of CIDP and the age of patients with antibodies to the nodes of Ranvier.[43] However, we did not find such correlation here, and generally, patients with anti-Nfasc155 IgG4 were younger.[6,35,37,44] Thus, patients' age may not be the critical factor influencing IgG4 monospecificity. Nonetheless, we cannot rule out that low GSH blood levels may participate in the persistence of monospecific antibodies. We did not measure GSH blood levels in our cohort because blood samples were not available and plasma levels of GSH are marginally low compared with blood levels.

Altogether, our data indicate that bivalent IgG4 are pathogenic in Nfasc155 autoimmune nodopathy and that promoting FAE using healthy IgG4 and GSH can decrease the pathogenic potential of these autoantibodies. It has to be kept in mind that promoting FAE may be detrimental in anti-MuSK myasthenia gravis.[20,21] Future works should examine whether FAE is beneficial or detrimental in anti-CNTN1 and anti-CASPR1 autoimmune nodopathy.

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Disclosure

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Appendix 2 *(continued)*

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IgG4 Valency Modulates the Pathogenicity of Anti–Neurofascin-155 IgG4 in Autoimmune Nodopathy

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