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BRIEF COMMUNICATION

G1m1 predominance of intrathecal virus-specific antibodies in multiple sclerosis

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Abstract

We have previously shown that plasmablasts of the G1m1 allotype of IgG1 are selectively enriched in the cerebrospinal fluid of G1m1/G1m3 heterozygous patients with multiple sclerosis, whereas both allotypes are equally used in neuroborreliosis. Here, we demonstrate a strong preference for the G1m1 allotype in the intrathecal humoral immune responses against measles, rubella, and varicella zoster virus in G1m1/G1m3 heterozygous multiple sclerosis patients. Conversely, intrathecally synthesized varicella zoster virus-specific IgG1 in varicella zoster virus meningoencephalitis comprised both allotypes. This implies that G1m1 B cells are selected to the central nervous system of multiple sclerosis patients regardless of specificity and suggests that an antigen-independent mechanism could drive the intrathecal humoral immune response.

Introduction

The efficacy of anti-CD20 therapy in multiple sclerosis (MS) has put B cells in the spotlight for their pathogenic involvement.[1] B cells can present antigens to T cells, undergo affinity maturation and clonal expansion, and differentiate into antibody-secreting cells.[2] In a majority of MS patients, antibody-secreting cells are present in the central nervous system, and locally produced immunoglobulin G (IgG) can be detected in the cerebrospinal fluid (CSF) as oligoclonal bands (OCB).[3] Curiously, an intrathecal humoral immune response targeting commonly encountered viruses, including measles, rubella, and varicella zoster virus (VZV) can be observed in most MS patients, as opposed to patients with other neuroinflammatory diseases.[4–8] These antibodies constitute a minor fraction of the intrathecally synthesized antibodies and are believed to be irrelevant to the pathophysiology of MS.[7] In contrast, no consensus has been found regarding an MS-specific target of the major oligoclonal IgG fractions.

It has been shown that genetic variants in the *IGHG1* gene on chromosome 14 coding for the G1m allotypes on the IgG1 constant chain influence IgG levels in the CSF and MS risk.[9–11] We recently demonstrated a dominance of plasmablasts carrying the G1m1 allotype in the CSF of G1m1/G1m3 heterozygous MS patients, as opposed to patients with neuroborreliosis.[12] However, the mechanism driving the G1m1 bias is not clear, and proposes a closer look to antigen specificity as a potential influencing factor. GM genes have previously been shown to regulate the magnitude of IgG responses to cytomegalovirus, and G1m allotypes have demonstrated differential binding to several viral Fc receptors.[13,14] To address whether the selection of G1m1 plasmablasts is influenced by antigen specificity, we here examined the

G1m allotypes of intrathecally synthesized antibodies against measles, rubella, and VZV in G1m1/G1m3 heterozygous MS patients.

Methods

Patient samples

Thirty paired CSF and serum samples of G1m1/G1m3 heterozygous, OCB positive, relapsing-remitting MS patients were collected from the Departments of Neurology at Akershus University Hospital, Oslo University Hospital and the Norwegian MS Registry and Biobank at Haukeland University Hospital, of which 28 patients had been included in our previous study.[12] Twenty-eight of the patients were recruited as they underwent lumbar puncture for diagnostic purposes, and two patients were included solely for research. All patients met the 2010 McDonald criteria for MS diagnosis, and 7 patients had clinical evidence of an acute relapse within 2 weeks before lumbar puncture. Paired CSF and serum samples of 10 G1m1/G1m3 heterozygous VZV meningoencephalitis patients were collected from the diagnostic biobanks of Oslo University Hospital and Akershus University Hospital and used as controls. The controls tested positive for PCR against VZV in the CSF, had increased mononuclear CSF cell count, and symptoms compatible with VZV meningoencephalitis. G1m allotypes were determined in MS patients and controls using ELISA as previously described.[12] Two MS samples were considered unsuitable for analysis, due to degradation. Approvals were issued by The Regional Ethical Committee South East (2009/23 S-04143a), and the Regional Ethical Committee West (046.03/2010.1821). We obtained written informed consent from all participants before inclusion.

Isoelectric focusing and affinity blotting

Viral antigens validated and approved for diagnostic use (Serion) of Measles (Strain Edmonston, Vero cell culture), VZV (Strain Ellen, HEL-299 cell culture) or Rubella (Strain HPV-77, Vero cell culture) were diluted to 10 μg/mL and used for overnight incubation of nitrocellulose paper (NCP; Amersham Protran Premium 0.45 μm NC; GE Healthcare). Control antigens (Serion) comprised the cell cultures used to obtain viral antigens (Vero and HEL-299 cell cultures, Serion).

We concentrated CSF samples (Vivaspin 500; Sartorius) and normalized CSF and serum IgG concentrations to 100 mg/L. Four microliter of paired samples were loaded on Isogel Agarose IEF Plate pH 3-10 (Lonza), and focused on a Multiphor II Electrophoresis System (GE Healthcare).[12] After incubating the antigen-coated NCP in 4% ECL Advance Blocking Agent (Amersham) for 50 min, we blotted it against the focused gel for 45 min. Next, the NCP was incubated overnight in mouse anti-human antibodies against IgG1 (1.0 μg/mL, HP6069, Invitrogen, Molecular Probes), G1m1 (31 ng/mL, 10H1), or G1m3 (0.20 μg/mL, HP6027, Abingdon Health). Controls for each antigen were selected from CSF of G1m1 and G1m3 homozygous MS patients. For visualization, we incubated the NCP in HRP-conjugated goat anti-mouse IgG antibodies (Southern Biotec) for 1 h and further applied SuperSignal West Pico (Thermo Fisher Scientific) according to manufacturer instructions. We exposed the NCP for 1 h in a G:BOX digital camera system (Syngene), and collected multiple images at different exposure times.

Two investigators analyzed the results independently, and there were no disagreements between the individual assessments. Intrathecal synthesis was defined as at least one band or distinct area exclusive to CSF or at least two times stronger than corresponding bands in serum.

Determining virus-specific humoral immune responses using ELISA

IgG1 concentrations were determined in all samples using the IgG1 Human ELISA Kit (Thermo Fisher Scientific) according to manufacturer instructions. We diluted all CSF and serum samples to 1 μg/mL IgG1 in PBS with 1% BSA and incubated paired duplicates of 50 μL for 120 min on ELISA plates coated with measles virus, VZV, or rubella virus antigens (Serion). After washing with TPBS, the samples were stained with mouse-anti human G1m1 (1.5 μg/ml, 10H1) or G1m3 (1.0 μg/mL, HP6027) and incubated for 90 min. The plates were washed again and incubated with rabbit anti-mouse IgG secondary antibodies conjugated with alkaline phosphatase (SuperClonal antibodies, A27032; Thermo Fisher Scientific). The signal was visualized with phosphatase substrate (Sigma-Aldrich) reactivity measured at 405 nm. Appropriate positive and negative control sera were included on all plates. The negative control sera consisted of sera from individuals not immunized with the relevant viruses (sera from non-vaccinated, non-pathogen exposed individuals). The optical density from the wells incubated with the negative serum (always < 0.1) was subtracted from readings on the same plate. To generate standard curves, we coated plates (Nunc, 436014; Thermo Fisher Scientific) with G1m1 or G1m3 myelomas in two-fold dilutions and processed them in parallel with the virus-coated plates using the same reagents. The readout for the standard curves was ng/ml, but since antibody activity cannot be assayed in concentration, we converted to arbitrary units (AU)/mL, where 1 AU/mL is analogous to 1 ng/mL.

Validation of antibody specificities

To validate the anti-G1m1 and the anti-G1m3 antibodies, we included an additional 16 individuals (10 MS patients and 6 patients with other inflammatory neurological diseases). From these individuals, IgG had previously been isolated and subjected to trypsin cleavage and mass spectrometry as described elsewhere.[15] In order to identify the two CH3 allotype sequences in the mass spectrometry raw data, we used MaxQuant software[16] with a database containing the CH3 sequences of both IgG1 allotypes. ELISA was used to determine the antibody reactivities against IgG in serum,[12] and the results were compared to the mass spectrometry data from trypsinized IgG from the same individuals. This revealed a 100% match between the antibody reactivities and the mass spectrometry data ($P < 0.0001$, binomial test, Table S1 and Fig. S1), confirming the antibody specificities.

Data analysis

Statistical tests are named in the figure legends. Tests were two-tailed, with the significance level set at 5%. GraphPad Prism 7 was used for analysis.

Results

To identify patients with intrathecal anti-viral humoral immune responses, we screened paired CSF and serum samples from 30 G1m1/G1m3 heterozygous OCB positive relapsing-remitting MS patients using isoelectric focusing and affinity blotting (representative blots are shown in Fig. S2A). Patients' characteristics are given in Table 1. Intrathecal synthesis of IgG1 against measles, rubella, or VZV was detected in 25/30 patients (83%) (Fig. S2B).

Table 1. Demographic and clinical data of all study participants.

	RRMS patients (n = 30)
Mean age in years (range; SD)	40 (18–63; 10)
Female/Male	17/13
Mean disease duration in months (range; SD)	84 (0–356; 95)[1]
EDSS (range; SD)	2 (0–6;1.4)[2]
Mean number of relapses (range; SD)	2 (1–5; 1)[2]
Mean duration since last relapse in months (range)	12.2 (0–148)[2]
Patients undergoing DMT at CSF collection	3/30 (10%)

RRMS, relapsing-remitting multiple sclerosis; SD, standard deviation; EDSS, Expanded Disability Status Scale; DMT, disease modifying treatment.
[1]Missing data from one patient
[2]Missing data from two patients.

Individually, there were positive findings against measles in 16/30 (53%), against VZV in 15/30 (50%) and against rubella in 16/30 (53%) patients. 17/30 (57%) demonstrated intrathecal antibody synthesis against at least two viral antigens, while 5/30 (17%) patients produced IgG1 specific to all three (Fig. S2B). All samples proved negative when blotted against control antigens. Similarly, paired CSF and serum samples from 10 controls with VZV meningoencephalitis were screened for intrathecal synthesis of IgG1 against VZV (Fig. S2C). We found 6/10 (60%) controls to have intrathecal production against VZV. When assessing the presence of bands, we analyzed multiple exposure times, to ensure identifying antibodies found in lower concentrations, depicted through weaker signal (Fig S3).

Next, we used isoelectric focusing and affinity blotting to determine the G1m allotypes of the virus-specific IgG1 OCB in MS patients and controls with VZV meningoencephalitis (Fig. 1A and B). In the vast majority of MS patients, we found a predominant usage of the G1m1 allotype in intrathecally synthesized antibodies against all tested viruses (Fig. 1B). We detected five exceptions, of which three against measles and two against rubella. In all exceptions, G1m3 antibodies were always accompanied by G1m1. In the six controls with VZV meningoencephalitis displaying an intrathecal IgG1 synthesis against VZV, we found that the G1m1 and G1m3 allotypes were equally present (Fig. 1A and B).

To quantify the anti-viral antibodies of both G1m allotypes, we used ELISA to analyze paired CSF and serum samples from the patients shown to display intrathecal anti-viral IgG1 synthesis (Fig. 2A). Anti-viral G1m1 levels were significantly higher in CSF compared to serum for all viruses tested, while G1m3 antibodies were significantly higher in the CSF compared to serum for measles virus, but not for rubella or VZV. When assessing the CSF:serum ratios of anti-viral antibodies, the ratio for G1m1 antibodies was significantly higher than that for G1m3 in all investigated viruses (Fig. 2A). In contrast, when quantifying the G1m allotypes of VZV-specific IgG1 in controls with VZV meningoencephalitis, we observed higher levels of both allotypes in the CSF from 6 of 10 controls (Fig. 2B), but the CSF:serum ratio of anti-VZV IgG1 were similar for the G1m1 and G1m3 allotypes (Fig. 2B). The G1m1:G1m3 ratio indicated an unequivocal increase in CSF of MS patients compared to serum, as well as compared to the CSF and serum of controls with VZV meningoencephalitis (Fig. 2C).

Discussion

The mechanism driving the selection of G1m1 positive plasmablasts to the CSF in MS is not known. It has been

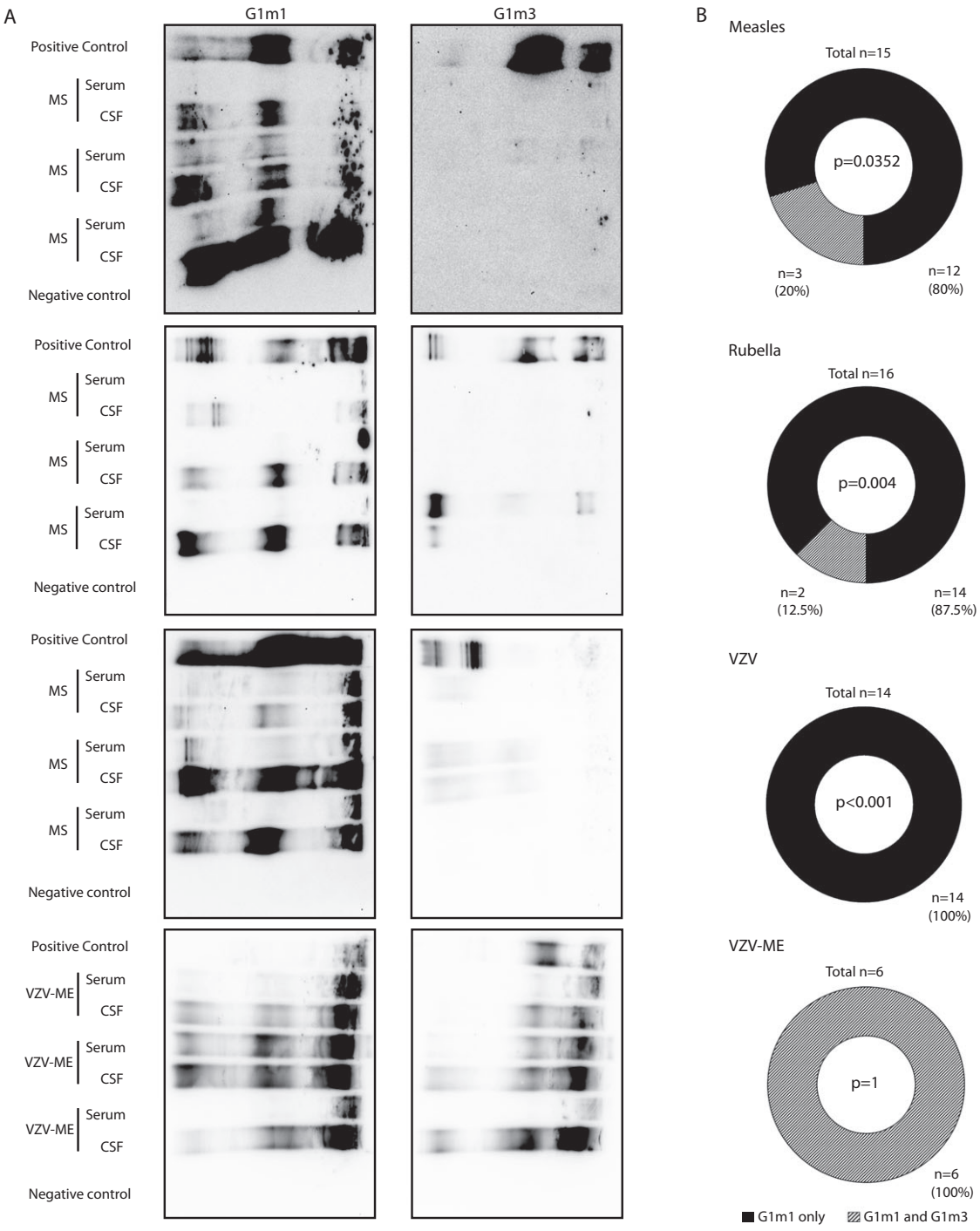

Figure 1. Intrathecal synthesis of IgG1 against measles, rubella and varicella zoster virus (VZV) shows G1m1 allotype predominance in MS patients and an even distribution in controls with VZV meningoencephalitis (VZV-ME). (A) The three upper panels show representative isoelectric focusing and affinity blotting against measles, rubella and VZV, determining IgG1 synthesis of G1m1 and G1m3 allotypes in three G1m1/G1m3 heterozygous MS patients. CSF samples from homozygous MS patients with virus-specific intrathecal synthesis were used as positive and negative controls. The bottom panel shows the results from three representative controls with VZV-ME after affinity blotting against VZV. (B) Isoelectric focusing and affinity blotting was performed on paired CSF and serum samples of G1m1/G1m3 heterozygous MS patients to determine G1m allotypes of intrathecally synthesized antibodies against measles, rubella and VZV. The same procedure was used to determine the G1m allotypes of intrathecally synthesized antibodies against VZV in G1m1/G1m3 heterozygous controls with VZV meningoencephalitis. The intrathecal synthesis of IgG1 of the G1m1 allotype alone was compared to that of IgG1 of both allotypes using Binomial test.

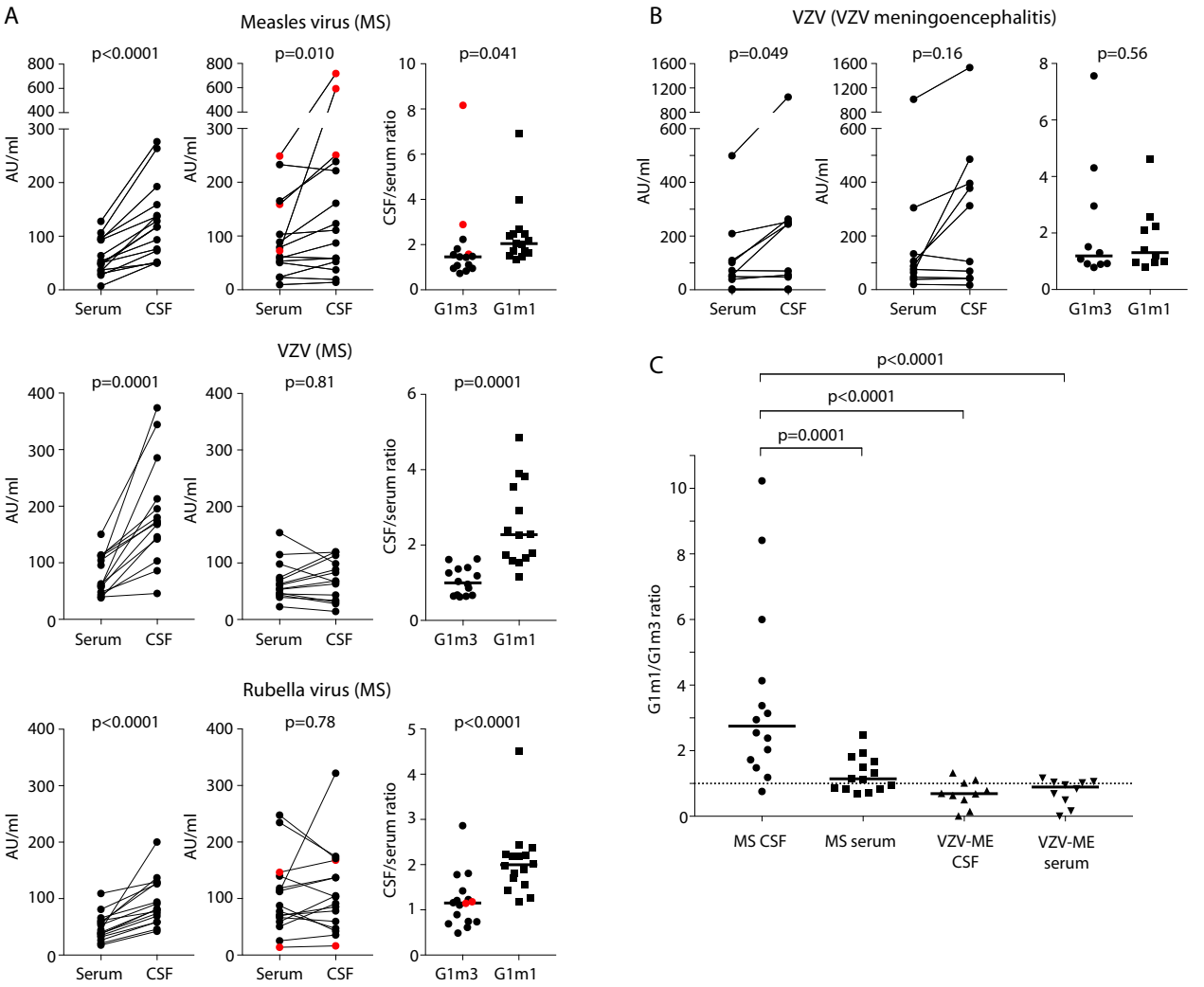

Figure 2. Anti-viral antibodies of the G1m1 allotype are enriched in cerebrospinal fluid (CSF) of MS compared to serum, and compared to CSF and serum of controls with varicella zoster virus meningoencephalitis (VZV-ME). (A) Measles-, rubella-, and VZV-specific G1m3 and G1m1 antibodies were quantified in paired serum and CSF samples of MS patients using ELISA, and the CSF:serum ratios were estimated. The bars depict the median values. Comparisons were made using the Wilcoxon signed-rank test. Exceptions displaying G1m3 usage according to isoelectric focusing and affinity blotting are marked in red. (B) VZV-specific G1m3 and G1m1 antibodies were quantified in paired serum and CSF samples of VZV-ME controls using ELISA, and the CSF:serum ratios were estimated. The bars depict the median values. Comparisons were made using the Wilcoxon signed-rank test. (C) G1m1/G1m3 ratios of VZV-specific antibodies in MS and VZV-ME samples of CSF and serum. The bars correspond to the median values. Statistical significance was established using Kruskal-Wallis test on all groups, Wilcoxon signed-rank test comparing patient CSF and serum, and Mann-Whitney *U* test comparing patients and controls.

hypothesized that GM genes could cause conformational changes in the antigen-binding site of the immunoglobulin variable regions,[17] or that the genetic variants are inherited together with polymorphisms in the variable region genes.[12] Any allotype-associated difference in the variable region of the IgG1 molecule could influence the affinity for a putative antigen and possibly explain the observed G1m1 selection to the CSF in MS. The present results argue against this possibility. Here, we demonstrate a strong dominance of the G1m1 allotype in intrathecally synthesized antibodies against measles, rubella, and VZV. This suggests that G1m1 positive plasmablasts are selected to the CSF of MS patients independently of their target antigen.

Several antigen-independent mechanisms explaining the selection of G1m1 positive B cells are conceivable. Preferential interactions between Fc receptors and the G1m allotypes have been suggested.[18] Additionally, G1m allotype combinations have been shown to influence the binding of IgG1 to the neonatal Fc receptor.[19] Another

possibility is the existence of an inflammatory microenvironment within meningeal B-cell follicles or deep cervical lymph nodes encouraging survival and/or differentiation of G1m1 B cells in favor of G1m3. Such mechanisms could involve the effect of cytokines on regulatory elements upstream of the *IGHG* switch regions.[9] Finally, B-cell adhesion molecules are essential for maintaining interactions with T follicular helper cells, and changes in T-B adhesiveness can alter B-cell selection for clonal expansion.[20]

The intrathecal polyspecific anti-viral immune response is detected in most MS patients, but rarely in other neuroinflammatory diseases.[6] These findings have also been confirmed in OCB negative contexts[21] and in clinically isolated syndrome patients who subsequently converted to MS.[22] The presence of an intrathecal humoral immune response against these viruses in MS is a conundrum. Most MS patients included in our study have been vaccinated during childhood against measles and rubella viruses, which makes the presence of these viruses within the CSF or CNS unlikely. In the absence of viral antigens,[23] it has been suggested that the mechanism might involve a local bystander activation of B cells against irrelevant specificities. The possibility of virus-specific B cells being cross-reactive to a yet undetermined self-antigen is not disproven. It might seem implausible, however, since the anti-viral fraction of intrathecally synthesized antibodies is directed against several different viruses and does not cross-react between the viruses.[4] Moreover, it would be relevant to investigate both the IgG1 allotype restriction and the intrathecal polyspecific anti-viral reaction in MS patients with other genetic backgrounds, such as Asian/Mongoloid populations, in which there has been a chromosomal crossover, leading to another combination of IgG1 allotypes in a proportion of the individuals. Here, we found similar levels of intrathecal virus-specific antibodies as previous studies.[4,24,25] Thus, it is unlikely that selection bias has influenced our results. Additionally, we found concordant results with quantitative and qualitative methods. In conclusion, preferential intrathecal G1m allotype usage in MS is not dependent on antigen specificity, supporting that the intrathecal synthesis of virus-specific IgG is maintained by the same mechanisms as the main fractions of the OCB.

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Author Contributions

Study concept and design: A.TB., F.V., T.H., C.A.V., A.L.; data acquisition and analysis: A.TB., F.V., T.H., C.A.V., F.V., A.L.; drafting the manuscript: A.TB., A.L; reviewing the manuscript for intellectual content: A. TB., F.V., T.H., C.A.V., A.L.

Conflicts of Interest

A.L. has received a research grant supporting the work from Sanofi Genzyme and speaker's fees from Merck Serono and Roche.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Validation of anti-G1m1 and anti-G1m3 antibody specificities in patients with multiple sclerosis (MS) and other inflammatory neurological diseases (OIND).
Figure S1. Optical density (OD) in ELISA for individuals predicted by mass spectrometry to carry the G1m1 allotype and/or the G1m3 allotype.
Figure S2. (A) Representative isoelectric focusing and affinity blotting against measles, rubella and VZV in G1m1/G1m3 heterozygous MS patients, determining virus-specific intrathecal IgG1 synthesis. (B) Positive virus-specific IgG1 findings using IEF and affinity blotting in G1m1/G1m3 heterozygous MS patients, according to virus specificity and number of different viruses. (C) Representative isoelectric focusing and affinity blotting against VZV in three G1m1/G1m3 heterozygous controls with VZV meningoencephalitis (VZV-ME).
Figure S3. Representative IEF and affinity blotting at four different exposure times.

