

Original Research paper

# Natalizumab exerts a suppressive effect on surrogates of B cell function in blood and CSF

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## Abstract

**Background:** Natalizumab for multiple sclerosis (MS) increases the risk of progressive multifocal leukoencephalopathy (PML).

**Objective:** We aimed to assess the effect of natalizumab on cellular composition and functional B cell parameters including patients with natalizumab-associated PML ( $n=37$ ).

**Methods:** Cellular composition by flow cytometry, levels of immunoglobulin (Ig)G/IgM by immunonephelometry, and oligoclonal bands by isoelectric focusing were studied in blood and cerebrospinal fluid.

**Results:** In MS patients treated with natalizumab without PML ( $n=59$ ) the proportion of CD19+ B cells was higher in blood, but lower in cerebrospinal fluid compared with MS patients not treated with natalizumab ( $n=17$ ). The CD4/CD8-ratio in cerebrospinal fluid was lower, and IgG and IgM levels as well as the IgG index dropped in longitudinal samples during natalizumab therapy. Oligoclonal bands persisted, but the total amount of the intrathecally produced IgG fraction, and the polyclonal intrathecal IgG reactivity to measles, rubella, and zoster declined. At the time of diagnosis of PML patients with natalizumab-associated PML had low total IgG levels in blood and cerebrospinal fluid.

**Conclusions:** Natalizumab impacts B and T cell distribution and exerts an inhibitory effect on surrogates of B cell function in periphery and in cerebrospinal fluid, potentially contributing to the increased risk of developing PML.

**Keywords:** Progressive multifocal leukoencephalopathy, JC virus, natalizumab, Tysabri, multiple sclerosis, disease-modifying therapies, immunosuppression

Date received: 15 June 2014; revised: 5 August 2014; 7 September 2014; accepted: 8 September 2014

## Introduction

Natalizumab, a monoclonal antibody against the  $\alpha 4$ -integrin, is approved for relapsing–remitting multiple sclerosis (MS) and prevents the transmigration of activated T cells across the blood–brain barrier into the brain tissue.<sup>1</sup> Reported changes during natalizumab therapy are an inversion of the CD4/CD8 ratio in cerebrospinal fluid (CSF), a reduction of antigen-presenting cells in the perivascular spaces,<sup>2,3</sup> and an effect on the immune repertoire of T cells in blood and CSF.<sup>4</sup>

However, the most prominent observation in the peripheral blood cell count of patients treated with

natalizumab is an increase in CD34+ hematopoietic progenitors<sup>5</sup> as well as of circulating pre-B and B cells.<sup>6,7</sup> This increase can be explained by an impaired homing,<sup>7,8</sup> and/or a diminished adhesion of these cells to the natural niches.<sup>9</sup> Adhesion signalling at natural niches provides pro-survival stimuli in particular to antibody-producing cells.<sup>10</sup> Inhibitory effects on B-cell function might be an important part of the mode of action of natalizumab, and potentially are also linked to the increased risk of developing progressive multifocal leukoencephalopathy (PML) or herpes virus complications.<sup>11–13</sup> Recent publications suggest that natalizumab might alter the humoral

Multiple Sclerosis Journal

2015, Vol. 21(8) 1036–1044

DOI: 10.1177/  
1352458514556296

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immune response toward JC virus (JCV) and varicella zoster virus (VZV),<sup>14</sup> decreases serum immunoglobulin (Ig)M and IgG levels,<sup>15</sup> and potentially also surrogates of intrathecal antibody synthesis.<sup>16,17</sup>

The aim of this study was to assess the effects of therapy with natalizumab in MS patients on the cellular composition and on B cell functional parameters in blood and CSF including patients with natalizumab-associated PML.

## Materials and methods

### Patients

The study was performed as part of the German pharmacovigilance study for natalizumab at the Department for Neurology, Heinrich-Heine University Duesseldorf, Germany. The study included 59 patients with relapsing–remitting MS treated with natalizumab for more than 18 months, and 17 age- and gender-matched patients with relapsing forms of MS not treated with natalizumab<sup>4</sup> who did not receive any disease-modifying therapy within the preceding 2 months of study inclusion. In addition, IgG levels in blood and CSF were available from 37 patients with natalizumab-associated PML, of which 20 were first sampled at time of diagnosis as defined by a first positive PCR for JCV DNA in CSF. The CSF anti-JCV IgG antibody index in this cohort of PML patients has been recently published.<sup>18</sup> The study was approved by the local institutional review board (protocol number 3315). All non-PML MS patients gave written informed consent to the scientific use of their blood and CSF samples and data. The local Ethics committee waived the requirement for written informed consent for the use of anonymized data of patients with PML available at the Institute for Virology.

### Flow cytometric analysis, immunonephelometry and isoelectric focusing

The cellular composition of blood and CSF was assessed by fluorescence-activated cell sorting (FACS), using fluorescence-labelled antibodies against CD14, CD19, CD3, CD4 and CD8 (all BD Bioscience, according to the manufacturer's protocol). Fresh ethylenediaminetetraacetic acid (EDTA) blood and CSF samples were used for analysis performed immediately after sampling. IgG, IgM and albumin were determined as published,<sup>18</sup> and the IgG and the IgM index, and the locally produced absolute IgG fraction (IgG-loc) were calculated. Detection of oligoclonal bands (OCBs) was performed by isoelectric focusing (IEF) on polyacrylamide gels followed by immunoblotting using an

IgG-specific antibody staining. Paired CSF and serum adjusted for protein concentrations were applied in the same assay as described earlier,<sup>19</sup> and OCBs were classified in five patterns as published.<sup>20</sup> In addition, the polyspecific intrathecal antibody responses to measles, rubella and zoster were studied by enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Lübeck, Germany), and the antibody specificity index was calculated as published.<sup>21</sup>

### Statistical analysis

Graph Pad Prism version 5.0 (GraphPad Software, San Diego, USA) was used for statistical analysis. 95% confidence intervals (CI) for proportions were calculated using the modified Wald method; for non-parametric measures the Mann–Whitney, or the Kurskal–Wallis test including Dunn's test to correct for multiple comparisons was applied. For longitudinal studies, the Wilcoxon matched pairs test was used; *p*-values <0.05 were considered significant.

## Results

### Patients' characteristics

MS patients treated with natalizumab and non-natalizumab-treated MS patients were age- and gender-matched.<sup>4</sup> Patients with natalizumab-associated PML<sup>18</sup> were older (*p*=0.003), and had longer duration of therapy with natalizumab (*p*<0.0001) compared with MS patients treated with natalizumab without PML (Table 1). In 8 of the 59 of the patients during long-term therapy with natalizumab, a CSF–serum pair obtained prior to natalizumab therapy was available, allowing us to study the intra-individual changes following natalizumab treatment initiation (median time between sampling: 30.5 months, range 21–55; no disease-modifying therapy within 4 weeks prior to natalizumab).

### The cellular composition of peripheral blood and CSF during therapy with natalizumab

Compared with MS patients not treated with natalizumab, natalizumab-treated patients had a higher relative number of CD19+ B cells in the peripheral venous blood (median: 16.4% vs. 6.7%; *p*<0.0001), and a lower proportion of CD19+ B cells in CSF (median: 0.4% vs. 2.4%; *p*<0.007). The proportions of CD14+, CD3+ cells and the CD4/CD8 ratio in blood did not significantly differ between MS patients treated or not treated with natalizumab. The CD4/CD8 ratio in CSF was found to be inverted (median: 0.66 vs. 4.47, *p*<0.0001),<sup>4</sup> and the relative number of

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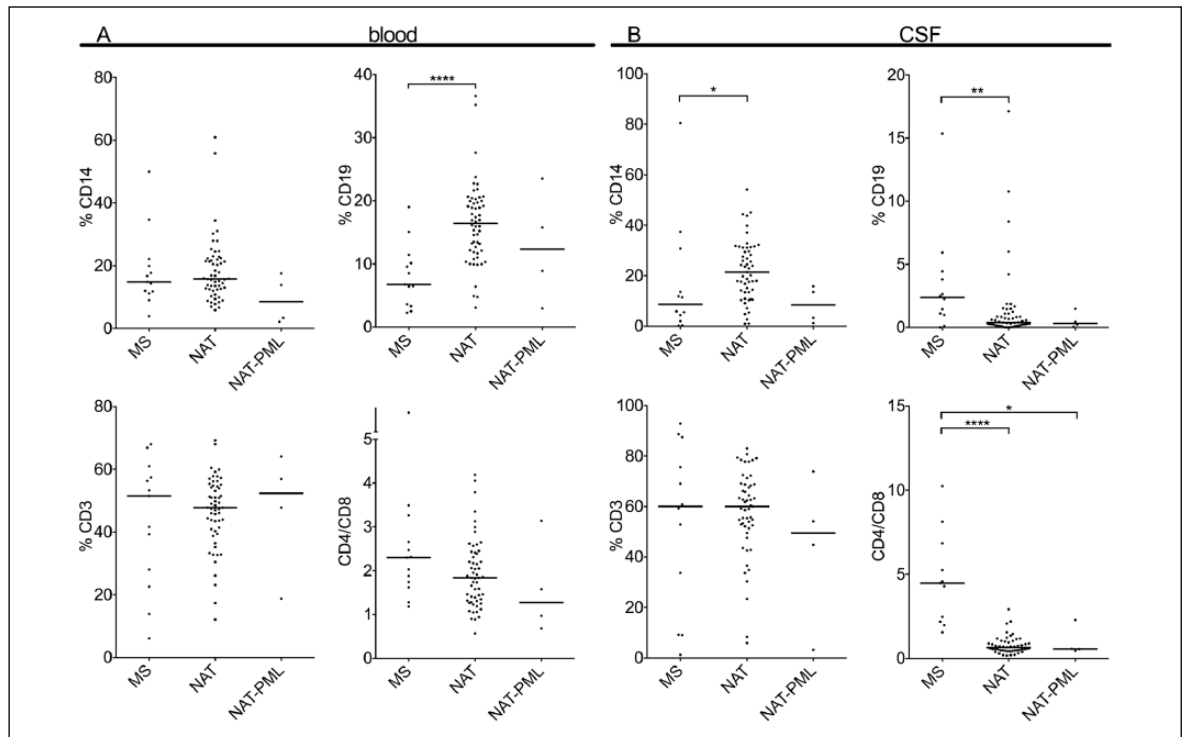
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**Table 1.** Patient characteristics.

	Total <i>n</i>	Age [years] median (range)	Sex <i>n</i> female (%)	Treatment duration [months] median (range)	EDSS median (range)	CSF [cells/ $\mu$ l] median (range)
MS <sup>6*</sup>	17	41.5 (22–70)	11 (65)	NA	3.0 (1–5.5)	3 (1–31)
NAT <sup>6</sup>	59	40 (23–60)	41 (69)	24 (19–50)	2.5 (0–6.5)	1 (1–6)
NAT-PML <sup>24</sup>	37	45 (28–63)	25 (68)	44 (19–75)	NA	NA

\*no disease-modifying therapy within the preceding 2 months prior to study inclusion; 12/17 were treatment naïve.



**Figure 1.** Cellular composition of blood (A) and cerebrospinal fluid (CSF; B) as assessed by FACS analysis. The relative number of CD14<sup>+</sup>, CD19<sup>+</sup>, CD3<sup>+</sup> cells and the CD4/CD8 ratio are shown. Bars represent the median. Statistics: Mann–Whitney. MS: patients with MS not treated with natalizumab; NAT: patients with MS treated with natalizumab; NAT-PML: patients with MS treated with natalizumab who developed PML.

CD14<sup>+</sup> cells to be increased in CSF (median: 21.4% vs. 8.7%;  $p=0.033$ , Figure 1).

In the limited number of PML patients ( $n=4$ ) with fresh EDTA and CSF cells available, CD19 proportions in CSF were found to be low (median: 0.33%;  $p=0.09$  for the comparison to non-natalizumab-treated MS patients), as well as the CD4/CD8 ratio (median: 0.57<sup>4</sup>;  $p=0.01$  for the comparison to non-natalizumab-treated MS patients) (Figure 1).

#### *IgG and IgM levels in blood and CSF during therapy with natalizumab*

IgG levels in patients with natalizumab-associated PML at the time of diagnosis of PML were lower

compared with non-natalizumab-treated MS patients in blood (median: 7.77 g/l vs. 9.66 g/l;  $p<0.001$ ) and CSF (median 18.40 mg/l vs. 26.35 mg/l;  $p=0.011$ ). We noted a similar trend for natalizumab-treated MS patients without PML ( $p=0.08$  in blood and CSF); no correlation between treatment duration with natalizumab and IgG levels in blood (spearman  $r$ : 0.15 (95% CI: -0.17–0.44)) or CSF (spearman  $r$ : 0.13 (95% CI: -0.19–0.43)) was observed. In samples obtained from patients with natalizumab-associated PML during the course of PML (following plasma exchange (PLEX)) the IgG index was found to be increased. This observation appeared more prominent for samples taken 4 weeks after PLEX initiation or later. IgM levels were lower in blood (median: 0.63 g/l vs. 1.40 g/l;  $p<0.001$ ) and CSF (median: 0.20 mg/l vs. 0.70

mg/l;  $p < 0.0001$ ) of patients treated with natalizumab compared with non-natalizumab-treated MS patients (Figure 2).

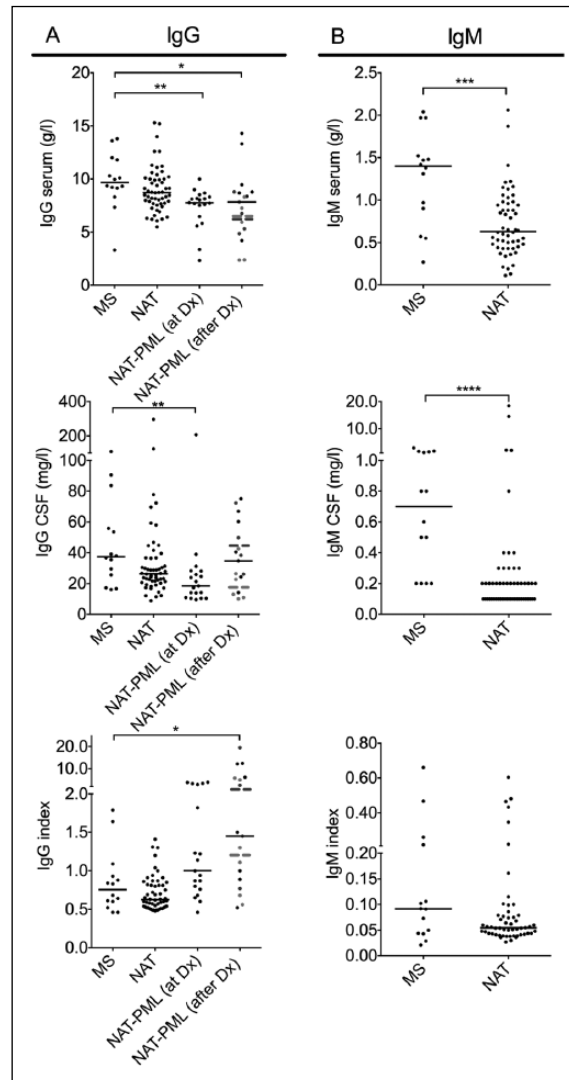
In longitudinal samples obtained before and during therapy with natalizumab, a decline in total IgG in blood (median of differences  $-1.66$  g/l;  $p = 0.008$ ) and CSF (median of differences  $-12.1$  mg/l;  $p = 0.008$ ), and total IgM in blood (median of differences  $-0.48$  g/l;  $p = 0.008$ ) and CSF (median of differences  $-0.20$  mg/l;  $p = 0.008$ ) was noted (Figure 3).

### The intrathecally produced IgG fraction during therapy with natalizumab

The IgG index (median of differences  $-0.18$ ,  $p = 0.031$ ) as a relative measure of the intrathecally produced IgG fraction declined in longitudinal samples collected before and during therapy with natalizumab (Figure 3). OCBs were detected in 49 out of 52 patients (94.2%; 95% CI: 83.7–98.6%) during therapy with natalizumab. In all six patients with sufficient material for longitudinal re-assessment OCBs remained detectable, but the OCB intensity declined (Figure 4(A)). In line with this observation, the absolute concentration of the locally produced IgG fraction (IgG-loc) declined in four of these six patients. This was associated with a decline in antibody index to measles, rubella and zoster in three of the four patients with quantifiable intrathecal IgG synthesis (Figure 4(B)).

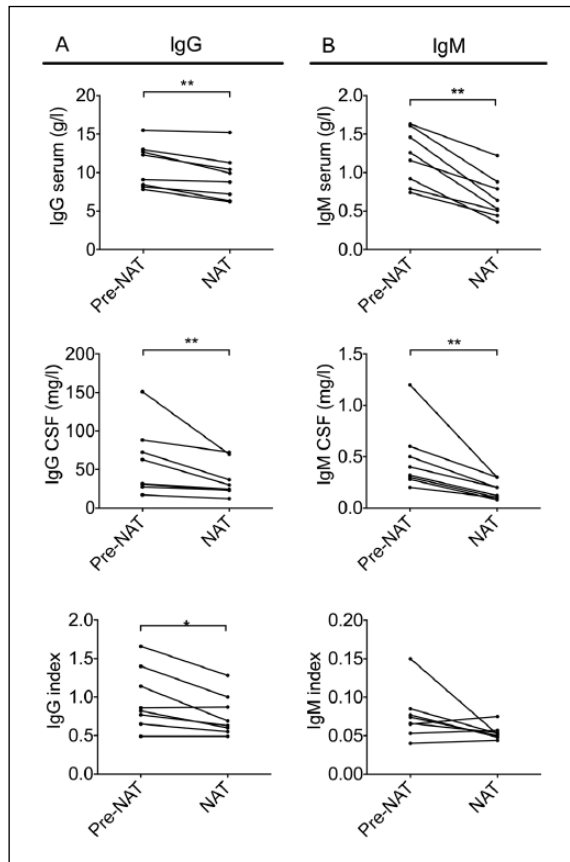
### Conclusions

We here report that natalizumab exerts a suppressive effect on various different functional parameters of antibody synthesis. Specifically, we noted a decrease of IgG and IgM levels in blood and CSF of longitudinal samples, which is in line with an earlier report on lowered IgG and IgM levels in blood only.<sup>15</sup> Partially conflicting findings in the literature on persistency of OCBs during natalizumab therapy are likely to be explained by differences in study design and methodology. While Harrer *et al.* analysed data aggregated from different laboratories and reported on disappearance of OCBs in 16% of individuals,<sup>17</sup> Mancuso and colleagues performed a smaller longitudinal single-centre study, noting complete disappearance of OCBs in as many as 55% of patients.<sup>16</sup> In our mono-centric approach, OCBs remained detectable in the majority of our cross-sectionally studied patients (94%), and in all six individuals with samples obtained before and during therapy with natalizumab. We confirmed longitudinal OCB persistency in a reference laboratory for OCB testing, and noted, despite the limited quality



**Figure 2.** Levels of total IgG in blood and cerebrospinal fluid (CSF) and the IgG index (A), and levels of IgM in blood and CSF and the IgM index (B) are shown. Bars represent the median. Statistics: Kruskal–Wallis (A); Mann–Whitney (B). MS: patients with MS not treated with natalizumab; NAT: patients with MS treated with natalizumab; NAT-PML (at Dx): Patients with natalizumab-associated PML and sampling at time of first positive qPCR for JCV DNA in CSF; NAT-PML (after Dx): Patients with natalizumab-associated PML and with sampling available after diagnosis of PML; red dots mark samplings within 4, blue dots samplings after 4 weeks of start with plasma exchange; the interrupted red or blue bars indicate the corresponding median.

related to pre-analytic quality issues, a decline of the intensity of some of the bands during therapy. Such a decline in intensity might result in findings below the detection threshold of other laboratories, and the reporting of a negative OCB status. The observed decline in OCB intensity was in line with a noted



**Figure 3.** Levels of total IgG in blood and cerebrospinal fluid (CSF) and the IgG index (A), and levels of IgM in blood and CSF and the IgM index (B). Pre-NAT: prior to therapy with natalizumab; NAT: during therapy with natalizumab. Statistics: Wilcoxon.

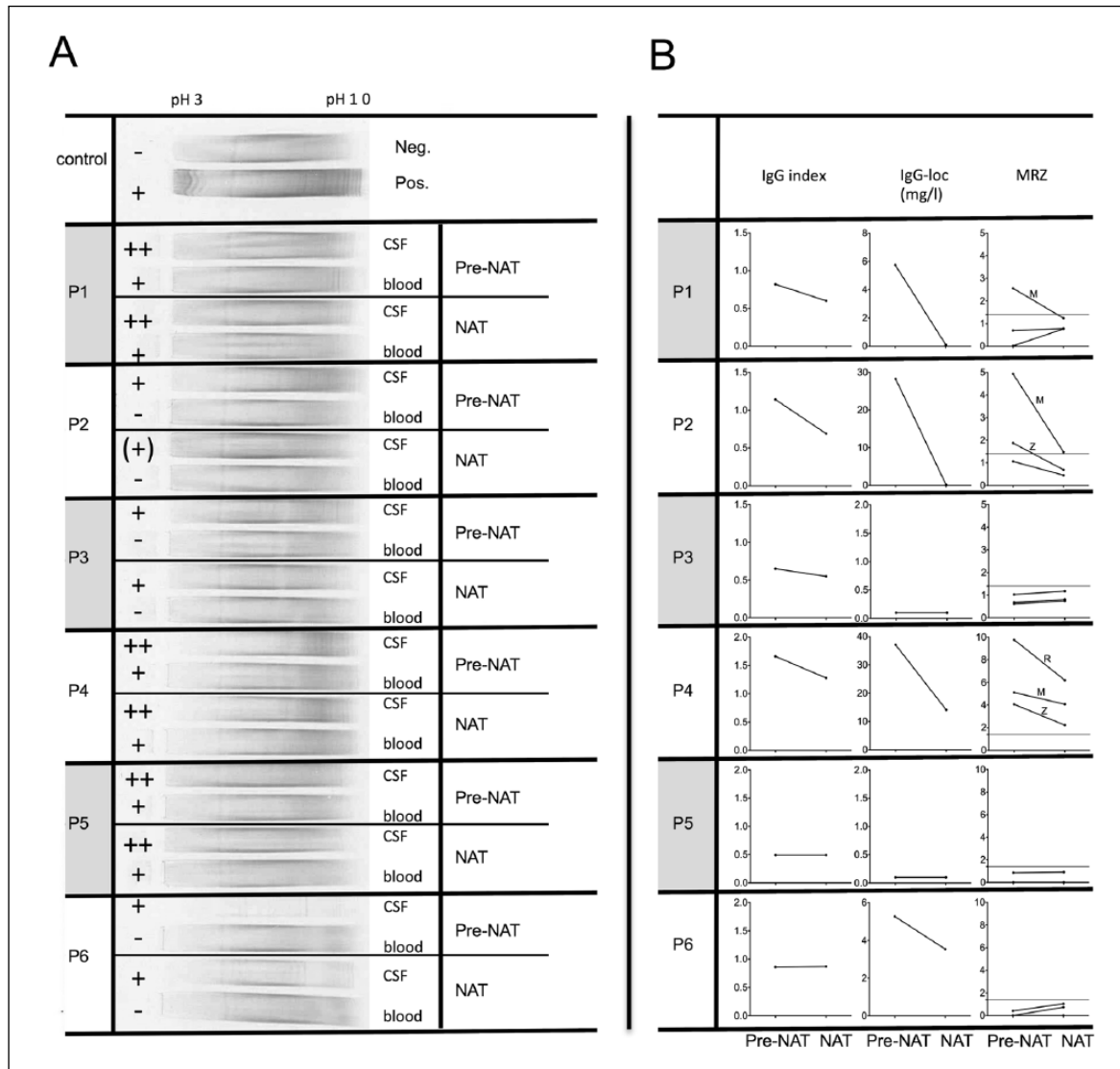
decrease of the IgG index, as well as the absolute IgG-loc concentration and the reduced polyspecific reactivity toward measles, rubella and zoster. In summary, our study in conjunction with previously published data is suggestive for a quantitative rather than a qualitative reduction of the intrathecally produced IgG fraction during therapy with natalizumab.

The majority of patients with untreated MS have an increased IgG index, OCBs in CSF, and a pathological antibody index toward measles, rubella, and zoster.<sup>22</sup> Thus, the decline in all these parameters observed in our study during natalizumab treatment implicate complex, most likely indirect, effects on B-lymphocyte function. Natalizumab targets the  $\alpha 4$ -integrin, which is expressed on B and T cells in a tissue-specific manner.<sup>23</sup> We did not mechanistically study how this mode of action may translate into a reduced peripheral and intrathecal antibody production. We assume that therapy-induced alterations of the B and T cell homeostasis might represent a possible explanation for the

changes observed. Within our current study, we noted a pronounced increase of the relative number of B cells in blood. Others have made similar observations.<sup>6,7</sup> This might not be surprising, considering the fact that various  $\alpha 4$ -dependent pathways control B cell homeostasis. VLA-4 ( $\alpha 4\beta 1$ )–VCAM-1 binding mediates homing of IgG-producing plasma cells to the bone marrow, and interacting of bone marrow stroma cells with developing B cells. Mature B cells use  $\alpha 4\beta 1$  to bind to VCAM-1 on follicular dendritic cells in the B cell follicles of secondary lymphoid organs, including the spleen, lymph nodes, and Peyer's patches. The  $\alpha 4\beta 7$ –MAdCAM-1 interaction is of importance for homing of naive and memory lymphocytes into the intestinal mucosa.<sup>24</sup> Overall, blockade of the  $\alpha 4$  integrin may directly impact B cell homing and adhesion to natural niches, which in turn might explain the increased numbers of B cells in the periphery, the loss of pro-survival stimuli in natural niches,<sup>25</sup> and a reduced peripheral antibody production during natalizumab therapy. Similar processes might also control the homing, adhesion, and interactions of lymphocytes in ectopic meningeal follicles considered relevant to the production of Ig in CSF.<sup>26</sup>

Disturbances in the B and T cell interactions and antigen recognition may also contribute to lower Ig levels. We noted a relative reduction of B cells and an inversion of the CD4/CD8 ratio in CSF, whereas monocytes/macrophages – reported to display a more moderate decrease in the  $\alpha 4$  and  $\beta 1$  surface expression in response to natalizumab as compared with B and T cells<sup>27</sup> – appeared to show a compensatory relative increase in CSF. Natalizumab is known to block transmigration of B cells, T cells, plasma cells, and antigen-presenting cells across the blood-CSF barrier.<sup>2,3</sup> The altered cellular immune surveillance in the central nervous system (CNS), together with altered antigen recognition, and B and T cell interactions, could secondarily contribute to a quantitative reduction of intrathecally produced Ig.

Reported natalizumab-induced alteration of gene expression is another possible explanation for changes in Ig production. Genes involved in B cell differentiation might be re-regulated by natalizumab,<sup>28</sup> a process in which epigenetic mechanisms could play a role.<sup>29</sup> The observation of an increased number of hematopoietic progenitors, pre-B and B cells in peripheral blood of MS patients during therapy with natalizumab, along with the finding of the transcription factor Spi-B being up-regulated in response to natalizumab therapy in CD34+ and CD19+ cells,<sup>30</sup> have been claimed to be relevant to pathogenesis of PML in patients with MS. In such a hypothesis, up-regulated



**Figure 4.** In six individuals (P1–P6) with samples pairs available before (Pre-NAT) and during natalizumab therapy (NAT) oligoclonal bands (OCBs) detected by isoelectric focusing on polyacrylamide gels are shown (A). In the same patients, the IgG index (left), the absolute IgG concentration locally produced in the central nervous system (IgG-loc, middle), and the antibody index to measles (M), rubella (R) and varizella zoster virus (Z, right) are depicted (B). Pre-analytic sample handling (stored samples, two freezing–thawing cycles prior to testing), and scanning procedure affected the quality of the OCB plot shown.

Spi-B would promote JCV reactivation via interaction with the non-coding control region of latent JCV in B cells or pre-B cells. B cells that harbour reactivated JCV would then carry the virus to the brain and promote CNS infection.<sup>31</sup> However, we previously failed to detect JCV DNA in peripheral blood mononuclear cells (PBMCs) or CD34<sup>+</sup> cells of patients treated with natalizumab,<sup>32</sup> although recently published data supports the view of B cells and/or CD34<sup>+</sup> progenitor cells as a possible reservoir for JCV.<sup>33,34</sup> However, it remains unclear how JCV-infected B cells, shown to have reduced expression of  $\alpha 4\beta 1$  during natalizumab

therapy,<sup>35</sup> and display impaired migration across endothelial barriers,<sup>8</sup> would reach the CNS. Indeed, B cells were barely detectable in CSF of our natalizumab-treated patients. In addition, gene expression studies in PBMCs that report up-regulation of genes involved in B cell activation<sup>28</sup> could possibly be biased by the pronounced increase of B cells in peripheral blood and the changes in the composition of B cell subpopulations discussed above.

Limitations to our study are the cross-sectional design, the longer treatment duration of patients with

natalizumab-associated PML compared with MS patients treated with natalizumab not developing PML, the lack of information on clinical disease activity scores throughout the study period (relapse rate, Expanded Disability Status Scale), and the fact that we did not assess antibody subclasses or specificity of antibodies. Nevertheless, our findings of lowered Ig levels in blood and CSF are consistent with the literature, and we are the first to show that patients with PML have also lowered overall IgG levels at time of diagnosis. In addition, we have previously observed that anti-JCV IgG antibody levels might decrease after treatment initiation with natalizumab.<sup>14</sup> It is unclear, to date, if these changes may predispose to the development of PML. Indeed, high and increasing anti-JCV antibody levels have been observed in patients with natalizumab-associated PML at time of diagnosis of PML.<sup>14</sup> This fits the widely accepted opinion that the humoral anti-JCV response alone might not be sufficient, while JCV-specific T-cell responses are considered key players in prevention of PML, and elimination of JCV in individuals with PML.<sup>12</sup> Nonetheless, the causative pathogen JCV appears to undergo intra-individually acquired viral-genetic changes in the coding and non-coding region.<sup>36,37</sup> During immunosuppression, the frequency of viral replication cycles at places of persistency, such as the renal tissue, might increase. This could be associated with a higher likelihood of the acquisition of pathogenic PML variants that eventually escape immune surveillance and cause PML. All arms of the adaptive immunity, including B cells as antigen-presenting cells, B cells for interactions with T cells, and high-affinity maturation of antibodies in germinal centres may be required to limit viral turnover, to neutralize virus in the blood stream, and to control virus in the CNS compartment that either reaches it via the blood stream or becomes locally reactivated from latently infected CNS cells.<sup>38</sup> Interestingly, we recently could show that the anti-JCV antibody response is strongly controlled by human leukocyte antigen class II variants.<sup>39</sup> Recently presented data on genome-wide transcript profiling in patients with PML versus non-PML showed a downregulation of genes involved in the regulation of immunoglobulins and B cell activation.<sup>40</sup> Overall, anti-JCV antibodies may not be sufficient to prevent PML, but B cells and their effector function in total might still play an important role in anti-JCV defence.

The post-PML samples of our current study show that during the course of PML following cessation of natalizumab and PLEX (most obvious in samples obtained after 4 weeks or later following PLEX initiation), total IgG levels in CSF appear to rise as well as

the IgG index. This may be a surrogate of reconstitution of adaptive immune competence in the CNS. Accordingly, we have previously shown that a majority of patients with natalizumab-associated PML have already developed a strong intrathecal anti-JCV antibody response at time of diagnosis of PML, which later increases and may aid in the early recognition of this dreaded complication.<sup>18</sup>

The extent to which changes in cellular as well as humoral immune composition described in this study may account for the development of PML in patients during selective immunosuppression needs to be studied in greater detail in larger patient cohorts. A more detailed assessment of global B cell effector functions may aid to elucidate the still incompletely understood pathogenesis of natalizumab-associated PML.

#### Conflict of interest statement

CW: speakers honoraria, Bayer Healthcare; TD: travel expenses Novartis Pharma, Genzyme; AKM: travel expenses, Biogen Idec; RG: speaker's honoraria, grant support, board honoraria, Biogen Idec, Bayer, Teva, Novartis, Merck Serono; TK: travel expenses, personal compensation (speaking fees) Bayer Healthcare/Teva Pharma, Merck Serono, Novartis, Sanofi-Aventis/Genzyme, Biogen Idec, grant support, Novartis Pharma; RH: board membership, consultancy, speaking fees, Novartis, Teva, Biogen Idec, Merck Serono, Bayer Schering, Sanofi-Aventis, Genzyme; grant support, Novartis, Teva, Biogen Idec, Merck Serono, Bayer Schering, Sanofi-Aventis; MM: speaker's honoraria, board honoraria Biogen Idec, Bayer, Teva, Novartis, Merck Serono, Genzyme, Sanofi-Aventis; MSt reports grants and personal fees from Biogen Idec, grants and personal fees from Novartis, grants and personal fees from Bayer Healthcare, grants and personal fees from Teva, personal fees from Sanofi-Aventis, personal fees from Baxter, personal fees from CSL Behring, personal fees from Grifols, outside the submitted work; TW: has received honoraria from Bayer, Biogen Idec, Genzyme, Novartis, Sanofi-Aventis for consulting and lecturing. As a member of the scientific advisory board of the PML consortium he is receiving honoraria for consulting; CK: speakers honoraria, consulting fees and grant support from Bayer Healthcare, Sanofi-Genzyme, Teva, Merck Serono, Boehringer Ingelheim, Biotronik, Biogen Idec, Novartis, CSL Behring, Pfizer, and Siemens; MPW: received honoraria for lectures and consultancy, Biogen Idec. AS: grant support, Biogen Idec; travel support, Biogen Idec, Genzyme/Sanofi, Merck Serono; lecture honoraria, Biogen Idec, Genzyme/Sanofi, Teva, Merck Serono; advisory board, Biogen Idec, Genzyme/Sanofi, Teva, Merck Serono; TO: grant support, personal fees, Biogen Idec, Novartis,

Genzyme, Merck. HPH: has received honoraria for consulting and speaking at symposia from Bayer, Biogen Idec, Genzyme, Merck Serono, Novartis Pharma, Roche and Teva Sanofi-Aventis, with approval by the Rector of Heinrich-Heine-University. HT: has received honoraria for consulting and lecturing, travel expenses for participating at symposia, and financial support for research from Bayer, Biogen Idec, Genzyme, Merck, Novartis, Roche Diagnostics, Siemens Diagnostics and TEVA. OA: personal fees, Biogen Idec. BCK: has received honoraria for lecturing, travel expenses for attending meetings, and financial support for research from Bayer Health Care, Biogen Idec, Genzyme/Sanofi-Aventis, Grifols, Merck Serono, Mitsubishi Europe, Novartis, and TEVA. MSt, VLeh, GvG, VS, VLim and DH have nothing to disclose.

### Funding

This work was supported by a local faculty grant (Research Commission of Heinrich Heine University, Duesseldorf [HHU]; OA) and a grant from the German Ministry for Education and Research (BMBF; German Competence Network MS [KKNMS], Natalizumab Pharmacovigilance Study, 01GI1002; BCK.). CW was supported by a European Committee for Treatment and Research in MS fellowship stipend. The research leading to these results received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement No. 115303, resources of which are composed of a financial contribution from the European Union's Seventh

Framework Program (FP7=2007–2013) and European Federation of Pharmaceutical Industries and Associations companies' in-kind contribution (to HPH, BCK, CW). The MS Center at the Department of Neurology, HHU is supported by the Walter and Ilse Rose Foundation. RH was supported by the German Research Foundation (DFG CRC TRR128, Z2) and BMBF (KKNMS). MSt is supported by the NiedersachsenResearchNetworkonNeuroinfectiology of the Ministry of Science and Culture of Lower Saxony and the BMBF (KKNMS). We highly appreciate the excellent technical support by Tatjana Males.

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