

Multiple Sclerosis Journal

<http://msj.sagepub.com/>

Immunological and clinical consequences of treating a patient with natalizumab

Nicholas Schwab, Karin G Höhn, Tilman Schneider-Hohendorf, Imke Metz, Max-Philipp Stenner, Samantha Jilek, Renaud A Du Pasquier, Ralf Gold, Sven G Meuth, Richard M Ransohoff, Wolfgang Brück and Heinz Wiendl

Mult Scler 2012 18: 335 originally published online 9 September 2011

DOI: 10.1177/1352458511421919

The online version of this article can be found at:

<http://msj.sagepub.com/content/18/3/335>

Published by:



<http://www.sagepublications.com>

Additional services and information for *Multiple Sclerosis Journal* can be found at:

Email Alerts: <http://msj.sagepub.com/cgi/alerts>

Subscriptions: <http://msj.sagepub.com/subscriptions>

Reprints: <http://www.sagepub.com/journalsReprints.nav>

Permissions: <http://www.sagepub.com/journalsPermissions.nav>

>> [Version of Record](#) - Mar 1, 2012

[OnlineFirst Version of Record](#) - Sep 9, 2011

[What is This?](#)

Immunological and clinical consequences of treating a patient with natalizumab

Multiple Sclerosis Journal
18(3) 335–344
© The Author(s) 2012
Reprints and permissions:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/1352458511421919
msj.sagepub.com


Nicholas Schwab^{1,2*}, Karin G Höhn^{1,2*},
Tilman Schneider-Hohendorf^{1,2*}, Imke Metz^{3*},
Max-Philipp Stenner^{2,#}, Samantha Jilek⁴, Renaud A Du Pasquier⁴,
Ralf Gold⁵, Sven G Meuth^{1,7}, Richard M Ransohoff⁶,
Wolfgang Brück³ and Heinz Wiendl^{1,2}

Abstract

Background: Long-term therapy with natalizumab increases the risk of progressive multifocal leukoencephalopathy (PML).

Objectives: We present a patient study through therapy, the diagnosis of PML (after 29 infusions), plasma exchange (PE) and development of immune reconstitution inflammatory syndrome (IRIS).

Methods: Routine diagnostics, magnetic resonance imaging (MRI), immunological status (flow cytometry, T-cell migration assays and T-cell repertoire analysis), and brain biopsy with immunohistological analysis.

Results: CD49d decreased after 12 months of treatment. At PML diagnosis, CD49d expression and migratory capacity of T cells was low and peripheral T-cell receptor (TCR) complexity showed severe perturbations. The distribution of peripheral monocytes changed from CCR5+ to CCR7+. After PE some changes reverted: CD49d increased and overshoot earliest levels, migratory capacities of T cells recovered and peripheral TCR complexity increased. With no clinical, routine laboratory or cerebrospinal fluid (CSF) changes, MRI 2 months after PE demonstrated progressive lesion development. Brain histopathology confirmed the presence of infiltrates indicative of IRIS without clinical signs, immunologically accompanied by CCR7/CCR5 recovery of peripheral monocytes.

Conclusion: Natalizumab-associated immunological changes accompanying PML were reversible after PE; IRIS can occur very late, remain asymptomatic and be elusive to CSF analysis. Our study may provide insights into the changes under treatment with natalizumab associated with JC virus control.

Keywords

antibodies (monoclonal), immune reconstitution inflammatory syndrome, JC virus, multiple sclerosis, progressive multifocal leukoencephalopathy

Date received: 17th February 2011; revised: 21st June 2011; accepted: 22nd July 2011

Introduction

Natalizumab (Tysabri®, Biogen-Dompé, Cambridge, MA, USA) is a recombinant humanized monoclonal antibody (mAb) directed against the adhesion molecule $\alpha 4$ -integrin (CD49d), expressed on the surface of circulating lymphocytes. Interaction of $\alpha 4$ -integrin with vascular cell adhesion molecule-1 (VCAM-1), expressed on endothelial cells, is required to allow activated immune cells to migrate into the central nervous system (CNS).¹ Therapeutic benefit of natalizumab in the treatment of multiple sclerosis (MS) has repeatedly been shown;² however, its use is associated with the occurrence of progressive multifocal leukoencephalopathy (PML).^{3–5} PML is a rare demyelinating disease of the CNS caused by JC virus infection of oligodendrocytes and astrocytes.⁶ Data indicate that the risk for PML increases with duration of treatment, showing a risk

¹Department of Neurology - Inflammatory Disorders of the Nervous System and Neurooncology, University of Münster, Münster, Germany.

²Department of Neurology, University of Würzburg, Würzburg, Germany.

³Department of Neuropathology, University of Göttingen, Göttingen, Germany.

⁴Divisions of Immunology and Allergy and of Neurology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.

⁵Department of Neurology, Ruhr University Bochum, Bochum, Germany.

⁶Neuroinflammation Research Center, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio.

⁷Institute of Physiology - Neuropathophysiology, University of Münster, Münster, Germany.

*these authors contributed equally; and

current address: Department of Neurology, University of Magdeburg, Magdeburg, Germany.

Corresponding author:

Prof. Heinz Wiendl, Department of Neurology – Inflammatory Disorders of the Nervous System and Neurooncology, University of Münster, Domagkstraße 13, 48149 Münster, Germany.
Email: heinz.wiendl@ukmuenster.de

elevation after 2 years of treatment (from an initial 1:1000 up to 1:100). However, it is still unclear how natalizumab interferes with JC virus control. Furthermore, few biomarkers are available to identify patients at risk for developing PML (e.g. the presence of anti-JCV antibodies in the serum of patients).⁷

We here provide serial immunological assessment of a patient on natalizumab, who developed PML 29 months after initiation of therapy. Immunological features were monitored in parallel to clinical, imaging, routine diagnostic and cerebrospinal fluid (CSF) measures. Our study might give some insight into the mechanisms of homeostatic JC virus control, its failure during natalizumab treatment and changes after plasma exchange (PE) and immune reconstitution inflammatory syndrome (IRIS). The findings in this patient might aid the search for biomarkers identifying patients at risk for PML development under natalizumab therapy.

Methods

Histological stainings

A formalin-fixed and paraffin-embedded brain biopsy was studied. Slices (4 µm) were stained with hematoxylin and eosin (H&E), Luxol-fast blue/periodic acid-Schiff (LFB/PAS) and Bielschowsky's silver impregnation. Immunohistochemical staining was performed with a biotin avidin technique. The following primary antibodies were used: anti-SV40 (JC virus, kindly provided by Professor Lüke, Deutsches Primatenzentrum Göttingen, Germany), PAb 2003 (JC virus, kindly provided by Dr Frisque, Pennsylvania State University, USA), anti-CD3 (T cells, Serotec, UK), anti-CD8 (cytotoxic T cells, Dako, Denmark), anti-CD20 (B cells, Dako, Denmark), anti-CD138 (plasma cells, Dako, Denmark), anti-myelin basic protein (anti-MBP, Boehringer Mannheim, Mannheim, Germany), anti-proteolipid protein (anti-PLP, Biozol, Eching, Germany), anti-myelin oligodendrocyte glycoprotein (anti-MOG, clone 8-18-C5, kindly provided by Professor Linington, University of Aberdeen, UK), anti-myelin-associated glycoprotein (anti-MAG, kindly provided by Professor Schwab, University of Zürich, Switzerland), anti-cyclic nucleotide phosphodiesterase (anti-CNPase, Sternberger, Maryland, USA), KiM1P (macrophages, kindly provided by Professor Radzun, University of Göttingen, Germany) and anti-MRP 14 (activated macrophages, BMA Biomedicals, Augst, Switzerland). DNA-DNA in situ hybridization with a JCV probe was performed by Dr König, University of Gießen, Germany.

Immune cell purification and isolation

Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Linz, Austria) within 24 h after blood draw. CD4+ or CD8+ T cells were

negatively isolated from PBMC using magnetic bead isolation (negative isolation procedure, MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. Purified T-cell subsets were over 95% pure as controlled by flow cytometry analysis.

Preparation of cells from CSF samples

CSF (8–10 ml) was obtained by lumbar puncture and processed as previously described.⁸

Reagents for flow cytometry

The following mAbs were used: CD4-FITC/-PE, CD8-FITC/-PE, CD14-APC, CD19-PE-Cy5, CD25-PE, CD45RA-FITC, CD49d-APC, CCR5-PE-Cy5, CCR7-FITC/-APC (all BD, Heidelberg, Germany). Staining was performed as previously described.⁹ Cells were analyzed with FACS-Calibur™ using Cell Quest™ (Becton Dickinson, Heidelberg, Germany) and FlowJo (Tristar, USA) software.

Human brain microvascular endothelial cell cultures and transmigration assay

Human brain microvascular endothelial cells (HBMEC) were purchased from ScienCell Research Laboratories (San Diego, CA, USA). Cells were cultured on filter membranes of Transwells® (3 µm pore size; Corning, NY, USA) until reaching confluence. Transmigration assays were performed as described previously.^{8,10,11} Briefly, HBMEC were cultured on the apical side of a filter membrane of Transwells® until confluence. In total, 2.5×10^5 of purified T cells in 0.1 ml of pre-warmed RPMI medium were added to the top of the HBMEC monolayers and 0.6 ml of medium was added to the outer chamber of the Transwells®. The cells were allowed to migrate for 18 h in a humidified cell culture incubator at 37°C and 5% CO₂.

RNA isolation and cDNA synthesis

RNA from blood and CSF immune cells was isolated with TriZol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

cDNA synthesis from peripheral blood T-cell RNA was performed using standard methods as previously described.¹² Reagents were obtained from Applied Biosystems (Foster City, CA, USA). For each sample, 250 ng of RNA was transcribed, using random hexamers and M-MLV reverse transcriptase.

For RNA isolated from CSF samples, a previously published sensitive TCR β-chain-specific cDNA primer, Cβ-RT was used,¹³ as well as SuperScriptIII™ reverse transcriptase (Invitrogen), according to the manufacturer's instructions.

T-cell receptor repertoire analysis by CDR3 spectratyping

Spectratyping was performed as described previously.¹⁴

This study has been approved by the local ethics committee of Würzburg and the patient has given written consent to participate in this study.

Results

Clinical case report

We report an HLA-A2+ 48-year-old, right-handed man with relapsing-remitting MS (RRMS), who received intravenous natalizumab therapy (300 mg every 4 weeks) over 29 months (initiated July 2007) because of active disease despite interferon- β treatment, under which he developed three clinical relapses, a progression on the Expanded Disability Status Scale from 2.0 to 4.5 and gadolinium-enhancing lesions on magnetic resonance imaging (MRI). Previous medical history includes mitoxantrone (October 2003–2005; overall: 85 mg/m²) as well as interferon- β treatment (Betaferon®, 1999–June 2003; Rebif®, January 2006–July 2007). No history of malignancies or signs of immunodeficiency were noted.

Under natalizumab treatment the patient showed a stable disease course over 2 years. One relapse occurred in May 2009, 24 months after treatment initiation. Corticosteroid treatment enabled complete recovery to be achieved, and natalizumab therapy was continued at this point. Some 5 months later the patient visited our center with new, multifocal neurological symptoms (sensory disturbances, numbness in hands, walking disturbances, pure alexia without agraphia). MRI scans showed a new occipital lesion, initially interpreted as a new MS lesion (Figure 1, 2 October 2009). Corticosteroids were initiated and led to an amelioration of the sensory symptoms. On a consecutive scan (4 days after the initial scan), typical signs of PML, including edema, demyelination and a rim zone of the lesion in the occipital cortex was noted (Figure 1); CSF assessment was performed in parallel and confirmed the diagnosis of PML by detecting low copy numbers of JC virus (8 copies/ml). The patient received PE of 30 ml/kg body weight (2.5 l) combined with cycles of immunoadsorption with a tryptophan column (overall: five cycles) in an effort to remove natalizumab.^{15,16} In addition we started treatment with mefloquine (250 mg once per week) and mirtazapine (30 mg per day), hypothesized to impede JC virus replication. Afterwards intravenous immunoglobulins (IVIg) were administered (five times, 6 g absolute), the corollary being that it might downregulate a potentially fulminant IRIS, although there is currently no direct evidence supporting this treatment. The patient showed a stable disease course with minor fluctuations of pure alexia without agraphia; other symptoms (sensory symptoms, walking disturbances) returned to baseline. No clinical, imaging or CSF

diagnostic signs of IRIS were notable. CSF was assessed several times (Table 1), but no cell elevations possibly indicating IRIS development were visible. Of note, JC virus was always detectable, but at very low copy numbers (assessments in three different labs, Virology, University of Würzburg; Virology, University of Düsseldorf; NIH reference lab, E. Major). In parallel, serial MRI scans showed stability of the occipital lesion over approximately 2 months (Figure 1). The patient was readmitted to the hospital 3 months after initial PML diagnosis for diagnostic and therapeutic reassessment. While the patient was clinically stable without any new neurological symptoms apart from residual pure alexia without agraphia, his MRI showed development of a massive progressive occipital lesion. It increased in signal intensity and size considerably within several days. We could not detect any gadolinium uptake, or any measurable clinical reflection of the lesion. JC virus copies were still detectable (6 copies/ml), and CSF cell count at that time point was very low (<1 cell/ μ l). To distinguish between reemerging PML with progressive lesion development, a rebound of MS after natalizumab withdrawal, or the development of a late IRIS, undetectable by CSF analysis (more than 8 weeks after the last PE), we decided to perform a diagnostic brain biopsy. In parallel we added cidofovir to the previous treatment with mirtazapine, mefloquine and corticosteroids. Histopathological assessment revealed an active demyelinating lesion and surrounding periplaque white matter, both heavily infiltrated by predominantly CD8+ T cells (see Figure 5). The latter finding in particular of the diffuse and extensive inflammation of the normal-appearing white matter by CD8+ T cells helped to diagnose an IRIS. JC virus reactivity was not detected. Within several weeks, during which the patient was feeling well, the occipital lesion started to resolve. Subsequently the patient was put on glatiramer acetate therapy in May 2010, which he has been on since then.

Serial analysis of immunological features during natalizumab treatment, PML, PE and development of IRIS – phenotype and dynamics of major immune cell subsets

We had the opportunity to analyze different immunological features of this patient over nearly 3 years (27 August 2007–15 June 2010). As we have followed this individual for a large part of his natalizumab therapy and then later through the PML and IRIS, we were able to assess the immune status pre-PML, at PML and post-PML. The patient's distribution of the major leukocyte subsets (CD4, CD8, CD19, CD14, Figure 2A) was assessed over various time points. Only 1 month after initiation of treatment, the patient's distribution of CD4 and CD8 already differed from that of healthy donors (HD). In addition, he had very low peripheral B-cell levels at this point. Interestingly, during the first 12 months of natalizumab treatment the CD4/

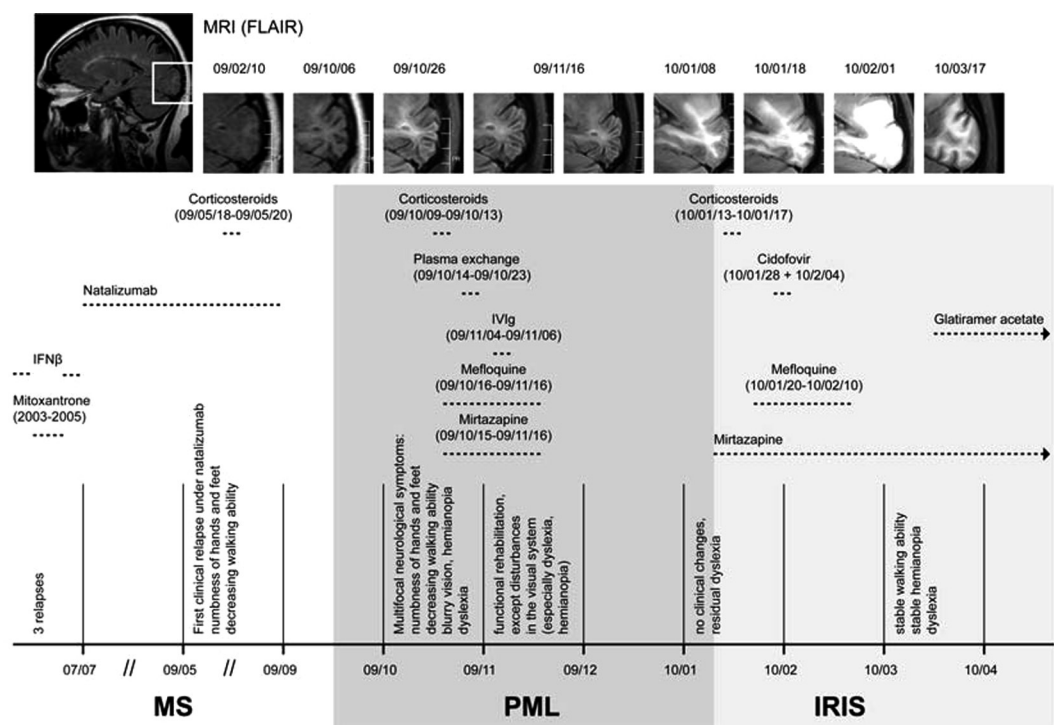


Figure 1. Overview of the clinical hallmarks, medication, and the magnetic resonance imaging scans over the course of the treatment with indication of the three phases MS/PML/IRIS. On a common time axis magnetic resonance images (upper part), therapies (middle part) and the neurological assessment (lower part) are shown. The three consecutive episodes are highlighted differently, referring to the time of natalizumab treatment (white), the time of progressive multifocal leukoencephalopathy (PML) (dark grey) and finally the subsequent immune reconstitution inflammatory syndrome (IRIS) (light grey).

Table 1. Cerebrospinal fluid clinical routine workup. Given are dates of cerebrospinal fluid draw, cell counts, amount of protein and virus load (assessed by NIH).

CSF taken	Cell count [x/μl]	Proteins [mg/dl]	Virus load NIH [copies/ml]
16 July 2007	<1	53.3	Not analyzed
9 October 2009	1	36.7	8
2 November 2009	<1	33.8	11
13 November 2009	1	33.9	Not analyzed
08 January 2010	2	41.5	26
18 March 2010	<1	35.8	6
14 June 2010	6*	87.5	Negative

*bloody puncture

CD8 ratio fell (from 0.86 to 0.48 during that time), and B-cell and monocyte counts were extremely low, but remained stable. During PML and IRIS, the distribution of these major peripheral subsets did not change significantly, the only exception being the comparatively low amount of T cells at the beginning of PML/time point of PML diagnosis (CD4: 12%, CD8: 38% of lymphocytes). After halting natalizumab treatment and during PE, levels of B cells continued to rise, eventually reaching levels of HD (20% on 15 June 2010).

CD4 and CD8 T cells were further divided into major subsets by their expression of CCR7, CD25 and CD45 (CD25+, naïve, central memory, effector memory T cells;

Figure 2B, C). The distribution of the CD4+ T-cell compartment changed during the first 12 months of therapy (effector cells from 60% to 75%, central memory cells from 27% to 12%, naïve cells from 8% to 5%). During PML, the peripheral distribution clearly shifted from CD4 effector cells to central memory cells, with a maximum of central memory cells (39%) before IRIS was diagnosed (18 November 2009). During IRIS, the proportions of the peripheral CD4 compartments returned to the state recorded before PML. The CD25+ CD4 T cells percentages before and after PML were stable, but lower during PML.

The CD8 compartment (Figure 2C) presented unusually from the start (e.g. only 4% naïve cells at baseline), but

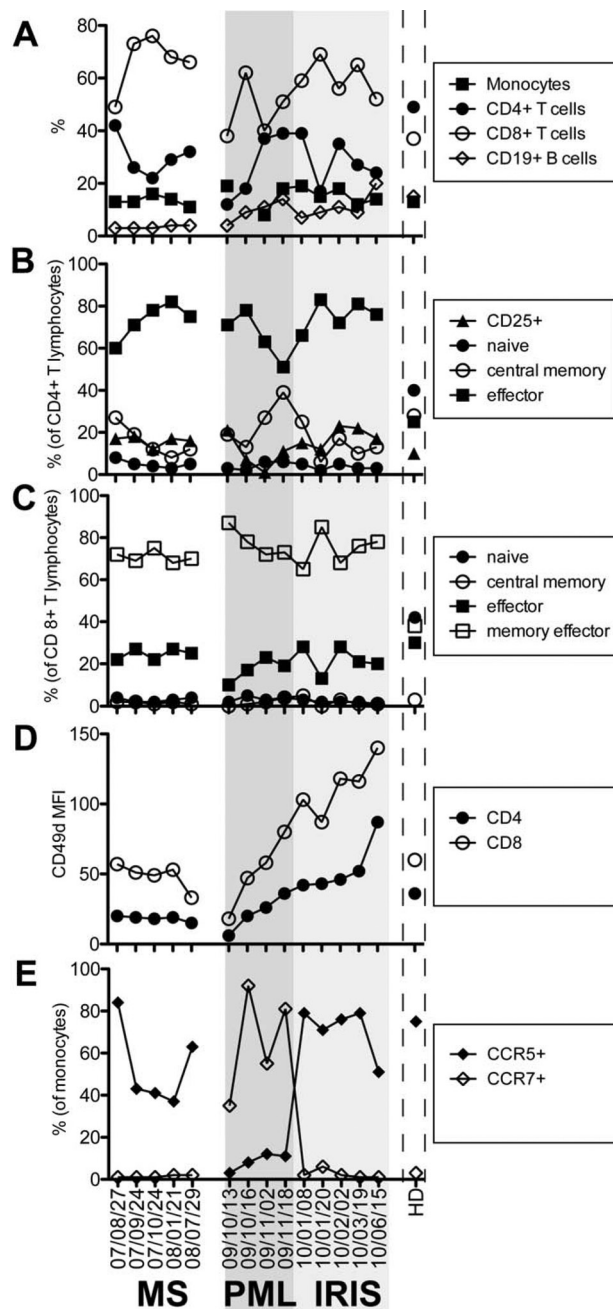


Figure 2. Development of immune subpopulations in the course of the natalizumab treatment, PML, and IRIS.

A: Distribution of monocytes (as % of leukocytes), CD4+ and CD8+ T cells, and B cells (as % of lymphocytes).

B, C: Memory–effector compartments of CD4+ T lymphocytes (B) and CD8+ T lymphocytes (C), activation status as measured by CD25 expression.

D: Expression of CD49d on CD4+ and CD8+ T cells.

E: Distribution of monocyte subpopulations (CCR5+ / CCR7+) over time.

The three phases are highlighted in white (multiple sclerosis, MS), dark grey (progressive multifocal leukoencephalopathy, PML) and light grey (immune reconstitution inflammatory syndrome, IRIS). An average of three healthy controls is shown to the right

showed more stability over the course of the natalizumab treatment. Upon onset of PML, however, the distribution had shifted from effector to memory effector cells (10% and 87%, respectively), which normalized after the PE and stayed stable afterwards.

We also studied expression of CD49d on T-cell subsets longitudinally (Figure 2D). CD49d expression is higher on CD8 than on CD4 T cells.¹⁷ During the first year of natalizumab therapy a slight downregulation of this surface marker was measurable. CD8+ T cells showed this more prominently than CD4 T cells (CD8: mean fluorescence index (MFI): 57 to 33; CD4+ T cells MFI: 20 to 15). Notably, upon onset of PML, both subsets showed an extremely low expression of CD49d (CD4: MFI 4, CD8: MFI 18). After PE, CD49d expression quickly recovered, indicating removal of natalizumab from the circulation. Interestingly, T cells continued to increase their CD49d expression, significantly exceeding levels at 1 month of treatment or levels of healthy controls. These results indicate that CD49d surface expression in this patient was extremely suppressed under natalizumab therapy. Recovery started soon after PE, but continued over several months (observation period 7 months after PML diagnosis) clearly showing a ‘rebound’ of $\alpha 4$ integrin expression.

We further assessed major subsets of monocytes, characterized by their cell-surface expression of CCR5 and CCR7. CCR7+ monocytes have been shown to be involved in the development of lymph node metastases in patients with lung cancer,¹⁸ esophageal cancer,¹⁹ and gastric cancer,²⁰ whereas CCR5 is upregulated in tissue-resident monocytes.²¹ At the beginning of natalizumab therapy, virtually no CCR7+ monocytes were detectable in peripheral blood, a finding consistent with the observations in healthy adults (own unpublished observations). We observed that onset of PML coincided with the appearance of CCR7+ monocytes in peripheral blood, while the number of CCR5+ monocytes decreased significantly (Figure 2E). The levels of CCR7+ and CCR5+ monocytes remained stable. Interestingly, the disappearance of peripheral CCR7+ monocytes and reappearance of CCR5+ in peripheral blood coincided with the onset of IRIS, which was demonstrated by MRI and later brain biopsy.

Migration of peripheral lymphocytes

Migratory capacity of lymphocytes is a prerequisite for immune reactions. To assess the ability of immune cells to transmigrate across the blood–brain barrier, we here used an *in vitro* system quantifying the transmigration of CD4 as well as CD8 T cells through human brain endothelial cells.^{8,17} The migratory capacity of CD4+ T cells was very low at the time of PML diagnosis (13 October 2009) with a mean of 134.5 ± 7.5 cells passing the endothelial barrier

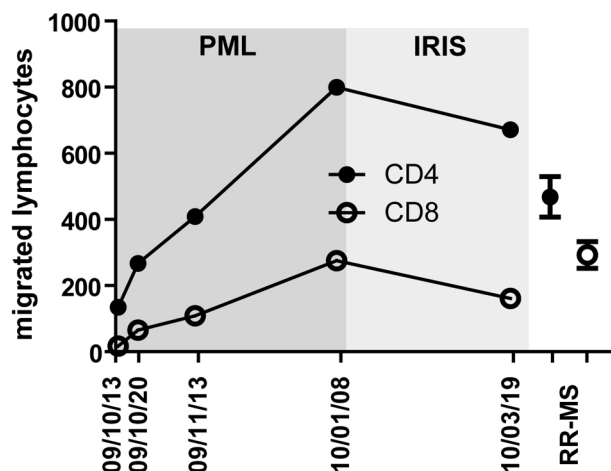


Figure 3. Migration of T cells during natalizumab therapy and at the time of PML diagnosis is severely inhibited, but recovers after plasma exchange.

(mean (untreated, stable RRMS patients): 468.2 ± 61.2 $n = 8$) (Figure 3). Moreover, the patient's CD8+ lymphocytes had a severely reduced capability to migrate, as there were very few cells detectable after having passed the layer within the 18-h time frame (17 ± 8 cells; mean (untreated, stable RRMS patients): 292.8 ± 40.5 $n = 8$), indicating a severe failure especially of CD8 migratory capacity. Recovery of the migratory capacity started soon after initiation of PE and continuously progressed. Maximum levels were reached approximately 2 months after PML diagnosis and cessation of natalizumab (CD4+: 799.5 ± 43.5 ; CD8+: 275 ± 23). Of note, the increase of migratory capacity correlated with the recovery of CD49d expression, indicating a close link between these two factors (Figure 2D and Figure 3).

Analysis of the T-cell receptor repertoire by CDR3 spectratyping

The peripheral T-cell receptor (TCR) repertoire of CD4 T cells before PML diagnosis was comparable with other MS patients or healthy adults. CD8 T cells showed a lower complexity score than HD or MS patients. At the stage of PML diagnosis, diversity of the TCR repertoire was very low in both populations, indicating a strong restriction of the TCR repertoire. However, this is probably not attributable to JCV-specific T cells, as the first detectable JCV-specific CD8+ T-cell response, as assessed by HLA-A*0201/JCV VP1_{p36} tetramer staining, became positive during IRIS (data not shown). Nevertheless, it cannot be ruled out that, at the time of PML diagnosis, CD8+ T cells reacted against JCV epitopes other than VP1_{p36}. Immediately after PE TCR complexity reached its minimum, possibly reflecting the systemic recovery of the patient's immune response and the generation of effector responses in the periphery. Thereafter, peripheral TCR diversity recovered within 2 weeks. Notably, while peripheral CD4 T cells reached nearly

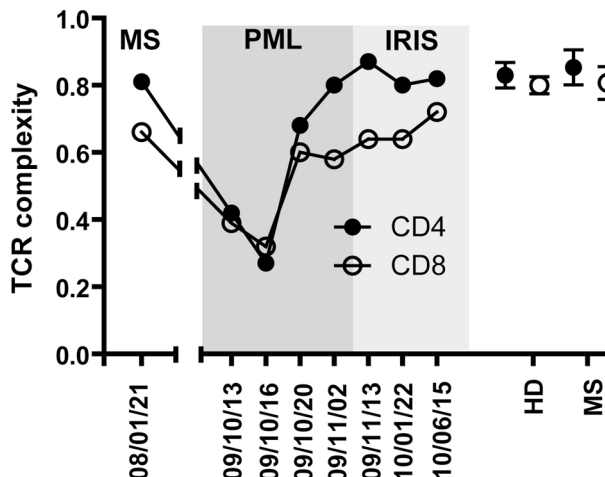


Figure 4. CDR3 spectratyping of blood-derived, magnetically isolated CD4+ and CD8+ T cells shows a slightly lower complexity during therapy, which is further reduced severely during PML, but recovers after plasma exchange. Complexity of the CD4 and CD8 T-cell receptor repertoire with a complexity ranging from 0 (no peak) to 1 (12 peaks/analyzed Vβ).

normal TCR complexity scores within approximately 4 weeks, it took 8 months for CD8 T-cell complexity scores to recover (15 June 2010) (Figure 4).

Dominant monoclonal expansions were only found in the CD8 compartment, while the CD4 TCR repertoire showed mild perturbations within a Gaussian distribution at any of the examined time points.

Histopathological examination of the biopsy brain specimen

A stereotactical brain biopsy was performed, because of the progressive lesion development observed on MRI, which could not differentiate between PML, IRIS or fast-developing MS lesions (no contrast enhancement). Conventional H&E (Figure 5A) as well as LFB/PAS (Figure 5B) stainings revealed two different areas: (1) a highly cellular active demyelinating lesion, and (2) surrounding non-demyelinated white matter. The lesion showed a sharp border to the adjacent white matter in which myelin was completely preserved. Both areas were characterized by a diffuse as well as perivascularly accentuated mononuclear inflammatory infiltrate. The demyelinating lesion in particular was diffusely infiltrated by cells of the macrophage/microglia lineage. The pronounced lymphocyte inflammation was evident in demyelinating areas as well as in the non-demyelinated white matter (Figure 5C). It was dominated by T cells (Figure 5C), which were predominantly CD8-positive T cells (Figure 5D). However, perivascular B cells and high numbers of parenchymal plasma cells were also found (Figure 5E and 5F). A reactive astrogliosis with astrocytes showing a bizarre morphology was evident. Oligodendrocytes appeared

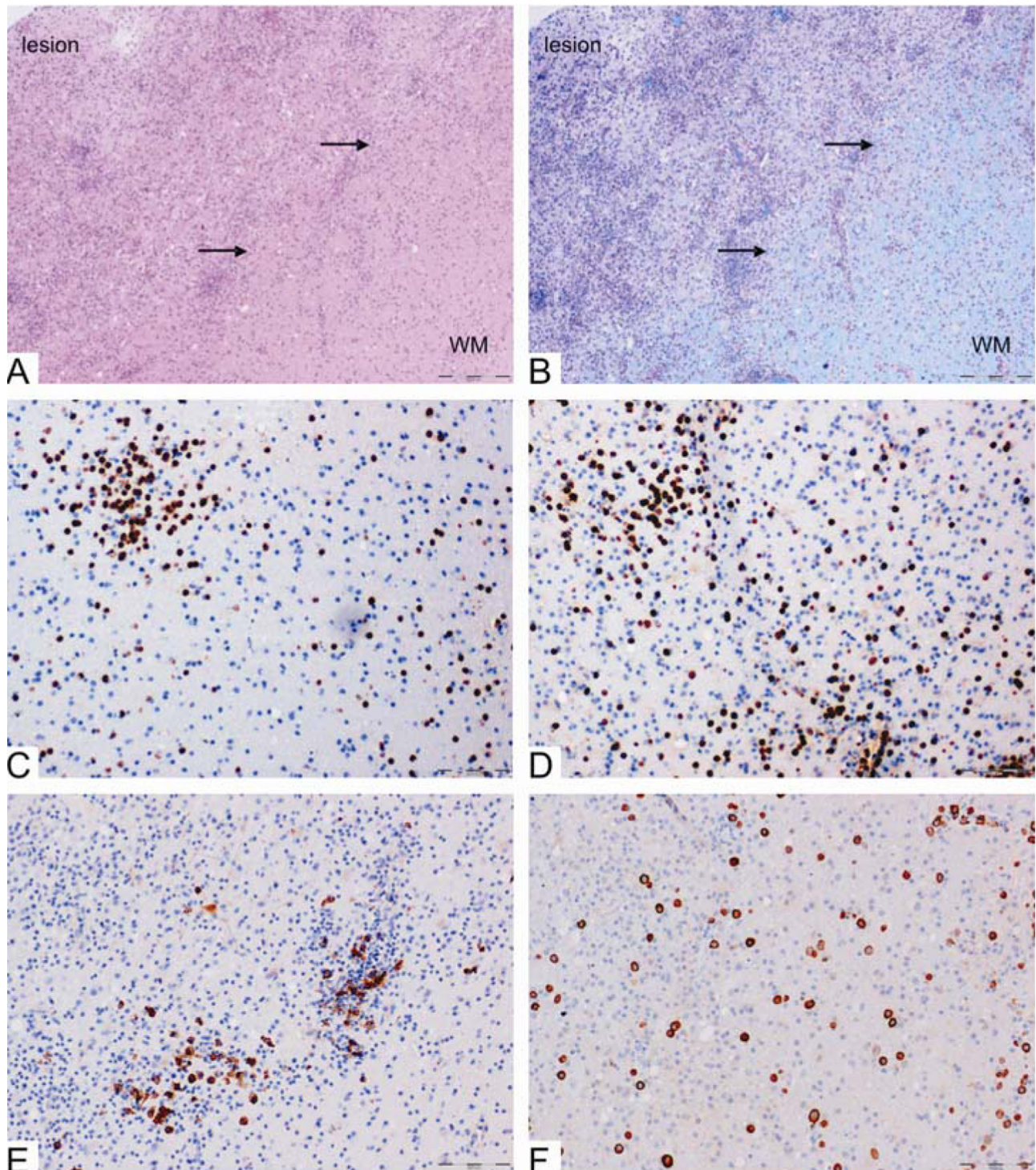


Figure 5. Inflammatory demyelinating lesion and adjacent white matter with pronounced, cytotoxic T-cell-dominated inflammation. A: H&E staining shows strong infiltration of inflammatory cells in central nervous system tissue. B: Myelin staining with LFB/PAS recognizes a sharply demarcated demyelinated lesion as well as adjacent white matter. Arrows in A and B indicate edge of demyelinated lesion. C, D: Within the non-demyelinated white matter a pronounced T-cell infiltrate is found (C, CD3), which consists almost exclusively of cytotoxic T cells (D, CD8). E, F: High numbers of B cells and plasma cells are also evident (E, CD20 and F, CD138). OM: A, B $\times 40$, C–F $\times 100$. WM = white matter

activated, but no PML-characteristic nuclear changes were seen. Immunohistochemistry for SV40 viral antigens and JCV proteins as well as an *in situ* hybridization for JCV DNA were negative (not shown).

Discussion

Our report follows and monitors the clinical and immunological course of a patient treated with natalizumab who developed PML, recovered after PE and developed a very delayed and mild, clinically undetectable IRIS. We try to retrace how natalizumab could induce JCV-associated PML and how cessation of natalizumab or removal by PE recovers immune functions. Further, we analyzed the immunological as well as histopathological aspects of IRIS, which in this case was asymptomatic and undetectable by CSF analysis and occurred 3 months after PE.

Changes associated with the onset of PML under natalizumab treatment included severe reduction of CD49d levels, loss of migratory capacity of T cells (more pronounced for CD8 than for CD4 T cells), severe perturbations of the peripheral TCR repertoire and the appearance of peripheral CCR7⁺ monocytes. A clear distinction between changes that regularly occur under treatment with natalizumab²² and those that are clearly associated with an elevated risk for PML seems difficult. Our data suggest that the suppression of CD49d and the reduction of migratory capacity of CD4 and, even more severe, of CD8 T cells could represent aspects that interfere with JC virus control in the CNS and thus might be linked to PML development, followed by the strong and prolonged rebound after cessation of natalizumab. Accordingly, along with the recovery of CD49d levels after PE, migratory function recovered.

Albeit our case report reveals some insights into the mechanisms associated with the loss of JC virus control, some caveats have to be considered before any general conclusions can be made. First, although this case report monitors longitudinal changes under therapy with natalizumab, it should be noted with caution that our patient already had phenotypical irregularities in his immune repertoire from the first month of natalizumab therapy onwards. Despite a history of mitoxantrone treatment (3 years prior to the initiation of natalizumab therapy), which is assumed to already increase the risk for PML development, there was however no history of any immune compromise. Parameters that were abnormal and could be associated with an increased risk of PML were the following: even at month 1 under therapy, our patient showed a reversed CD4/CD8 ratio, very low peripheral B-cell counts and almost no peripheral naïve T cells. In the course of the first year of therapy, these abnormalities intensified. Further, expression of CD49d decreased on all T cells and the first disturbances in the CD8 TCR repertoire could be noted. At the beginning of PML (15 months after the last

measurement) CD49d expression was nearly undetectable and CD8 T cells had lost their capacity to transmigrate the blood–brain barrier.

JCV-specific CD8 T cells are critical for the control of JC virus: JCV-specific CD8⁺ T cells have been shown to correlate with survival during PML.^{23,24} Therefore, inhibiting the migration of these cells into the CNS could be a prerequisite for its development. As CD49d is important for a functioning immune response and CNS immune surveillance,²⁵ this effect of natalizumab is disconcerting, especially when the recovery time after PE in our case was followed by a rebound effect that boosted CD49d levels to unusual heights over the course of more than 8 months.

A second interesting part of our report is the first histopathological evidence for a natalizumab-associated IRIS without obvious clinical signs. Histopathological data about lesion development are available from HIV patients with PML, who are at risk (5%) of developing IRIS after initiation of antiretroviral therapy.^{26–28} However, no detailed histopathological analysis of an IRIS syndrome after natalizumab therapy has been reported until now. Histopathology shows a dense lymphoplasmacytic infiltrate in the demyelinating lesion and the surrounding non-demyelinated white matter, which is dominated by CD8⁺ T cells. The latter finding in particular, of the severe and diffuse CD8 T cell-dominated inflammation in the normal-appearing, non-lesional white matter, makes a rebound MS lesion after stopping natalizumab and PE as well as immunoabsorption unlikely. Thus, the typical IRIS histopathology is shown here. We could not detect JC virus in the brain biopsy, but demyelinating lesions without viral detection in HIV patients with IRIS after PML have also been described.²⁸ PML shows oligodendrocytes with nuclear inclusions at the lesion edge (so-called ground glass oligodendrocytes). The oligodendrocytes are lost within the lesions due to lytic viral infection. However, in our tissue oligodendrocytes are preserved within the lesion, as known from MS patients, and ground glass oligodendrocytes are not found. Bizarre astrocytes – present in our biopsy – are typical for PML lesions,²⁹ but are also known from acute MS lesions as so-called Creutzfeldt-Peters cells.³⁰ Strikingly, our patient did not show any clinical deterioration despite typical IRIS pathology. This is paralleled by the description of a clinically stable HIV patient with PML and a marked lymphoplasmacytic infiltration in biopsy tissue after initiation of antiretroviral therapy.²⁷

Our report reveals immunological changes following long-term immunotherapy with natalizumab. Despite the pressing need for biomarkers indicating a risk for PML under this therapy, thus far only few candidates have evolved (for example, Gorelik et al.⁷). Ideally, the immune functions of potential patients would be tested before and under therapy. According to our data, patients with an overly strong reduction of their CD49d-dependent T-cell

CNS migration system could represent a subgroup at increased risk for a loss of CNS immune surveillance. The validation of these risk candidates needs further studies in larger cohorts of prospectively followed patients under long-term natalizumab therapy.

Funding

This study was funded by the German ministry of education and research (BMBF), and performed within the German competence network of MS (KKNMS), project "pharmacovigilance Study natalizumab", to HW and RG.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

The authors would like to thank Theresa Moritz and Andrea Staudigel for excellent technical assistance.

References

- Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L and Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* 1992; 356: 63–66.
- Rudick RA, Stuart WH, Calabresi PA, Confavreux C, Galetta SL, Radue E-W, et al. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. *N Engl J Med* 2006; 354: 911–923.
- Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2006; 354: 899–910.
- Wenning W, Haghighi A, Laubenberger J, Clifford DB, Behrens PF, Chan A, et al. Treatment of progressive multifocal leukoencephalopathy associated with natalizumab. *N Engl J Med* 2009; 361: 1075–1080.
- Ransohoff RM. Natalizumab and PML. *Nat Neurosci* 2005; 8: 1275.
- Koralnik IJ. Progressive multifocal leukoencephalopathy revisited: Has the disease outgrown its name? *Ann Neurol* 2006; 60: 162–173.
- Gorelik L, Lerner M, Bixler S, Crossman M, Schlain B, Simon K, et al. Anti-JC virus antibodies: implications for PML risk stratification. *Ann Neurol* 2010; 68: 295–303.
- Huang Y-H, Zozulya AL, Weidenfeller C, Metz I, Buck D, Toyka KV, et al. Specific central nervous system recruitment of HLA-G(+) regulatory T cells in multiple sclerosis. *Ann Neurol* 2009; 66: 171–183.
- Schwab N, Zozulya AL, Kieseier BC, Toyka KV and Wiendl H. An imbalance of two functionally and phenotypically different subsets of plasmacytoid dendritic cells characterizes the dysfunctional immune regulation in multiple sclerosis. *J Immunol* 2010; 184: 5368–5374.
- Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol* 2005; 6: 889–894.
- Lee BP and Imhof BA. Lymphocyte transmigration in the brain: a new way of thinking. *Nat Immunol* 2008; 9: 117–118.
- Wiendl H, Malotka J, Holzwarth B, Weltzien HU, Wekerle H, Hohlfeld R, et al. An autoreactive gamma delta TCR derived from a polymyositis lesion. *J Immunol* 2002; 169: 515–521.
- Seitz S, Schneider CK, Malotka J, Nong X, Engel AG, Wekerle H, et al. Reconstitution of paired T cell receptor alpha- and beta-chains from microdissected single cells of human inflammatory tissues. *Proc Natl Acad Sci U S A* 2006; 103: 12057–12062.
- Schwab N, Bien CG, Waschbisch A, Becker A, Vince GH, Dornmair K, et al. CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. *Brain* 2009; 132: 1236–1246.
- Khatri BO, Man S, Giovannoni G, Koo AP, Lee JC, Tucky B, et al. Effect of plasma exchange in accelerating natalizumab clearance and restoring leukocyte function. *Neurology* 2009; 72: 402–409.
- Schröder A, Lee D-H, Hellwig K, Lukas C, Linker RA and Gold R. Successful management of natalizumab-associated progressive multifocal leukoencephalopathy and immune reconstitution syndrome in a patient with multiple sclerosis. *Arch Neurol* 2010; 67: 1391–1394.
- Stenner MP, Waschbisch A, Buck D, Doerck S, Einsele H, Toyka KV, et al. Effects of natalizumab treatment on Foxp3+ T regulatory cells. *PLoS ONE* 2008; 3: e3319.
- Takanami I. Overexpression of CCR7 mRNA in nonsmall cell lung cancer: correlation with lymph node metastasis. *Int J Cancer* 2003; 105: 186–189.
- Ding Y, Shimada Y, Maeda M, Kawabe A, Kaganai J, Komoto I, et al. Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clin Cancer Res* 2003; 9: 3406–3412.
- Mashino K, Sadanaga N, Yamaguchi H, Tanaka F, Ohta M, Shibuta K, et al. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res* 2002; 62: 2937–2941.
- Ubogu EE, Callahan MK, Tucky BH and Ransohoff RM. CCR5 expression on monocytes and T cells: modulation by transmigration across the blood-brain barrier in vitro. *Cell Immunol* 2006; 243: 19–29.
- Niino M, Bodner C, Simard ML, Alatab S, Gano D, Kim HJ, et al. Natalizumab effects on immune cell responses in multiple sclerosis. *Ann Neurol* 2006; 59: 748–754.
- Du Pasquier RA, Clark KW, Smith PS, Joseph JT, Mazullo JM, De Girolami U, et al. JCV-specific cellular immune response correlates with a favorable clinical outcome in HIV-infected individuals with progressive multifocal leukoencephalopathy. *J Neurovirol* 2001; 7: 318–322.
- Du Pasquier RA, Kuroda MJ, Zheng Y, Jean-Jacques J, Letvin NL and Koralnik IJ. A prospective study demonstrates an association between JC virus-specific cytotoxic T lymphocytes and the early control of progressive multifocal leukoencephalopathy. *Brain* 2004; 127: 1970–1978.
- Rose DM, Han J and Ginsberg MH. Alpha4 integrins and the immune response. *Immunol Rev* 2002; 186: 118–124.
- Gray F, Bazille C, Adle-Biasset H, Mikol J, Moulignier A and Scaravilli F. Central nervous system immune

- reconstitution disease in acquired immunodeficiency syndrome patients receiving highly active antiretroviral treatment. *J Neurovirol* 2005; 11(Suppl 3): 16–22.
27. Miralles P, Berenguer J, Lacruz C, Cosín J, López JC, Padilla B, et al. Inflammatory reactions in progressive multifocal leukoencephalopathy after highly active antiretroviral therapy. *AIDS* 2001; 15: 1900–1902.
28. Vendrely A, Bienvenu B, Gasnault J, Thiebault JB, Salmon D and Gray F. Fulminant inflammatory leukoencephalopathy associated with HAART-induced immune restoration in AIDS-related progressive multifocal leukoencephalopathy. *Acta Neuropathol* 2005; 109: 449–455.
29. Richardson-Burns SM, Kleinschmidt-DeMasters BK, DeBiasi RL and Tyler KL. Progressive multifocal leukoencephalopathy and apoptosis of infected oligodendrocytes in the central nervous system of patients with and without AIDS. *Arch Neurol* 2002; 59: 1930–1936.
30. Kuhlmann T, Lassmann H and Brück W. Diagnosis of inflammatory demyelination in biopsy specimens: a practical approach. *Acta Neuropathol* 2008; 115: 275–287.