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Central role of JC virus-specific CD4$^+$ lymphocytes in progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome

Lilian Aly,[1,*] Sara Yousef,[1,*] Sven Schippling,[1] Ilijas Jelcic,[1,2] Petra Breiden,[1] Jakob Matschke,[3] Robert Schulz,[4] Silvia Bofill-Mas,[5] Louise Jones,[6] Viktorya Demina,[7] Michael Linnebank,[2] Graham Ogg,[6] Rosina Girones,[4] Thomas Weber,[8] Mireia Sospedra[1,†] and Roland Martin[1,2,†]

1 Institute for Neuroimmunology and Clinical Multiple Sclerosis Research (inims), Centre for Molecular Neurobiology (ZMNH), University Medical Centre Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg, Germany
2 Department of Clinical Neurology and Multiple Sclerosis Research, Neurology Clinic, University Hospital Zürich, University of Zürich, Frauenklinikstrasse 26, 8091 Zürich, Switzerland
3 Institute of Neuropathology, University Medical Centre Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
4 Department of Neurology, University Medical Centre Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
5 Department of Microbiology, Faculty of Biology, University of Barcelona, Diagonal, 645, Barcelona, Spain
6 Weatherall Institute of Molecular Medicine, MRC Human Immunology Unit, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DS, UK
7 Life Science Inkubator, Ludwig-Erhard-Allee 2, 53175 Bonn, Germany
8 Department of Neurology, Marienkrankenhaus, Alfredstrasse 9, 22087 Hamburg, Germany

*These authors contributed equally to this work.
†These authors contributed equally to this work.

Correspondence to: Roland Martin, MD,
Department of Clinical Neuroimmunology and Multiple Sclerosis Research,
Neurology Clinic, University Hospital Zürich, University of Zürich,
Frauenklinikstrasse 26, 8091 Zürich,
Switzerland
E-mail: roland.martin@usz.ch

Progressive multi-focal leucoencephalopathy and progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome are caused by infection of the central nervous system with the JC polyoma virus. Both are complications of monoclonal antibody therapy in multiple sclerosis and other autoimmune diseases. Progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome can obscure the diagnosis of progressive multi-focal leucoencephalopathy and lead to severe clinical disability and possibly death. Different from progressive multi-focal leucoencephalopathy, in which demyelination results from oligodendrocyte lysis by JC virus in the absence of an immune response, tissue destruction in progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome is caused by a vigorous immune response within the brain. The cells and mediators that are involved in progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome are as yet poorly understood. We examined two patients with multiple sclerosis, who developed progressive multi-focal leucoencephalopathy and later progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome under natalizumab therapy. Due to initially negative JC viral deoxyribonucleic acid testing in the cerebrospinal fluid, a diagnostic brain biopsy was performed in one patient. Histopathology revealed brain inflammation characterized by a prominent T cell infiltrate (CD4$^+$ > CD8$^+$ T cells), but also B/plasma cells and monocytes. Despite very low

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JC viral load, both patients showed high intrathecal anti-JC virus antibodies. Brain-infiltrating CD4$^+$ T cells were studied regarding antigen specificity and function. CD4$^+$ T cells were highly specific for peptides from several JC virus proteins, particularly the major capsid protein VP1. T cell phenotyping revealed CD4$^+$ Th1 and bifunctional Th1-2 cells. The latter secrete large amounts of interferon-γ and interleukin-4 explaining the strong brain inflammation, presence of plasma cells and secretion of intrathecal anti-VP1 antibodies. The functional phenotype of brain-infiltrating JC virus-specific CD4$^+$ T cells was confirmed and extended by examining brain-derived JC virus-specific CD4$^+$ T cell clones. Our data provide novel insight into the pathogenesis of progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome and indicate that JC virus-specific CD4$^+$ T cells play an important role in both eliminating JC virus from the brain, but also in causing the massive inflammation with often fatal outcome.

Keywords: PML; PML-IRIS; CD4$^+$ T cells; JC polyoma virus; immunopathogenesis

Abbreviations: IRIS = immune reconstitution inflammatory syndrome; JC virus = John Cunningham virus; PML = progressive multi-focal leucoencephalopathy; VLP = virus-like particles

Introduction

Progressive multi-focal leucoencephalopathy (PML) was first described by Astrom *et al*. (1958). Crystalline inclusions in oligodendrocytes indicated a viral infection (Zurhein and Chou, 1965), and the polyoma John Cunningham (JC) virus was identified as the causative agent (Padgett *et al*., 1971). PML is an opportunistic infection that occurs in states of acquired or hereditary immunocompromise such as HIV infection, or rarely during autoimmune diseases (Major, 2010). Infection with JC virus is highly prevalent in healthy adults, and $\geqslant$60% of the population carries a latent/persistent infection (Egli *et al*., 2009). Recently, PML has emerged as a serious adverse event in monoclonal antibody therapy of autoimmune diseases, in particular patients with multiple sclerosis treated with natalizumab (anti-VLA-4) (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould *et al*., 2005; Anonymous, 2010; Jilek *et al*., 2010). Other monoclonal antibodes such as rituximab (anti-CD20), infliximab [anti-tumour necrosis factor (TNF)-α] and the IgG1-TNF receptor 2 fusion protein etanercept that are used to treat rheumatoid arthritis have also been associated with PML (Major, 2010). Efalizumab (anti-leucocyte function-associated antigen-1) has been withdrawn from the market (Pugashetti and Koo, 2009). With more than 100 cases with PML reported in patients with multiple sclerosis receiving natalizumab, the PML incidence is between 1:500 and 1:1000 and jeopardizes the use of this highly effective treatment (Anonymous, 2010).

The pathogenesis of PML is characterized by a lytic infection of oligodendrocytes and abortive infection of astrocytes in the absence of a notable immune reaction (Koralnik, 2006; Major, 2010). Although the mechanisms of controlling JC virus infection are as yet incompletely understood, both humoral and/or cellular immune responses probably play a role (Weber *et al*., 1997; Du Pasquier *et al*., 2001, 2004*a*; Khanna *et al*., 2009). Accordingly, the presence of JC virus-specific CD8$^+$ cytotoxic T cells has been linked to the recovery from PML, while these cells were absent in cases with PML with fatal outcome (Koralnik *et al*., 2002; Du Pasquier *et al*., 2004*a*, 2006). The observations that PML occurs preferentially in situations of decreased CD4$^+$ T cell numbers or compromised CD4$^+$ cell functions such as AIDS and idiopathic CD4$^+$ lymphopenia (Stoner *et al*., 1986; Gillespie *et al*., 1991; Zonios *et al*., 2008) and often resolves following restoration of CD4$^+$ numbers, indicate that both CD4$^+$ and CD8$^+$ JC virus-specific T cells are important for host protection.

Different from the general immunosuppresion, in AIDS, monoclonal antibody-based therapies inhibit specific immune functions such as cell migration across the blood–brain barrier during natalizumab therapy, or eliminate certain immune cells such as CD20-expressing B cells in the case of rituximab (Lutterotti and Martin, 2008). With respect to anti-VLA-4 therapy, it is assumed that PML results from compromised immune surveillance of the CNS, since activated T cells and CD209$^+$ dendritic cells cannot cross the blood–brain barrier (Yednock *et al*., 1992; Stuve *et al*., 2006; Del Pilar Martin *et al*., 2008), thus compromising local antigen presentation in the CNS (Del Pilar Martin *et al*., 2008). Alternatively, inhibition of VLA-4/vascular cell adhesion molecule-1 (VCAM-1) interactions, which serve as a retention signal for haematopoietic precursor cells in the bone marrow, may lead to release of JC virus from its natural niches (Tan *et al*., 2009*a*), increased viral replication and occurrence of JC virus variants with tropism for CNS cells (Houff *et al*., 1988; Ransohoff, 2005). Cessation of monoclonal antibody therapy in PML re-establishes immune surveillance for JC virus-infected cells in the CNS and leads to clinically apparent inflammatory responses in the brain. Inflammation can be visualized by contrast-enhancing lesions on MRI due to opening of the blood–brain barrier. The latter manifestation of PML has been termed PML-immune reconstitution inflammatory syndrome (IRIS) (Koralnik, 2006; Tan *et al*., 2009*b*). PML-IRIS can lead to rapid deterioration of the patient's clinical state and death in ~30–50% of cases (Tan *et al*., 2009*b*). Its cellular and molecular pathogenesis, i.e. which T cell subtypes, antibodies or cytokines are involved, are currently poorly understood. Diagnosing PML and PML-IRIS as early as possible and identifying effective therapies based on the underlying disease mechanisms are important goals not only in multiple sclerosis, but also in a number of other autoimmune diseases, during acquired immunodeficiencies, during malignancies and in transplant medicine.

In the present study, we characterized in detail the brain tissue infiltrate with respect to cellular composition as well as the humoral anti-JC virus-specific immune response in the CSF.

Furthermore, we established from a brain biopsy *ex vivo* bulk T cell cultures and $CD4^+$ T cell clones, which we then studied regarding antigen specificity, functional phenotype, relative abundance and T cell receptor expression. This is the first study that investigates the antigen specificity and phenotype of brain-infiltrating T cells in PML-IRIS.

Materials and methods

Patients

Patients with multiple sclerosis developing PML and PML-IRIS under natalizumab therapy

Clinical, neuroimaging (MRI), virological and clinical chemistry findings are summarized in Supplementary Table 1 and Fig. 1 (Patient 1); Supplementary Table 2 and Fig. 2 (Patient 2).

HLA-class II types: DRB1*13:01, DRB1*16:01; DRB3*02:02; DRB5*02:02; DQA1*01:02, DQA1*01:03; DQB1*05:02, DQB1*06:03 (Patient 1); and DRB1*11:03, DRB1*15:01; DRB3*02:02; DRB5*01:01; DQA1*01:02, DQA1*05:XX (X indicating not typed to the exact subtype); DQB1*03:01, DQB1*06:02 (Patient 2).

Other patients with PML, PML-IRIS and inflammatory neurological controls

Brain biopsy tissue from a 48-year-old male with AIDS-related PML and subsequent PML-IRIS, and CSF cells from two patients with PML based on hereditary immunodeficiencies, i.e. a 63-year-old male with idiopathic $CD4^+$ lymphopenia and a 20-year-old male with hyper-IgE syndrome, were examined. Furthermore, CSF cells from a 50-year-old male suffering from neurosyphilis and a 32-year-old female with relapsing–remitting multiple sclerosis were examined (for clinical case summaries see Supplementary material).

Neuropathology

Small tissue fragments of a total volume of ~0.1 ml were obtained by open biopsy. Following fixation in buffered formalin for 2 h, tissue was embedded in paraffin. Microtome sections of 4 μm were stained with haematoxylin and eosin, van Gieson's trichrome, periodic acid-Schiff, Turnbull's stain for siderin and Luxol. Immunohistochemical staining was performed on an automated Ventana HX immunohistochemistry system, benchmark (Ventana-Roche Medical Systems) following the manufacturer's instructions using the following antibodies: anti-CD45/LCA (Dako; M701), anti-CD3 (Dako; M1580), anti-CD45R0 (Dako; M0742), anti-CD20 (Dako; M0755), anti-CD79a (Dako; M7050), anti-CD68 (Immunotech/Beckmann-Coulter), anti-HLA-DR (Dako; M775), anti-NF (Zymed/Invitrogen; 80742971), anti-GFAP (Dako; Z334) and anti-p53 (Dako; M7001).

Brain tissue processing and expansion of brain-, cerebrospinal fluid- and peripheral blood-derived mononuclear cells

A biopsy of ~0.033 ml was cut into small pieces and disrupted by incubation in a solution containing 1 mg/ml collagenase A (Roche Diagnostics) and 0.1 mg/ml DNAse I (Roche) at 37°C in a water bath for 45 min. The resulting cell suspension was washed three times, and brain-derived mononuclear cells were separated using a Percoll density gradient centrifugation (GE Healthcare). Cells were re-suspended in a 30% Percoll solution and carefully underlayered with a 78% Percoll solution. After centrifugation brain-derived mononuclear cells were gathered from the interface of the gradient. Brain tissue from the patient with AIDS PML-IRIS was processed in a similar way.

CSF-derived mononuclear cells were obtained directly from a diagnostic spinal tap, and peripheral blood mononuclear cells separated by Ficoll density gradient centrifugation (PAA).

Brain, CSF and peripheral blood-derived mononuclear cells were expanded in 96-well U-bottom microtitre plates by seeding 2000 cells per well together with 2×10^5 non-autologous, irradiated peripheral blood mononuclear cells (3000 rad) and 1 μg/ml of phytohaemagglutinin-L (Sigma). Medium consisted of Roswell Park Memorial Institute (RPMI) (PAA) containing 100 U/ml penicillin/streptomycin (PAA), 50 μg/ml gentamicin (BioWhittaker, Cambrex), 2 mM L-glutamine (GIBCO, Invitrogen) and 5% heat-decomplemented human serum (PAA). After 24 h, 20 U/ml of human recombinant IL-2 (hrIL-2, Tecin, Roche Diagnostics) were added and additional hrIL2 was added every 3–4 days. After 2 weeks, cells were pooled and analysed, cryopreserved or restimulated with 1 μg/ml phytohaemagglutinin, 20 U/ml hrIL-2 and allogeneic irradiated peripheral blood mononuclear cells.

Flow cytometry analysis of brain-derived mononuclear cells

Brain-derived mononuclear cells directly from brain digestion were stained with the following antibodies for surface markers: CD45 (AmCyan, 2D1, BD Pharmingen), CD56 (Alexa 488, B159, BD Pharmingen), CD3 (PeCy7, UCHT1, eBioscience), CD4 (APC, RPA-T4, eBioscience), CD8 (PB, DK25, Dako), CD45RO (FITC, UCHL1, eBioscience), CD19 (FITC, HIB19, BD Pharmingen), CD38 (APC, HIT2, BD Pharmingen) and CD27 (APC-Alexa 750, CLB-27/1, Invitrogen). Analysis was performed on a LSRII (BD Biosciences) flow cytometer.

Proteins and peptides

For the identification of JC virus-specific T cells, 204 (13–16-mer) peptides covering the entire JC viral proteome, were applied. Peptides were synthesized and provided by Peptides and Elephants GmbH. These 204 peptides overlap by five amino acids and include 35 common single amino acid mutations (Supplementary Table 3). To account for amino acid variations that occur among the different JC virus genotypes and strains, amino acid sequences of each JC virus encoded protein including Agno, VP1, VP2, VP3, large T antigen and small T antigen, from all 479 JC virus genomic sequences available in GenBank (by March 2008), were aligned and those polymorphisms, which were prevalent in >1% of the all retrieved sequences, were defined as common mutations.

In order to determine which individual peptides are recognized by CNS-derived T cells, a 2D seeding scheme was applied. Peptides were arranged in a set of 82 pools and each pool contained five different peptides (Supplementary Table 4). By the combination of different peptides in each well according to a rectangular matrix and each individual peptide appearing in exactly two pools, in which the residual peptides differed, immunogenic candidate peptides could be identified at the intersections of the positive pools.

JC virus VP1 protein forms virus-like particles (VLP), and VP1 and VLP are therefore used as interchangeable terms. VP1 protein forming

VLP (VP1/VLP) was generated by the Life Science Incubator, as previously described (Goldmann *et al*., 1999).

Twenty-mer myelin peptides with an overlap of 10 amino acids and covering myelin basic protein (16 peptides), myelin oligodendrocyte glycoprotein (25 peptides) and proteolipid protein (27 peptides) were synthesized and provided by PEPScreen, Custom Peptide Libraries, SIGMA (Supplementary Table 5). Tetanus toxoid (Novartis Behring) was used as positive control.

Proliferative assays

Recognition of JC virus peptides, VP1/VLP and tetanus toxoid were tested by seeding duplicates in 96-well U-bottom microtitre plates 2–2.5 $\times$ 10^4 brain-, CSF- or peripheral blood-derived phytohaemagglutinin-expanded cells per well and 1 $\times$ 10^5 autologous irradiated peripheral blood mononuclear cells with or without peptides for 72 h. Unmanipulated peripheral blood mononuclear cells were tested at 2 $\times$ 10^5 cells/well in a 7-day primary proliferation. In addition to tetanus toxoid, phytohaemagglutinin-L stimulation was added as positive control. All JC virus peptides were either tested in pools or as individual peptides at a final concentration of 2 μM per peptide for peptides in pools and at a concentration of 10 μM for individual peptides. VP1/VLP was tested at 2 μg/ml, tetanus toxoid at 5 μg/ml and phytohaemagglutinin at 1 μg/ml. Proliferation was measured by ^{3}H-thymidine (Hartmann Analytic) incorporation in a scintillation beta counter (Wallac 1450, PerkinElmer). The stimulatory index was calculated as:

$$\text{Stimulatory index} = \text{Mean counts per minute (peptide)} / \text{Mean counts per minute (background)}$$

Responses were considered as positive when the stimulatory index >3, counts per minute >1000 and at least three standard deviations (SDs) above average background counts per minute. Myelin peptides were tested as individual peptides at 5 μM, as described above.

Generation of brain-derived VP1/VLP-specific T cell clones

Brain-derived phytohaemagglutinin-expanded cells (2.5 $\times$ 10^4) were seeded in 96-well U-bottom microtitre plates with 1 $\times$ 10^5 autologous irradiated peripheral blood mononuclear cells with or without VP1/VLP protein. After 48 h of culture, plates were split into mother and daughter plates. Proliferation was measured in daughter plates by methyl-^{3}H-thymidine incorporation. VP1/VLP-responsive cultures were identified in mother plates, and IL-2 was added every 3–4 days until Day 12. T cell clones were established from positive cultures by seeding cells from VP1/VLP-responsive wells under limiting dilution conditions at 0.3 and 1 cell/well in 96-well U-bottom microtitre plates, and addition of 2 $\times$ 10^5 allogeneic, irradiated peripheral blood mononuclear cells and 1 μg/ml of phytohaemagglutinin-L in complete RPMI. After 24 h, 20 U/ml of human recombinant IL-2 was added. VP1/VLP specificity was then confirmed by seeding 2.5 $\times$ 10^4 cells from growing colonies with autologous irradiated peripheral blood mononuclear cells with or without VP1/VLP protein for 72 h. Specific cultures were restimulated every 2 weeks with 1 μg/ml phytohaemagglutinin-L, 20 U/ml hrIL-2 and allogeneic irradiated peripheral blood mononuclear cells and hrIL2 was added every 3–4 days.

T cell receptor analysis

T cell receptor Vβ chain expression was assessed in phytohaemagglutinin-expanded cells and T cell clones by 22 anti-TCRBV monoclonal antibodies (Immunotech) (Muraro *et al*., 2000) in combination with CD4 (APC, eBioscience) and CD8 (PB, PB, DakoCytomation).

Determination of precursor frequency in central nervous system-derived mononuclear cells

Frequencies of VP1/VLP-specific cells were determined by limiting dilution. Twenty, 200, 2000 or 20 000 brain-derived phytohaemagglutinin-expanded cells were seeded in quadruplicates in 96-well U-bottom microtitre plates with 1 $\times$ 10^5 autologous-irradiated peripheral blood mononuclear cells with or without VP1/VLP protein. After 72 h, proliferation was measured by methyl-^{3}H-thymidine incorporation. Frequencies were calculated as previously described (Taswell, 1981). Observed data included: r, the number of negatively responding cultures or wells of each dose i; n, the total number of wells per dose i and λ the number of cells in the dose i. Calculated data were $p_i = r_i/n_i$, the fraction of negatively responding cultures of each dose i. The frequency was calculated using the following formula: $f = -(\ln p_i)/\lambda_i$.

Cytokine production

For intracellular cytokine staining, phytohaemagglutinin-expanded cells and T cell clones were analysed 12 days after last restimulation. Cells were stimulated with phorbol myristate acetate (50 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) in the presence of Brefeldin A (10 μg/ml, eBioscience) for 5 h. Cells were then stained with LIVE/DEAD® Fixable Dead Cell Stain Kit (AmCyan, Molecular Probes, Invitrogen), fixed and permeabilized with the corresponding buffers (eBioscience) and stained for CD3 (PE, DakoCytomation), CD8 (PB, DakoCytomation), IFN-γ (FITC, BDPharmingen), IL-4 (PE-Cy7, eBioscience) and IL-17 A (Alexa Fluor®-647, eBioscience) at room temperature. IFN-γ-, IL-4- and IL-2 levels were also determined by ELISA following the manufacturer's protocol (Biosource) in culture supernatants of phytohaemagglutinin-expanded cells and in T cell clones 72 h after stimulation with phytohaemagglutinin or VP1/VLP.

Quantification of messenger RNA expression levels by real time-polymerase chain reaction

For messenger RNA gene expression assays, the primer and probe sets (Tbet, Hs00203436_m1 and Gata3, Hs00231122_m1) were purchased from Applied Biosystems. Ribosomal RNA (18S) was used as endogenous control, and the relative gene expression was calculated by the ΔΔCt method using brain-derived phytohaemagglutinin-expanded cells as calibrator.

Enzyme-linked immunosorbent assay for VP1/VLP-specific antibodies

The titre of VP1/VPL-specific immunoglobulin G antibodies in CSF and sera from both patients with PML-IRIS was determined as previously described (Weber *et al*., 1997). Briefly, ELISA plates were coated with 100 μl VP1/VLP (1 mg/μl) and incubated with serial dilutions of CSF or sera. Human IgG was captured by a biotin-conjugated anti-human Fc antibody (eBioscience) and detected by an avidin horseradish peroxidase (eBioscience). Antibody titres in CSF as well as serum were

adjusted to the total amount of IgG in the particular compartment. Results were expressed as arbitrary units, which were standardized using the same human serum as standard.

HLA-A*0201/JC virus $VP1_{36}$ and $VP1_{100}$ tetramers and tetramer staining

HLA-A*02:01 tetrameric complexes were synthesized as previously described (Ogg *et al*., 1998). Briefly HLA-A*02:01, β2 microglobulin and epitope peptide were refolded and isolated using size exclusion chromatography. Site-specific biotinylation was achieved through addition of the BirA target sequence to the last C terminal extracellular domain of the HLA-A*0201 molecule. Tetrameric complexes were generated using Extravidin-PE (Sigma). Phytohaemagglutinin-expanded brain-infiltrating cells were stimulated with anti-CD2/CD3/CD28 MACs beads (Miltenyi Biotec) and at Day 5 after stimulation, cells were washed and resuspended to a concentration of 5×10^6 cells/ml; 100 µl were stained with 3 µl of Phycoerythrin-coupled tetrameric HLA-A*02:01/JCV $VP1_{36}$ or HLA-A*02:01/JCV $VP1_{100}$. After 30 min incubation at 37°C, the cells were washed and stained with anti-CD3 (PB, eBiolegend) and anti-CD8 (FITC, Dako) for additional 30 min on ice. Cells were then washed and fixed with 0.5% paraformaldehyde before analysis by flow cytometry.

Results

Two cases of natalizumab-associated PML-IRIS

Two male patients of 41 and 43 years with relapsing–remitting multiple sclerosis presented with clinical signs (visual field defect in Patient 1; monoparesis in Patient 2) and imaging findings suspicious of PML in July 2009 and January 2010, respectively (Fig. 1A; Supplementary Figs. 1 and 2; Supplementary Tables 1 and 2) after 28 and 40 months, respectively, of natalizumab treatment. Natalizumab was stopped immediately, and several rounds of plasmapheresis performed. Both patients subsequently developed PML-IRIS with patchy or band-like areas of contrast enhancement on MRI (Fig. 1A) and worsened clinical findings of complete loss of vision in Patient 1, and hemiplegia, hemianopia and neuropsychological deterioration in Patient 2. With respect to diagnostic workup, JC virus loads in urine, serum and CSF samples were kindly quantified by Prof. H.H. Hirsch (Transplantation Virology, Institute for Medical Microbiology, Department of Biomedicine, University of Basel, Switzerland) and by Dr E.O. Major [Laboratory of Molecular Medicine and Neuroscience (LMMN), NINDS, NIH Bethesda, USA]. Details regarding polymerase chain reaction methodologies are provided in the Supplementary Material. Patient 1 was immediately diagnosed as PML based on a positive CSF JC virus viral load, although it was low (Fig. 1B). Diagnosis in Patient 2 was more complicated with repeatedly negative polymerase chain reaction results for CSF JC virus viral load until the third positive testing, just above threshold levels (Fig. 1B and Supplementary Fig. 3; 12 copies; threshold 10 copies in the NIH reference laboratory). In contrast to the low or borderline JC virus CSF viral loads, antibody testing for JC virus major capsid protein (VP1/VLP)-specific antibodies in serum and CSF, which was established during this study, revealed strong intrathecal antibody response with 95- to 180-fold higher VP1/VLP-specific antibody titres in the CSF compared with serum after adjusting total IgG concentrations to the same levels (Fig. 1C). Hence, different from the polymerase chain reaction testing for viral DNA, the intrathecal antibody response left no doubt of CNS infection by JC virus at the time of PML-IRIS. The analysis of the IgG subclasses in Patient 2 demonstrated that intrathecal antibodies are mainly IgG1 and IgG3 (Fig. 1D). These data indicate a strong JC virus-specific humoral immune response that is confined to the CNS compartment and directed primarily against the major structural JC virus protein VP1/VLP. Whether minor components of the antibody response target other JC virus proteins remains to be determined.

Due to the difficulties in diagnosing PML, Patient 2 underwent a diagnostic brain biopsy to confirm or refute PML (Supplementary Fig. 3). Neuropathological examination failed to show the typical signs of PML, i.e. nuclear inclusions in hyperchromatic oligodendrocytes and bizarre astrocytes, but rather a paucity of CNS cells and massive perivascular and parenchymal lymphomononuclear infiltrates (Fig. 2A), reactive gliosis with stellate astrocytes (Fig. 2B) and predominance of diffuse and destructive parenchymal infiltrates of foamy macrophages (Fig. 2C). The majority of cells stained positive for HLA-DR, which is usually exclusively found on activated microglia and absent in normal brain tissue (Fig. 2D). T cells (Fig. 2E) and B cells (Fig. 2G) were present in the infiltrate, and a high proportion of the latter stained positive for the plasma cell marker CD138 (Fig. 2H). Part of the biopsy tissue was processed, and CNS-derived mononuclear cells were also characterized by flow cytometry. Cells (96.5%) expressed the pan-haematopoietic cell marker CD45 (data not shown) and among them 42.4% expressed the pan-T cell marker $CD3^+$ (Fig. 2F). Of these, 24.1% were $CD8^+$ and 70.4% $CD4^+$ T cells (Fig. 2F). Almost all T cells expressed the memory marker CD45RO (Fig. 2F). Twenty-nine per cent of CNS-infiltrating cells expressed the B cell marker CD19 (Fig. 2I), and among these 86.1% were positive for CD27/CD38 (Fig. 2I), i.e. they were memory B cells/plasma cells. A marker for microglia/monocytes/macrophages was not included in the fluorescence-activated cell sorting panel, but from the immunohistochemistry studies these cells probably constitute another important part of immune cell infiltrate. Accordingly, a diagnosis of inflammatory demyelinating disease, rather than PML, was made. Subsequent immunohistochemistry for JC virus was negative, but sparse nuclear signals for JC virus DNA were found by the second attempt of *in situ* hybridization (data not shown), which together with the low JC virus viral load and strong intrathecal antibody response confirmed the initial suspicion of PML and pointed towards IRIS rather than the underlying demyelinating disease as being responsible for the neuropathological findings.

Antigen specificity of brain-infiltrating T cells

Next, we characterized the antigen specificity and frequency of brain-infiltrating T cells. Brain-derived mononuclear cells were

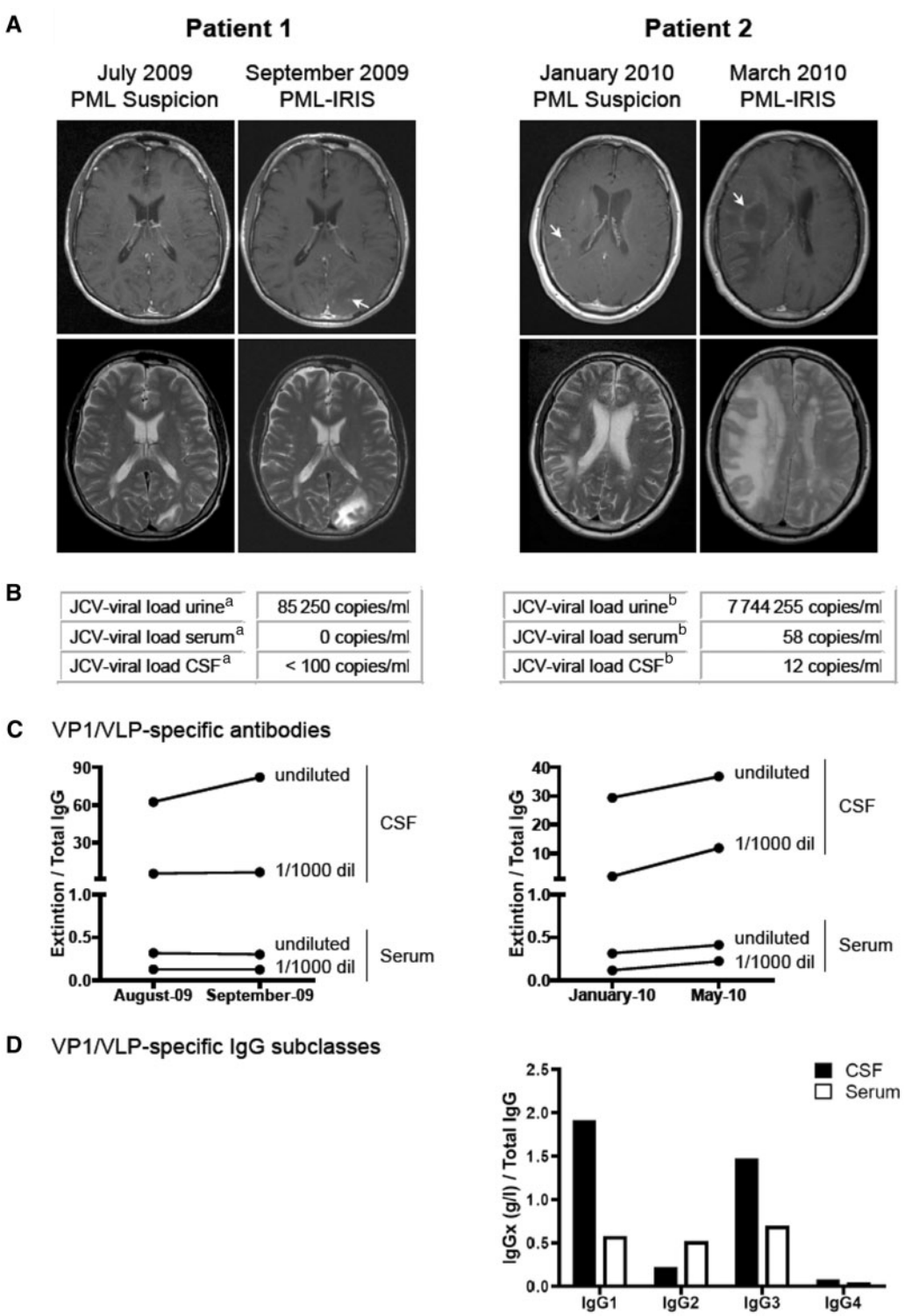

Figure 1 (**A**) MRI images at the time of suspected PML and during PML-IRIS in Patients 1 and 2. (*Top*) T_1-weighted images after administration of gadolinium contrast agent. Patient 1 shows minimal patchy enhancement (white arrowhead) in the left occipital T_1-hypointense area, and Patient 2 shows first a patchy, and later band-like enhancement in various locations (white arrowheads). (*Bottom*) T_2-weighted images with a circumscript left occipital T_2-hyperintense lesion that expands between July and September 2009 in Patient 1. In Patient 2, the parieto-occipital T_2-hyperintense lesion at the time of suspected PML later extends to almost the entire right hemisphere, (**B**) JC viral load in urine, serum and CSF in Patients 1 and 2. (**C**) ELISA of CSF and serum, obtained at the same time, against JC virus VP1/VLP in PML-IRIS Patient 1 (*left*), and in PML-IRIS Patient 2 (*right*). (**D**) Analysis of the IgG subclasses in Patient 2 in CSF and serum. [a]Results provided by Prof. H. Hirsch, Department of Biomedicine, Institute for Medical Microbiology, University of Basel, Switzerland. [b]Results provided by Dr Eugene O. Major, Laboratory of Molecular Medicine and Neuroscience, NINDS, NIH, Bethesda, USA.

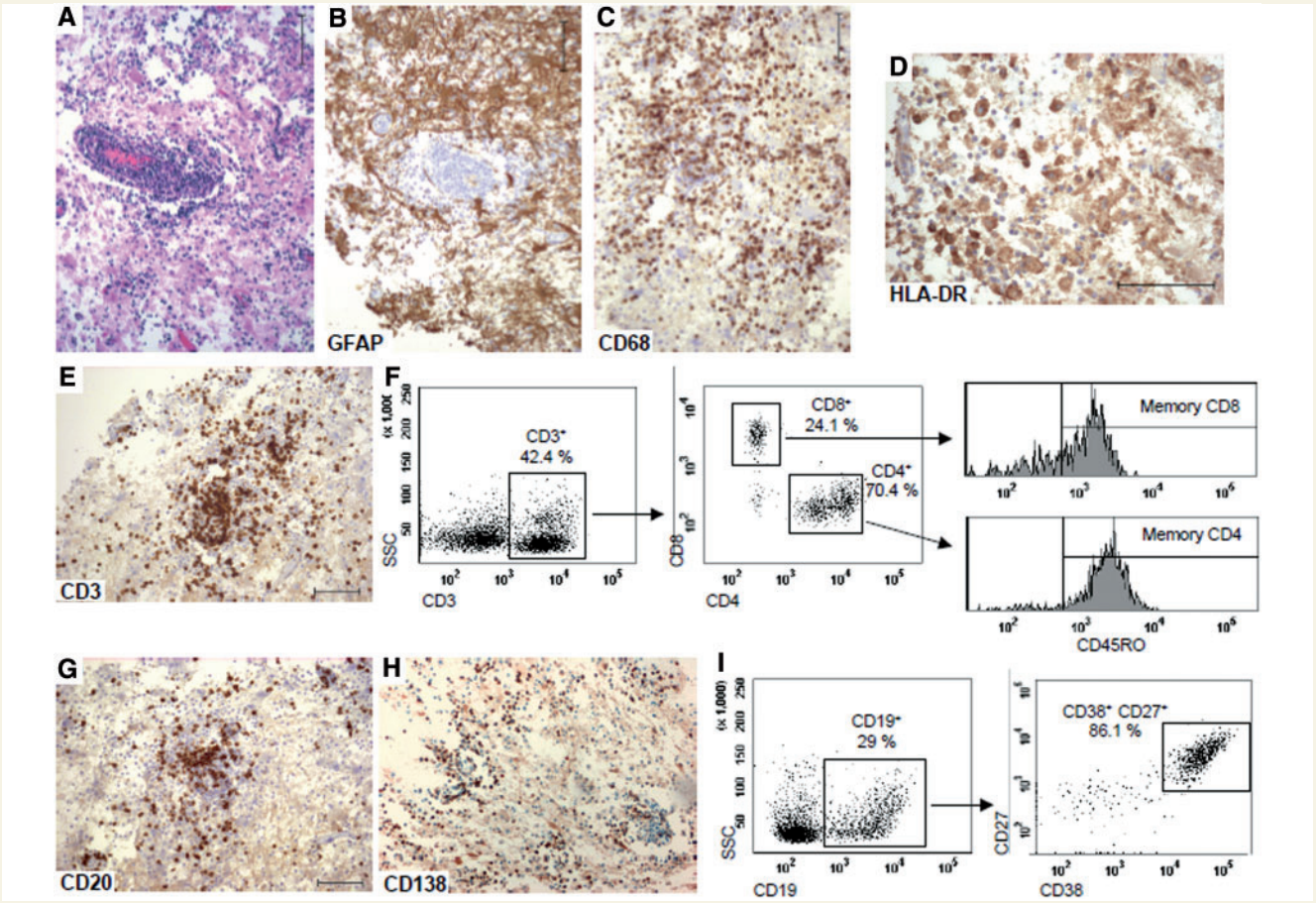

Figure 2 (**A**) PML-IRIS with massive perivascular and parenchymal infiltration (haematoxylin). (**B**) Reactive gliosis with numerous stellate astrocytes (anti-glial fibrillary acidic protein) and (**C**) diffuse and destructive parenchymal infiltration of foamy macrophages (anti-CD68). (**D**) The vast majority of infiltrating and resident cells express HLA-DR. (**E**) Perivascular and parenchymal infiltrates are rich in T cells (anti-CD3). (**F**) Brain-infiltrating cells (42.4%) of $CD45^+$ are $CD3^+$ T cells (boxed population on *left*), and of these 24.1% and 70.4%, respectively, $CD8^+$ and $CD4^+$ T cells, respectively (*middle*). In both T cell populations, almost all cells express the memory marker CD45RO (*right*). (**G**) Perivascular and parenchymal infiltrates are also rich in B cells (anti-CD20) and (**H**) can further be characterized as plasma cells (anti-CD138). (**I**) Flow cytometric analysis shows that 29% of $CD45^+$ cells are B cells (anti-CD19; *left*), and of these 86.1% are memory B cells/plasmablasts or plasma cells (*right*). The hallmarks of PML, bizarre giant astrocytes and oligodendrocytes with enlarged hyperchromatic nuclei, were absent. Scale bar = 100 μm.

first expanded as bulk populations by an unbiased stimulus (phytohaemagglutinin; Supplementary Fig. 4A). While our culture conditions favoured the expansion of $CD4^+$ over $CD8^+$ T cells (Supplementary Fig. 4B), the relative composition of $CD4^+$ T cells remained stable as demonstrated by staining with monoclonal antibodies against T cell receptor variable chains Vβ1–Vβ22 (Supplementary Fig. 4C). Due to almost 3-fold excess of memory $CD4^+$ over $CD8^+$ T cells at the time of brain biopsy, we focused our attention on $CD4^+$ cells and assessed their specificity for JC virus. For this purpose, expanded brain T cells were tested against recombinant JC virus capsid protein VP1/VLP and against tetanus toxin protein in proliferative assays. We directly compared brain-derived versus CSF- or peripheral blood-derived T cells as well as versus unmanipulated peripheral blood mononuclear cells. As shown in Fig. 3A, brain-derived T cells responded with a stimulation index >600 against VP1/VLP protein with no response against tetanus toxoid. Stimulatory indices against VP1/VLP and tetanus toxoid in the CSF were 7 and 14, respectively, and in phytohaemagglutinin-expanded peripheral blood mononuclear cells the responses to VP1/VLP and tetanus toxoid were negative and moderately positive (stimulatory index of 6.5), respectively. Unmanipulated peripheral blood mononuclear cells showed a significantly stronger response to tetanus toxoid compared with VP1/VLP in a 7-day primary proliferation assay.

Functional phenotype of brain-infiltrating $CD4^+$ T cells

We then examined if intracerebral $CD4^+$ T cells belonged to one of the major T helper (Th) subtypes, Th1, Th2 or Th17 cells, based on their cytokine secretion pattern. Expanded bulk T cell populations from the brain, CSF and peripheral blood mononuclear cells, as well as unmanipulated peripheral blood mononuclear cells, were examined by intracellular cytokine staining against IFN-γ, IL-4 and

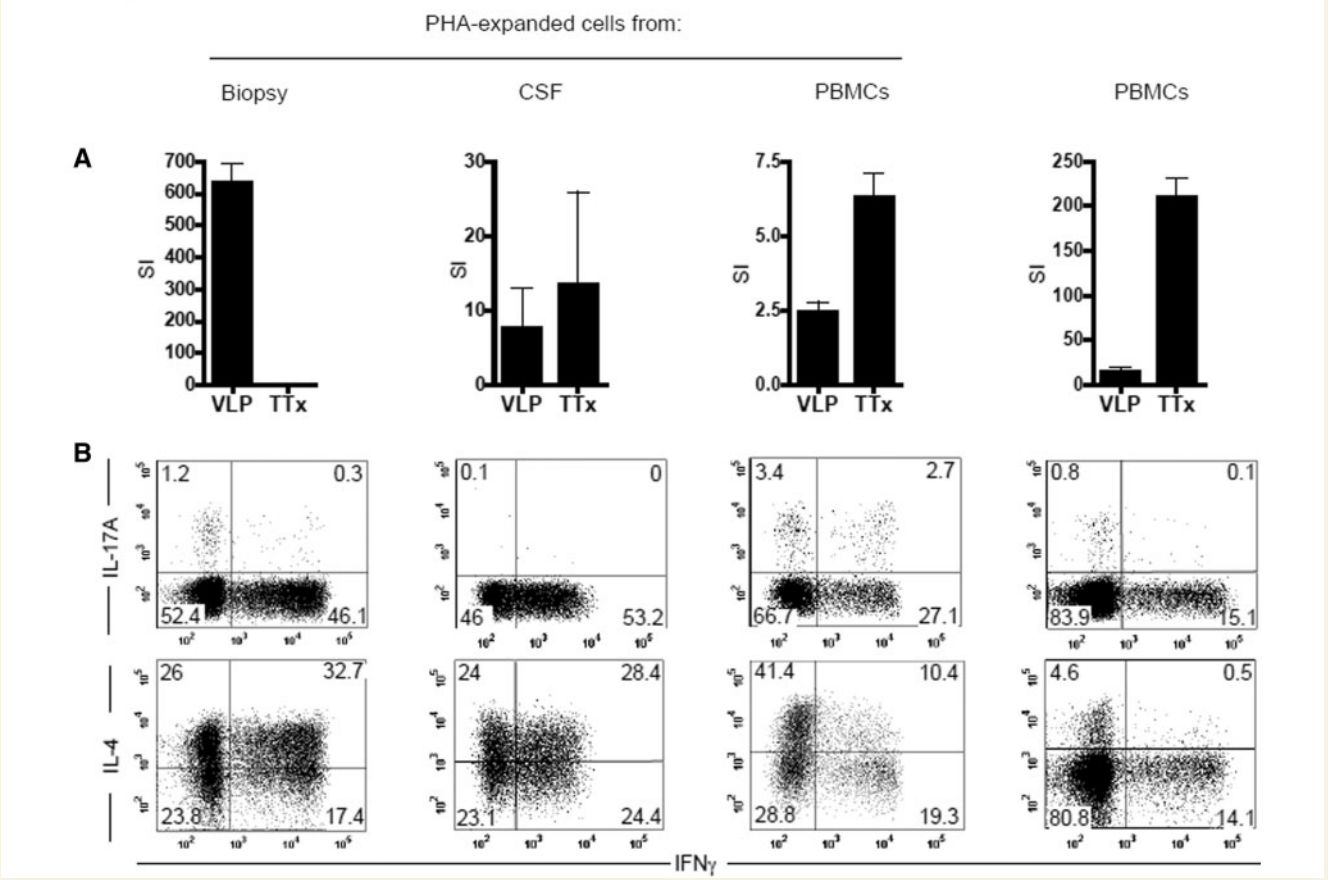

Figure 3 (**A**) Phytohaemagglutinin-expanded bulk mononuclear cell populations from the brain biopsy (*left*), CSF (*second from left*) and peripheral blood mononuclear cells (PBMCs) (*third from left*) as well as unmanipulated peripheral blood mononuclear cells (*right*) were tested against JC virus VP1/VLP protein and tetanus toxoid protein (TTx). Results show the mean stimulatory index ± SEM. Note the different scales for the *y*-axis. (**B**) *Ex vivo* quantification of Th1-, Th2-, Th17- and Th1-2 cells in phytohaemagglutinin-expanded $CD4^+$ T cells from brain biopsy (*left*), CSF (*second from left*), peripheral blood mononuclear cellss (*third from left*) and in unmanipulated peripheral blood mononuclear cells (*right*). Numbers represent the percentage of positive cells. Th1 were identified as $CD4^+$ IFN-γ^+ IL-$17A^-$/IL-4^-; Th17 cells as $CD4^+$ IL-$17A^+$ IFN-γ^-; Th2 as $CD4^+$ IL-4^+ IFN-γ^-; and Th1-2 as $CD4^+$ IFN-γ^+ IL-4^+. PHA = phytohaemagglutinin.

IL-17, the signature cytokines of Th1-, Th2- and Th17 cells. IL-17-producing cells were hardly detectable (Fig. 3B), while IFN-γ-secreting cells made up between 46.1% and 53.2% in cells from the brain and CSF (Fig. 3B). When combining intracellular staining for IFN-γ and IL-4, the situation was remarkably different. In the brain- and CSF-derived population, Th1-, Th2- and bifunctional Th1-2 cells (secreting both IL-4 and IFN-γ) were similar in frequency (Fig. 3B), while Th2 cells predominated in the peripheral blood-derived, phytohaemagglutinin-expanded cells (Fig. 3B). Brain-derived $CD4^+$ T cells (32.7%) had a bifunctional Th1-2 phenotype.

We then examined whether bifunctional Th1-2 $CD4^+$ T cells were also abundant in the brain- and CSF-derived population from another patient with PML-IRIS and two more patients with PML. We analysed the phenotype of brain-derived expanded bulk T cell populations from the patient with AIDS, who developed PML/PML-IRIS. We found that 15.7% of intracerebral $CD4^+$ T cells had a bifunctional Th1-2 phenotype (Fig. 4A), while IL-17-producing cells were hardly detectable and IFN-γ-secreting cells made up >50% (Fig. 4A). We also analysed the phenotype of CSF-derived expanded bulk T cell populations from two patients with PML, one with hyper-IgE syndrome and the other with idiopathic CD4 lymphocytopenia. CSF expanded $CD4^+$ T cells (26.9%) in the patient with PML/hyper-IgE syndrome and 14.4% in the patient with PML/idiopathic CD4 lymphocytopenia had a bifunctional Th1-2 phenotype. These percentages were higher than those observed in control patients not suffering from PML. We analysed the phenotype of CSF-derived, expanded bulk T cell populations from two control patients, one with neurosyphilis and the other with relapsing–remitting multiple sclerosis. In both patients, the percentage of $CD4^+$ T cells with a bifunctional Th1-2 phenotype was ~4% (Fig. 4B).

Fine specificity and frequency of brain-infiltrating T cells

To determine which specific JC virus peptides are recognized by brain-infiltrating T cells, 204 15-mer peptides spanning all JC virus proteins (Agno, VP1, VP2, VP3, large-T and small-T) were synthesized and arranged in a set of 82 pools, where each peptide

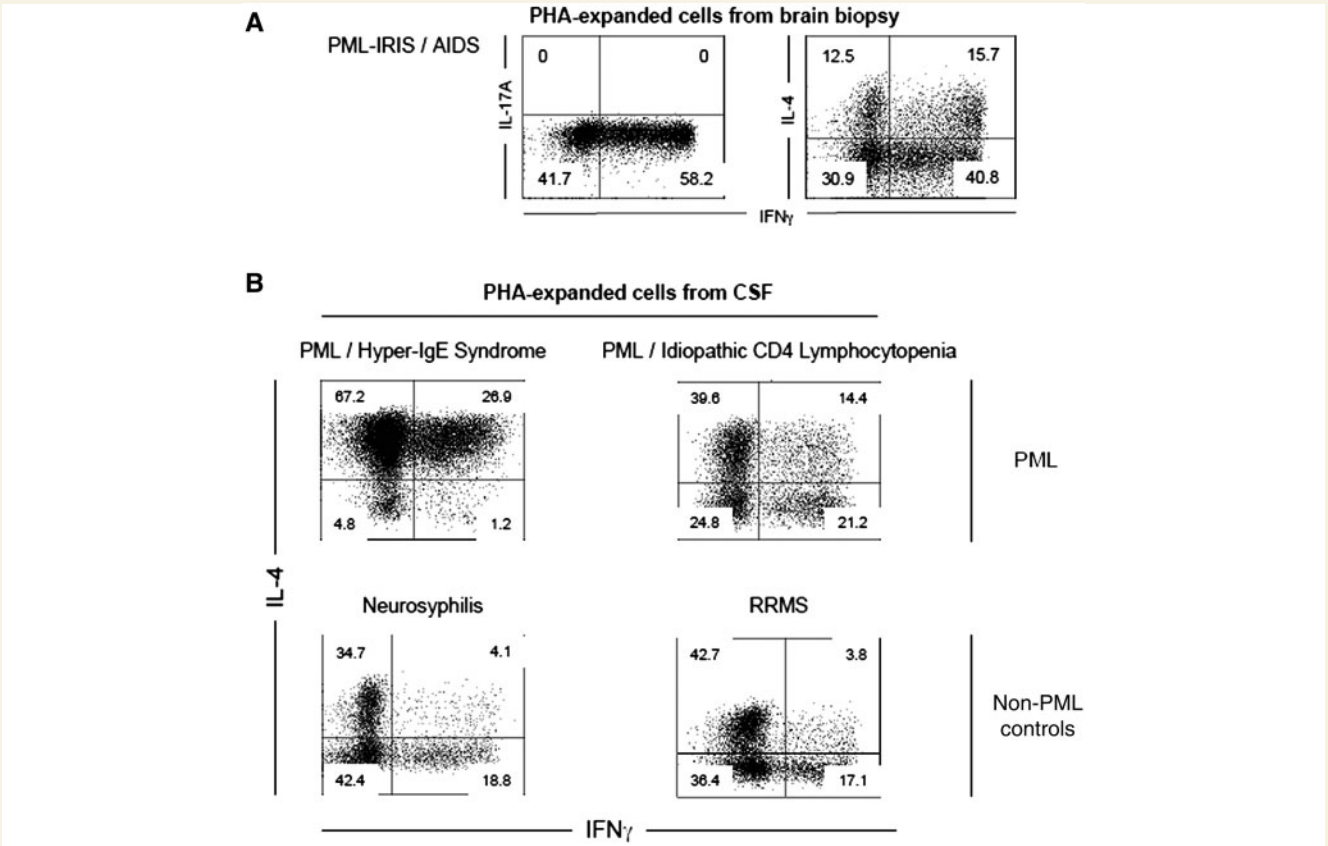

Figure 4 (**A**) *Ex vivo* quantification of Th1-, Th2-, Th17- and Th1-2 cells in brain-derived expanded bulk T cell populations from the patient with AIDS, who developed PML/PML-IRIS. (**B**) *Ex vivo* quantification of Th1-, Th2- and Th1-2 cells in CSF-derived expanded bulk T cell populations from two patients with PML, one with hyper-IgE syndrome (*first upper*) and the other with idiopathic CD4 lymphocytopenia (*second upper*), and from two control patients, one with neurosyphilis (*first lower*) and the other with relapsing–remitting multiple sclerosis (*second lower*). Numbers represent the percentage of positive cells. Th1 were identified as $CD4^+$ IFN-γ^+ IL-4^-; Th2 as $CD4^+$ IL-4^+ IFN-γ^-; and Th1-2 as $CD4^+$ IFN-γ^+ IL-4^+. PHA = phytohaemagglutinin.

appeared twice, but in two different pools (Supplementary Table 4). Brain-derived T cells responded to multiple pools (Fig. 5A; pools 1–41, and Supplementary Fig. 5A; all 82 pools). We identified 15 immunogenic candidate peptides (Supplementary Fig. 5B) that were then tested individually (Supplementary Fig. 5C) and led to the identification of 11 stimulatory peptides (peptides with stimulatory index >10) (Fig. 5B). The response was directed against peptides **4** ($Agno_{25}$, the number denotes the first amino acid of the 15-mer peptide; peptides with stimulatory index >25 in bold), **20** ($VP1_{34}$), **23** ($VP1_{54}$), **27–29** ($VP1_{74}$) (all $VP1_{74}$ peptides; 28 and 29 are variants of peptide 27 with single amino acid mutations), 72 ($VP1_{310}$), **73** ($VP1_{319}$), **76** ($VP1_{335}$), **191** ($LTAg_{668}$) and 195 ($sTAg_{82}$). Thus, brain-derived T cells responded to several JC virus proteins (Agno, VP1, LTAg, sTAg), however, by far the strongest against VP1 (six peptides). That VP1 is the prime target is supported by an even stronger response against entire VP1/VLP protein (Fig. 3A) and by the higher precursor frequencies of VP1-specific T cells (between 1/294 and 1/714 T cells responding to peptides $VP1_{34}$, $VP1_{319}$ and $VP1_{74}$) when compared with cells responding to $Agno_{25}$ (1/14492) and $LTAg_{668}$ (1/1449) (Fig. 5C). When we examined phytohaemagglutinin-expanded CSF- and peripheral blood-derived T cells, CSF cells showed only weak responses against Pool 39 and peptide $LTAg_{668}$ contained in this pool, and peripheral blood mononuclear cells were negative (Supplementary Fig. 6A and B). Remarkably, peptide $VP1_{34}$, the peptide that elicits the strongest response with respect to stimulatory index (Fig. 5B) and precursor frequency (Fig. 5C), contains the JC virus epitope $VP1_{36}$, one of the two epitopes together with $VP1_{100}$ that are recognized by HLA-class I-restricted $CD8^+$ T cells in the context of HLA-A*02:01 (Du Pasquier *et al*., 2003). Patient 2 with PML-IRIS was HLA-A*02:01^+ (HLA-class I and -class II types in 'Material and methods' section). We determined the frequency of $CD8^+$ T cells specific of these two HLA-A*02:01 JC virus epitopes in the phytohaemagglutinin-expanded brain-infiltrating $CD8^+$ T cells by tetramer staining. Phytohaemagglutinin-expanded brain-infiltrating $CD8^+$ T cells (0.8%) were specific for $VP1_{36}$ and 0.6% for $VP1_{100}$ (Fig. 5D). With respect to HLA-class II, Patient 2 expressed the multiple sclerosis-associated HLA-DR haplotype DRB1*15:01 and DRB5*01:01. The JC virus-specific $CD4^+$ T cell response was largely restricted by DRB1*15:01/B5*01:01, when VP1/VLP protein was presented by APCs from a DRB1*15:01/B5*01:01 homozygous donor (data not shown). These data demonstrate that, similar to intrathecal antibodies, the $CD4^+$ T cell response is

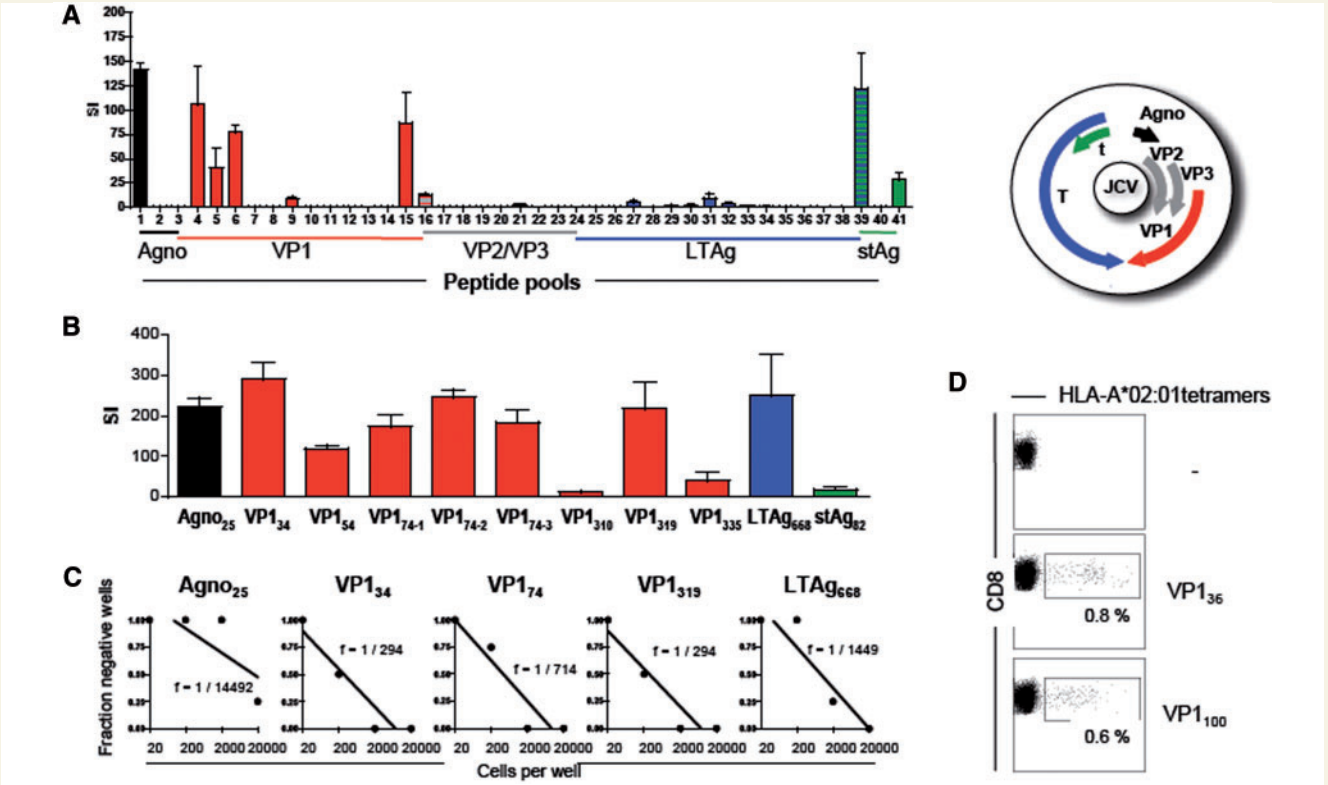

Figure 5 (**A**) Proliferative response of brain-derived phytohaemagglutinin-expanded cells against 204 overlapping 15-mer peptides spanning all open reading frames of JC virus (covering Agno, VP1, VP2, VP3, large-T and small-T proteins) and organized in 41 pools of five peptides each. Results show the mean stimulatory index ± SEM. The different colours of the bars correspond to the different open reading frames. Schematic representation of the five open reading frames in the JC virus genome (*top right*). (**B**) Proliferative response of brain-derived bulk mononuclear cell populations against individual JC virus peptides. Results show the mean stimulatory index ± SEM. Note the different scales of the *y*-axes (**A**) and (**B**). (**C**) Precursor frequency of T cells specific of the five JC virus peptides inducing the strongest proliferative responses in phytohaemagglutinin-expanded cells from brain biopsy. (**D**) Percentage of $CD8^+$ T cells that bind HLA-A*02:01-$VP1_{36}$ tetramers (*middle*) and HLA-A*02:01-$VP1_{100}$ tetramers (*lower*). SI = stimulatory index.

mainly directed against the major structural JC virus protein VP1 and that peptide $VP1_{34}$ contains an epitope for both virus-specific $CD4^+$ and $CD8^+$ T cells. Such a focus of $CD4^+$ and $CD8^+$ T cells on the same immunodominant epitope has previously been shown for an influenza nucleoprotein peptide (Carreno *et al.*, 1992).

Since PML is characterized by oligodendrocyte lysis and release of myelin and since the patient suffers from multiple sclerosis, it was of interest to examine if brain-derived T cells responded to myelin proteins. Phytohaemagglutinin-expanded brain-derived T cells were tested against overlapping peptides spanning the major myelin proteins, myelin basic protein, proteolipid protein and myelin oligodendrocyte glycoprotein, but none of the myelin peptides was recognized despite a strong response against JC virus VP1/VLP protein (Supplementary Fig. 7).

Fine specificity and functional phenotype of JC virus-specific $CD4^+$ T cell clones

Previous data have shown that $CD4^+$ differentiate into certain T helper phenotypes such as Th1 cells (IFN-γ producers), Th17 cells secreting IL-17, Th2 cells expressing the signature cytokine IL-4 or T regulatory cells based on the expression of certain transcription factors (Zhu *et al.*, 2010). The differentiation into Th1 or Th2 cells is considered mutually exclusive and controlled by the transcription factors T-bet (Th1) and Gata-3 (Th2) (Zhu *et al.*, 2010). Based on these data, our finding of committed memory cells with a bifunctional (Th1-2) phenotype was highly unexpected, and we therefore established VP1/VLP-specific T cell clones to examine this point at the clonal level. VP1/VLP-specific T cell clones were generated as described above. Initially 21 VP1/VLP-specific single cell-derived cultures were generated by limiting dilution and characterized for T cell receptor V beta expression, functional phenotype and fine specificity (Table 1). This characterization allowed the identification of 11 presumed different T cell clones. The number of single cell growing cultures corresponding to each T cell clone gives an indication of the frequency of each T cell clones in the brain infiltrate (Fig. 6A). T cell clone-4 was most abundant and represented by five colonies emerging from single growing wells, followed by T cell clone-2 with three single cell growing cultures, T cell clones-1, -8, -9 and -10 with two single cell growing cultures and finally T cell clone-3, -5, -6, -7 and -11 with only one single cell growing culture. The fine specificity of

Table I Characterization of VP1 specific brain-infiltrating TCC

TCC #	Well #	Th phenotype	TCR Vβ	Fine specificity
TCC-1	17A	Th1-2	Vβ2	$VP1_{34}$
	18A	Th1-2	Vβ2	$VP1_{34}$
TCC-2	16A	Th1	Vβ2	$VP1_{34}$
	28A	Th1	Vβ2	$VP1_{34}$
	18B	Th1	Vβ2	$VP1_{34}$
TCC-3	29A	Th1	Vβ18	$VP1_{54}$
TCC-4	10A	Th1-2	Vβ5.1	$VP1_{74\text{-}1}$, $VP1_{74\text{-}2}$, $VP1_{74\text{-}3}$
	14A	Th1-2	Vβ5.1	$VP1_{74\text{-}1}$, $VP1_{74\text{-}2}$, $VP1_{74\text{-}3}$
	27A	Th1-2	Vβ5.1	$VP1_{74\text{-}1}$, $VP1_{74\text{-}2}$, $VP1_{74\text{-}3}$
	30A	Th1-2	Vβ5.1	$VP1_{74\text{-}1}$, $VP1_{74\text{-}2}$, $VP1_{74\text{-}3}$
	19B	Th1-2	Vβ5.1	$VP1_{74\text{-}1}$, $VP1_{74\text{-}2}$, $VP1_{74\text{-}3}$
TCC-5	3A	Th1	Vβ -	$VP1_{91}$
TCC-6	11B	Th1	Vβ -	$VP1_{143}$
TCC-7	12B	Th1-2	Vβ2	$VP1_{229}$
TCC-8	21A	Th1-2	Vβ -	$VP1_{319}$
	25A	Th1-2	Vβ -	$VP1_{319}$
TCC-9	36A	Th1	Vβ -	$VP1_{319}$
	1B	Th1	Vβ-	$VP1_{319}$
TCC-10	19A	Th1-2	Vβ5.3	$VP1_{335}$
	3B	Th1-2	Vβ5.3	$VP1_{335}$
TCC-11	24A	Th1	Vβ -	$VP1_{335}$

the 11 T cell clones is summarized in Fig. 6B. Each T cell clone was tested against 64 15-mer peptides spanning VP1 protein and led to the identification of the following stimulatory peptides: $VP1_{34}$ (recognized by T cell clone-1 and -2), $VP1_{54}$ (recognized by T cell clone-3), $VP1_{74}$ (all $VP1_{74}$ peptides, recognized by T cell clone-4), $VP1_{91}$ (recognized by T cell clone-5), $VP1_{143}$ (recognized by T cell clone-6), $VP1_{229}$ (recognized by T cell clone-7), $VP1_{319}$ (recognized by T cell clone-8 and -9) and $VP1_{335}$ (recognized by T cell clone-10 and -11). Taking into account both the number of different T cell clones recognizing a specific peptide and the frequency of each T cell clone in the brain infiltrate, the immunodominant peptides recognized by VP1 specific brain infiltrating T cell clones were $VP1_{34}$, $VP1_{74}$, $VP1_{319}$ and $VP1_{335}$ confirming the fine specificity obtained using the brain-derived bulk cell population (Fig. 6C). Intracellular cytokine staining of these T cell clones revealed Th1-2 and Th1 phenotypes (Fig. 7A). Five T cell clones representing the 57% of the brain-derived VP1-specific single cell growing cultures showed a Th1-2-, and six T cell clones representing the 43% of the brain-derived VP1-specific single cell growing cultures, a Th1 phenotype (Fig. 7B). Specificity and functional phenotype did not correlate. For three of the immunodominant peptides ($VP1_{34}$, $VP1_{319}$ and $VP1_{335}$), we found T cell clones with both phenotypes. The T helper phenotype of Th1-2 and Th1 T cell clones was confirmed by measuring IL-4 and IFN-γ protein secretion by ELISA and by determining the expression of messenger RNAs of the transcription factors Gata3 and T-bet. Th1-2 T cell clones ($n = 5$) secreted IL-4 in addition to IFN-γ, while IL-4 secretion was barely detectable in Th1 T cell clones ($n = 6$) (Fig. 7C). Cytokine secretion profiles were not due to stimulation with phytohaemagglutinin, since the same stable patterns were observed after specific stimulation with VP1/VLP protein by intracellular cytokine staining or ELISA measurements from culture supernatants (Supplementary Fig. 8A and B). Transcription factor expression confirmed the phenotype of the T cell clones. Th1-2 T cell clones ($n = 5$) expressed messenger RNA for Gata3 and T-bet, while Th1 T cell clones ($n = 6$) only expressed T-bet (Fig. 7D).

Discussion

The viral aetiology of PML was shown almost 40 years ago, but still relatively little is known about the immune mechanisms that control JC virus infection. $CD8^+$ JC virus-specific cytotoxic T cells have been related to recovery from PML (Koralnik *et al*., 2002; Du Pasquier *et al*., 2004*a*), and two viral epitopes have been identified in HLA-A*02:01-positive individuals (Du Pasquier *et al*., 2004*a*, *b*). In contrast, limited information is available on the fine specificity and characteristics of JC virus-specific $CD4^+$ T cells in PML and even less in PML-IRIS (Jilek *et al*., 2010). The virus-specific T cell response at the site of infection, i.e. the CNS parenchyma, has not been examined at all.

Despite the limitation to a few patients and the bulk of the information deriving from Patient 2, our data provide novel insights into this subject and lead us to propose the following pathogenetic events during PML-IRIS under natalizumab treatment. The anti-VLA-4 antibody inhibits immune surveillance of JC virus infection at immunoprivileged sites, such as the brain, by blocking cell migration (Stuve *et al*., 2006) and local antigen presentation in the CNS (Del Pilar Martin *et al*., 2008). As a result, pathological neurotropic JC virus variants may lead to PML in a small number (1/500–1/1000) of treated patients with multiple sclerosis for reasons that are not yet understood (Ransohoff, 2005; Major, 2010). As soon as PML is suspected and natalizumab is stopped or actively removed by plasmapheresis, fully functional and activated T cells regain access to the CNS compartment, initiate the strong inflammation that is typical for PML-IRIS and effectively eliminate virus-infected cells by a number of mechanisms including $CD4^+$ and $CD8^+$ T cells and antibody-forming plasma cells.

Among the CNS-infiltrating T- and B cells, $CD4^+$ T cells with either Th1- or the above bifunctional Th1-2 phenotype are probably the most critical element based on the following findings. Their parallel secretion of Th1- (IFN-γ) and Th2 (IL-4) cytokines probably explains the expression of HLA-class II molecules on resident cells such as virus-infected astrocytes and microglia, but also on infiltrating immune cells (Fig. 2D), since IFN-γ is the strongest inducer of HLA-class II. Although colocalization studies of HLA-DR with an astrocytic marker such as glial fibrillary acidic protein could not be performed due the paucity of material, the widespread expression of HLA-DR (Fig. 2D) strongly suggests that these are also positive. In analogy to multiple sclerosis and its animal model experimental autoimmune encephalitis, where local reactivation of immigrating T cells has been demonstrated (Flugel *et al*., 2001), we assume that JC virus-specific Th1-2 and also Th1 cells are probably locally reactivated by recognition of JC virus peptides on JC virus-infected, HLA-class II positive astrocytes, microglia/macrophages or recruited dendritic cells. Furthermore, the secretion of large quantities of IL-4 leads to activation and expansion of

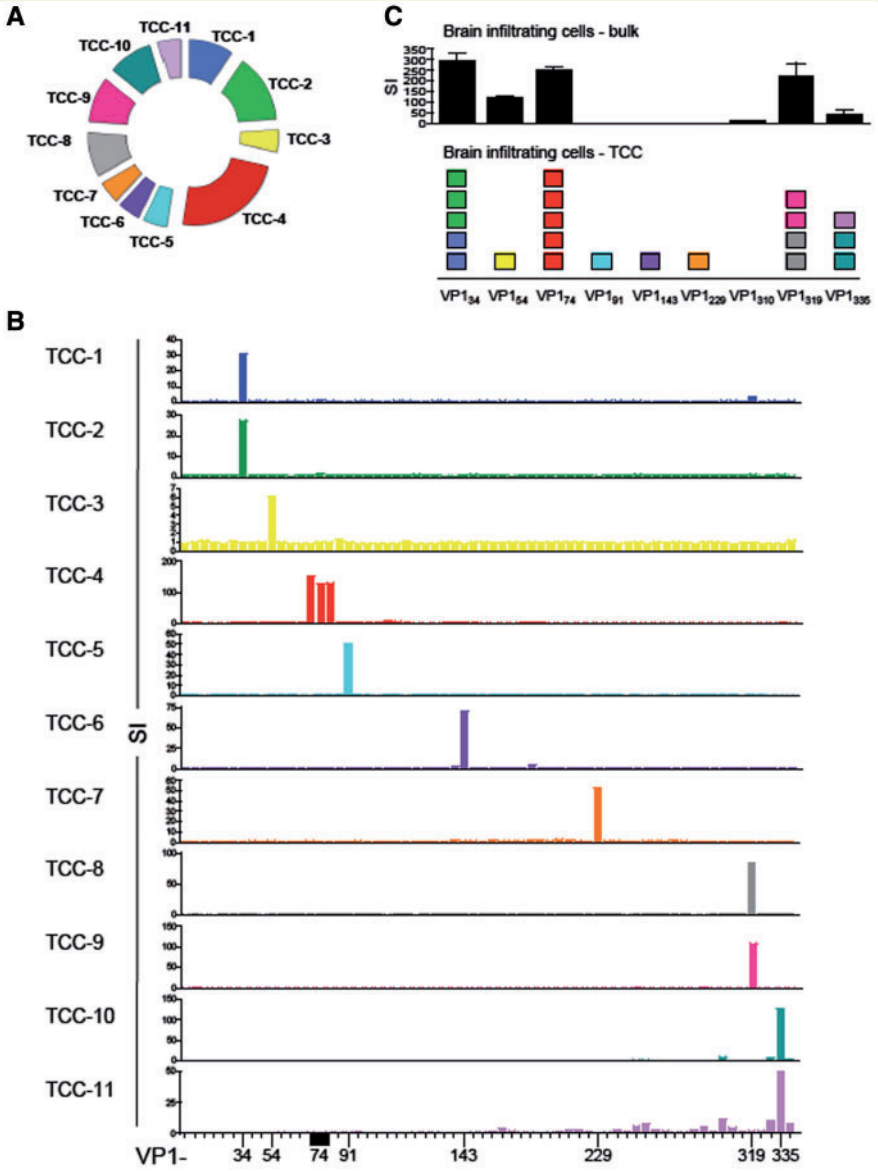

Figure 6 (**A**) Doughnut representing the frequency of each individual T cell clone in the brain biopsy. (**B**) Proliferative response of T cell clones against 64 individual VP1 peptides. Results show the mean stimulatory index ± SEM. (**C**) Schematic representation of the immunodominant peptides identified for the brain-derived bulk population (*upper*, show the mean stimulatory index ± SEM) and for the different T cell clones (*lower* graph; the different colours correspond to the different T cell clones and each single cell growing culture is represented by a square).

memory B cells/plasmablasts in the CNS compartment (Fig. 2G–I) with the consequence of virus-specific antibody secretion. Locally produced JC virus capsid protein (VP1)-specific IgG antibodies may recognize virus-infected oligodendrocytes, which could then be lysed by complement- or antibody-mediated cellular cytotoxicity. The relative increase in the CSF of IgG1 and IgG3 antibodies, which bind complement with high affinity and have been described in the context of other viral infections (Cavacini *et al*., 2003), supports this notion. The exact sequence of events, whether the functional priming of Th1 and Th1-2 cells occurs in the periphery or the CNS and whether IFN-γ and IL-4 jointly contribute to the strong IgG1 and IgG3 antibody secretion, cannot be proven by our data. However, the observation in patients with HIV with PML that higher JC virus-specific IgG production and JC virus-specific T cell responses at diagnosis were associated with better survival (Khanna *et al*., 2009) supports the above reasoning. Since infected oligodendrocytes do not express HLA-class II, but effectively express HLA-class I, it can be expected that JC virus-specific, HLA-A2-restricted CD8$^+$ cytolytic T cells (Koralnik *et al*., 2001, 2002) also contribute by killing JC virus-infected oligodendrocytes and/or astrocytes. That these previously described cells in the peripheral blood of patients with AIDS with PML are probably also participating in the local eradication of JC virus in the brain is supported by our observation of CD8$^+$

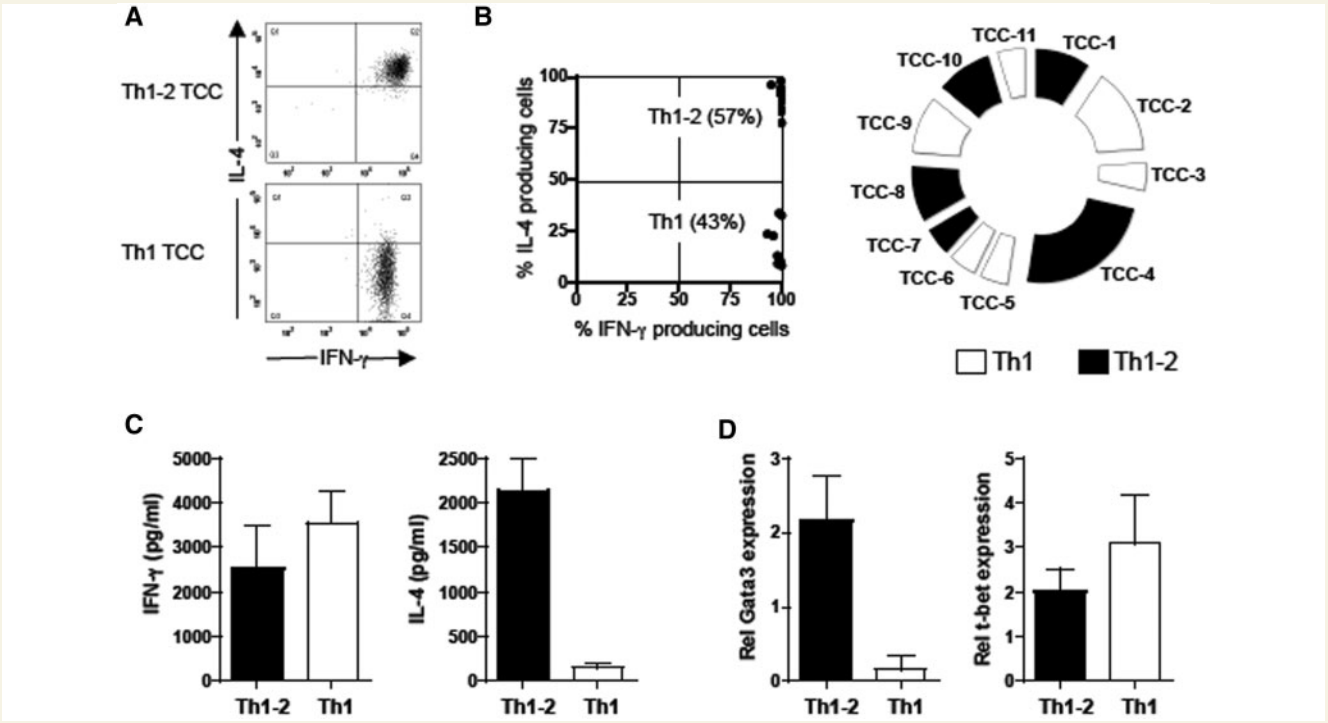

Figure 7 (**A**) Representative flow cytometry analysis of intracellular IFN-γ and IL-4 production by a Th1-2 (*top*) and a Th1 (*bottom*) VP1/VLP-specific CD4$^+$ T cell clone. (**B**) The dot plot represents the percentage of VP1-specific, brain-derived single cell cultures with Th1-2 and Th1 phenotype by intracellular cytokine staining. Each dot corresponds to one of the 21 single cell cultures analysed. The doughnut represents the functional phenotype of each T cell clone (TCC). (**C**) ELISA detection of IFN-γ and IL-4 production in culture supernatants of Th1-2 T cell clones ($n = 5$, black bars) and Th1 T cell clones ($n = 6$, white bars) 72 h after stimulation with phytohaemagglutinin. Results show the mean ± SEM. (**D**) Real-time polymerase chain reaction analysis for transcription factors Gata3 and T-bet of Th1-2 T cell clones ($n = 5$, black bars) and Th1 T cell clones ($n = 6$, white bars). Values are relative expression compared with brain-derived phytohaemagglutinin-expanded cells (calibrator = 1). Results show the mean ± SEM.

T cells specific for JC virus $VP1_{36}$ and JC virus VP_{100} as defined by peptide-loaded HLA-A*02:01 tetramers. Infected astrocytes may not only serve as local antigen presenting cells for CD4$^+$ virus-specific T cells, but may also be killed by Th1-2 cytolytic cells (Hemmer *et al*., 1997), but this together with the question of DR expression by astrocytes will require further examination.

The above pathogenetic scenario accounts for the effects of IFN-γ- and IL-4, i.e. the widespread expression of HLA-class II molecules in the brain as well as the strong intrathecal antibody response against JC virus, however, it is still puzzling that a large fraction of brain-infiltrating cells show a Th1-2 phenotype. Previously, these cells were referred to as Th0 cells and considered an intermediate differentiation step before naive cells develop into memory cells committed to either Th1 or Th2 lineage (Mosmann and Coffman, 1989). This notion has, however, already been contended early based on following the cytokine patterns of single clones (Kelso, 1995). Today, Th1- and Th2 cells are understood to have mutually exclusive fates (Ansel *et al*., 2006). However, individual T cell clones with dual cytokine secretion have been described as Th0 cells in measles virus infection (Howe *et al*., 2005) and among disease-exacerbating autoreactive T cells during altered peptide ligand-based therapy of multiple sclerosis (Bielekova *et al*., 2000). Our present observation of stable Th1-2 clones based on intracellular cytokine staining, cytokine secretion and transcription factor expression point to a defined T helper cell subpopulation in the CNS rather than an intermediate or transient differentiation stage. Due to the abovementioned ill-defined role of Th0 cells and the prior controversy about their existence as terminally differentiated cells, we propose here to refer to IFN-γ/IL-4 T helper cells as bifunctional Th1-2 cells. The context and signals that lead to this Th1-2 differentiation need further examination. In a recently published study in a viral infection model, the authors demonstrated that non-protective Th2 cells could be converted to stable IFN-γ/IL-4-expressing and protective CD4$^+$ cells by concerted action of antigen-specific T cell receptor signal, type I and –II interferons and IL-12 (Hegazy *et al*., 2010; Zhu and Paul, 2010). To our knowledge, our present report is the first evidence for the existence of a stable GATA-3$^+$T-bet$^+$ and IL-4$^+$IFN-γ^+ Th1-2 phenotype *in vivo* in humans. It is conceivable that these cells were reprogrammed in the brain, and they could well explain the unusually strong immune response and fulminant course of PML-IRIS. The putative protective role of Th1-2 bifunctional CD4$^+$ T cells in JC virus infection and where their differentiation occurs requires further investigation.

Regarding the fine specificity of brain-infiltrating T cells our data are interesting in several aspects. The JC virus-specific T cell response is overall broad since peptides from almost all JC virus proteins are recognized, which is consistent with our efforts to

map immunodominant epitopes of JC virus for peripheral blood-derived $CD4^+$ T cells in healthy donors and patients with multiple sclerosis (Aly *et al*., in preparation). However, >50% of peptides recognized by brain-derived $CD4^+$ T cells are part of the major structural protein VP1. Furthermore, VP1-specific T cells dominate with respect to strength of proliferation and precursor frequency. It is intriguing that $VP1_{34-48}$ contains not only a major epitope for cytotoxic, HLA-A*02:01-restricted $CD8^+$ T cells (Du Pasquier *et al*., 2003), which we found in the brain of the patient with PML-IRIS by tetramer staining, but also for HLA-DRB1*15:01/DRB5*01:01-restricted $CD4^+$ T cells. Furthermore, the recognition of peptide $VP1_{74}$ and two variants thereof with single amino acid substitutions indicates that recognition of this epitope may be relevant to protect the host from immune evasion during persistent JC virus infection. This has been shown previously for human immunodeficiency (Borrow *et al*., 1997) and lymphocytic choriomeningitis virus infections (Ciurea *et al*., 2001). The vigorous intrathecal antibody response against VP1 further underscores the role of this structural protein. Therefore, we speculate that VP1 is important for protective immune responses against JC virus-infected brain cells and that these are mediated by antibodies, $CD4^+$ and $CD8^+$ T cells. The strength of this response is probably in part determined by the HLA type of Patient 2, who expresses both the major multiple sclerosis risk allele DRB1*15:01/DRB5*01:01 and A*02:01, which present an identical VP1 epitope to $CD4^+$ and $CD8^+$ T cells. He may therefore have experienced a particularly pronounced T cell-mediated immune response in the brain with its immunopathological consequences of massive PML-IRIS, brain swelling (Fig. 1A, Supplementary Fig. 2C), and neurological worsening. As already pointed out by others (Cinque *et al*., 2003) the JC virus-specific immune response is a double-edged sword. Without a functional immune response brain cells are lysed by uncombated viral infection. On the other hand, if unleashed, the vigorous JC virus-specific response during PML-IRIS causes brain inflammation and oedema, and while it effectively eliminates JC virus from the CNS, it may lead to death of the patient if not at least temporarily attenuated by immunosuppression (Tan *et al*., 2009*b*).

The cellular and humoral JC virus-specific immune response in the brain during PML-IRIS not only complicates the treatment, but may also cloud the diagnosis of PML in the first place. Different from current routine, which relies on CSF JC virus viral load and, if a biopsy is performed, on immunohistochemistry and *in situ* hybridization for JC virus antigen and DNA, respectively, the intrathecal antibody response against VP1 appears more robust and should be examined. In both patients with PML-IRIS in this study, intrathecal VP1-specific antibody titres were extremely high despite almost undetectable JC virus DNA by polymerase chain reaction and *in situ* hybridization. The important role of JC virus antibody testing is supported by prior observations of high antibody titres in patients with AIDS with PML (Weber *et al*., 1997), but also by recent data in natalizumab-treated patients with multiple sclerosis (Gorelik *et al*., 2010).

Another important and unexpected observation of this study is that, unlike the JC virus-specific antibody response, pathogenetically relevant T cells are confined to the CNS parenchyma itself, and the CSF is of little use for investigating T cell specificity and function. This finding is probably highly relevant not only to PML-IRIS, but also to multiple sclerosis, where most studies have focused on CSF as a surrogate for the responses within the CNS from obvious reasons, i.e. because CNS tissue is rarely available to investigators. Future research should therefore make every possible effort to examine biopsy or autopsy tissue if it can be acquired. When studying the brain-infiltrating $CD4^+$ T cells of this patient with multiple sclerosis with PML-IRIS, we were further surprised to see that none of the peptides from three major myelin proteins were recognized, suggesting that bystander activation or recruitment of myelin-specific T cells during massive brain inflammation does not occur or is a minor part of the inflammatory response, while the majority of cells are exquisitely specific for the causal agent.

Based on our data in natalizumab-related PML-IRIS we suggest that immunomodulatory therapies that attenuate the vigorous T cell response should be implemented as soon as PML-IRIS is diagnosed. Unfortunately, there is no treatment that is supported by sufficient clinical testing. However, the use of drugs that inhibit viral replication such as mefloquine (Brickelmaier *et al*., 2009), but also type I interferons (O'Hara and Atwood, 2008) appears reasonable. While experience from a phase III trial, in which patients with multiple sclerosis received the combination of IFN-β and natalizumab, raised the suspicion that it may increase the risk for PML (Kleinschmidt-DeMasters and Tyler, 2005), transient interferon-β therapy could be useful in the case of natalizumab-associated PML-IRIS. It is well tolerated, probably has antiviral properties against JC virus (O'Hara and Atwood, 2008), blocks blood-brain barrier opening via inhibition of matrix metalloproteases (Waubant *et al*., 2003) and shedding of adhesion molecules (Calabresi *et al*., 1997), and has protective effects in the CNS during autoimmune encephalitis (Prinz *et al*., 2008). This suggestion needs clinical testing, and further therapies should also be assessed.

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Supplementary material

Supplementary material is available at *Brain* online.

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