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Broadly neutralizing human monoclonal JC polyomavirus VP1–specific antibodies as candidate therapeutics for progressive multifocal leukoencephalopathy

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Abstract

In immunocompromised individuals, JC polyomavirus (JCPyV) may mutate and gain access to the central nervous system resulting in progressive multifocal leukoencephalopathy (PML), an often fatal opportunistic infection for which no treatments are currently available. Despite recent progress, the contribution of JCPyV-specific humoral immunity to controlling asymptomatic infection throughout life and to eliminating JCPyV from the brain is poorly understood. We examined antibody responses against JCPyV major capsid protein VP1 (viral protein 1) variants in the serum and cerebrospinal fluid (CSF) of healthy donors (HDs), JCPyV-positive multiple sclerosis patients treated with the anti-VLA-4 monoclonal antibody natalizumab (NAT), and patients with NAT-associated PML. Before and during PML, CSF antibody responses against

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Author contributions: Ivan J., B.C., M.S., J.G., and R.M. designed the study. Ivan J. and R.M. drafted the manuscript. Ivan J., B.C., Ilijas J., R.N., T.S., J.G., and R.M. corrected the manuscript. R.N. and R.M. initiated the study. Ivan J., B.C., W.F., B.R. and L. Senn performed experiments and acquired and analyzed data. In particular, Ivan J. acquired data on VP1 variant recognition using VP1 pentamer ELISA and flow cytometry; B.C. isolated and characterized VP1-specific monoclonal antibodies with respect to affinity, epitopes, and BCPyV cross-reactivity; W.F. performed microscopy; and B.R. completed the neutralization assay. Ilijas J. followed the disease course and sample collection of PML and PML-IRIS patients, performed sequence analysis, and designed the phylogenetic tree based on antibody sequences. Ivan J. performed statistical analysis using GraphPad Prism. L. Ströh designed and purified the recombinant VP1 pentamers as well as elaborated structural images of VP1. M.S. and T.S. were involved in the experimental design and supervision of several aspects of the study. Ivan J., B.C., Ilijas J., W.F., B.R., M.S., J.G., and R. M. interpreted the data.

Competing interests: B.C., Ivan J., R.M., and J.G. are listed as inventors on a patent of the human monoclonal antibodies against JCPyV VP1 for the treatment of PML. J.G. and R.N. are employees and shareholders of Neurimmune. B.C. and L. Senn are employees of Neurimmune. The remaining authors declare no competing interests.

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JCPyV VP1 variants show “recognition holes”; however, upon immune reconstitution, CSF antibody titers rise, then recognize PML-associated JCPyV VP1 variants, and may be involved in elimination of the virus. We therefore reasoned that the memory B cell repertoire of individuals who recovered from PML could be a source for the molecular cloning of broadly neutralizing antibodies for passive immunization. We generated a series of memory B cell-derived JCPyV VP1-specific human monoclonal antibodies from HDs and a patient with NAT-associated PML-immune reconstitution inflammatory syndrome (IRIS). These antibodies exhibited diverse binding affinity, cross-reactivity with the closely related BK polyomavirus, recognition of PML-causing VP1 variants, and JCPyV neutralization. Almost all antibodies with exquisite specificity for JCPyV, neutralizing activity, recognition of all tested JCPyV PML variants, and high affinity were derived from one patient who had recovered from PML. These antibodies are promising drug candidates for the development of a treatment of PML.

INTRODUCTION

JC polyomavirus (JCPyV) establishes lifelong persistent infection of the kidney in a large fraction of the healthy population without known clinical consequences (1). In acquired or hereditary immunodeficiency such as AIDS, cancer, CD4 lymphopenia, or monoclonal antibody therapy, archetypal JCPyV may acquire mutations. Whether these mutations are a prerequisite for central nervous system (CNS) entry or occur in the CNS is not clear. Mutated JCPyV variants (JCPyV_{PML/GCN}) cause lytic infection of glial cells or cerebellar granule neurons resulting in progressive multifocal leukoencephalopathy (PML) and granule cell neuronopathy (GCN), respectively (2–5). Immunomodulatory or immunosuppressive treatments with specific monoclonal antibodies including efalizumab, rituximab, and particularly natalizumab (NAT) have been shown to increase the risk for PML/GCN, underscoring that JCPyV infection of the CNS may occur when immune surveillance by virus-specific T cells and/or antibodies is perturbed (5–8). Both PML and GCN may be fatal if the underlying immune suppression is not resolved.

To overcome PML, measures to boost general immune competence such as infusion of recombinant interleukin-2 (IL-2) (9) and IL-7 (10, 11) and administration of polyvalent intravenous immunoglobulins (12), as well as infusion of JCPyV-specific cytotoxic T cells (13) and a combination of active vaccination with JCPyV VP1 and recombinant IL-7 (14), have been applied and have shown promise. These data, together with the abovementioned studies, indicate that specific immune recognition by T cells and antibodies is critical for terminating PML. In patients with AIDS and those with NAT-associated PML (NAT-PML), restored CD4⁺ T cell function and recovering immune surveillance of the CNS after NAT washout not only can lead to a so-called PML immune reconstitution inflammatory syndrome (IRIS), which is characterized by massive infiltration of T and B cells leading to prominent inflammation of the JCPyV-infected CNS tissue that efficiently eliminates JCPyV, but also often results in acute neurological deterioration and additional brain damage and can even lead to death due to tissue swelling (15–18).

Attempts to treat PML with the antivirals mefloquine and mirtazapine have all failed (19), and no effective therapy is currently available. Observations from AIDS patients who can

recover from PML once CD4⁺ numbers and virus-specific immunoglobulin G (IgG) titers rise under antiretroviral therapy (5, 7), as well as data on PML from NAT-treated multiple sclerosis (MS) patients who eliminate JCPyV once the anti-VLA-4 monoclonal antibody has been washed out, suggest that regaining immunocompetence and allowing JCPyV-specific T cells access to the brain are critical factors to recover from PML. According to immunological studies, JCPyV-specific CD8⁺ and CD4⁺ T cells and, associated with the latter, also JCPyV-specific IgG titers have been implicated in recovery from PML (6–8, 17), although the specific contributions of each component of the adaptive immune system are not fully understood.

PML incidence steadily declined in HIV-infected individuals with the introduction of antiretroviral therapy, but emerged as a major medical concern during monoclonal antibody therapy, particularly in NAT-treated MS patients (5). More than 560 PML cases have been reported (20). In more than 20% of patients, PML was fatal, and in a large fraction, it led to severe residual neurological deficits (21). In both AIDS patients and NAT-PML patients (17), the major capsid protein of JCPyV VP1 has been identified as the most important target antigen for CD4⁺ and CD8⁺ T cells in several studies (17, 22–24). Whereas the role of JCPyV-specific antibodies has remained less clear, the abovementioned data indicate that CD4⁺ T cell numbers and function and JCPyV-specific IgG titers correlate with recovery from PML of AIDS patients (7). In NAT-treated MS patients, risk stratification strategies have been based on previous exposure to immunosuppressive drugs, length of NAT treatment, and JCPyV-specific antibody indices (25, 26). About 54% of MS patients are JCPyV-seropositive (26–28). Both JCPyV-specific serum antibody levels and intrathecal antibody production are increased in patients at the onset of NAT-PML (28, 29). The latter finding and the fact that pre-PML samples revealed sustained higher JCPyV-specific antibody indices over time (26) question the role of JCPyV-specific antibodies in the prevention of PML development and the clearance of JCPyV infection from the brain. However, no data are available on the functional properties of JCPyV-specific antibodies with respect to neutralization, affinity, and target epitopes, neither for HDs, MS patients, nor PML patients.

Besides the quality and strength of the adaptive immune response of the infected host, the characteristics of the virus play an important role in PML. Archetypal or wild-type virus, which causes lifelong, clinically inapparent infection of the epithelial cells in the kidney and urinary tract, has to undergo mutations to be able to cause PML (30). PML-associated JCPyV genotype variants show characteristic mutations of VP1 and rearrangements of the noncoding regulatory region (NCCR), which are probably responsible for preferential tropism for glial cells or neurons and propagation in the CNS (31–33). Furthermore, rearranged NCCRs constitutively activate early gene expression and increase viral replication rate (34). It is not known, however, whether mutations in VP1 of PML-causing JCPyV variants influence the immune surveillance by JCPyV VP1-specific antibodies.

On the basis of the above considerations, it is highly desirable to establish prophylactic and therapeutic immunization regimens to avoid the onset of PML or eliminate the virus once it has infected the CNS. Such active and passive vaccinations should either stimulate protective adaptive immunity or, in the case of ongoing PML, be administered as a

treatment. In patients with underlying hereditary or acquired immunodeficiency, we reasoned that active immunization with JCPyV VP1 may only be effective if combined with measures that allow a certain degree of immune reconstitution. Our recent data from compassionate use vaccinations of PML patients with idiopathic CD4 lymphopenia or immunodeficiency after cancer and stem cell transplantation support this rationale (14). Administration of human recombinant IL-7 as a globally immune-reconstituting agent combined with active immunization with JCPyV VP1 and a Toll-like receptor 7/9 agonist as adjuvant (imiquimod) led to the clearance of JCPyV from the CNS and recovery from PML (14). However, active immunization with JCPyV VP1 may only be effective either if the patient has residual immune function or if the latter is restored by cytokines such as IL-7 and IL-2 (9–11). If JCPyV-specific antibodies play a role in neutralizing free infectious virions and/or clearing the virus from the CNS during PML, we assumed that the therapeutic administration of JCPyV-specific antibodies, that is, passive vaccination, may be an effective and safe measure that could immediately be applied once PML has been diagnosed. Support for this assumption stems from a patient with idiopathic CD4 lymphopenia, whom we successfully vaccinated with JCPyV VP1/virus-like particles (VLPs), imiquimod, and recombinant IL-7, as previously shown (14). In this patient, the JCPyV-specific antibody response rapidly increased after vaccination and broadened with respect to recognition of PML variants including the one infecting the patient, and in parallel to the increasing antibody response, the patient's cerebrospinal fluid (CSF) JCPyV viral load declined to zero (35).

The present study aimed to develop a better understanding of the biological role of JCPyV-specific antibodies in the onset/termination of PML. Further, we speculated that NAT-treated MS patients who developed PML and, after IRIS, eliminated the virus should harbor antibodies that are suitable for passive vaccination. For this purpose, we evaluated the antibody response in the sera and CSF of healthy donors (HDs), NAT-treated MS patients, NAT-PML patients, and NAT-associated PML-IRIS (NAT-PML-IRIS) patients against various JCPyV VP1 variants to identify donors for the molecular cloning of JCPyV-specific human monoclonal antibodies from memory B cells after recovery from PML. We characterized 30 human JCPyV VP1-specific monoclonal antibodies with respect to their functionality and specificity profiles and selected lead candidates for the further development of a potential treatment of PML patients or patients at risk for PML.

RESULTS

Humoral immune response against JCPyV VP1 variants is compromised in PML

We developed a capture enzyme-linked immunosorbent assay (ELISA) using recombinant JCPyV VP1 variants including the prototypic neurovirulent MAD1 strain and a kidney isolate strain (WT3), as well as three of the most frequently occurring PML-associated VP1 variants harboring the mutations L55F, S267F, or S269F in the background of VP1_{MAD1} (36, 37). Equivalence in purity and quantity of the recombinant VP1 proteins was demonstrated by gel electrophoresis (Fig. 1A). An HD serum was selected as reference standard with equivalent concentration-dependent binding to all VP1 variants (Fig. 1B). Screening with VP1_{MAD1} revealed an increase of anti-JCPyV VP1 antibodies in the serum of NAT-PML

patients and even higher levels in NAT-PML-IRIS patients, compared to HDs and JCPyV-seropositive MS patients under NAT treatment (Fig. 1C and Table 1), consistent with a previous report (28). The sera were further analyzed for binding activity to VP1 variants after normalization of their response against prototypic MAD1. Responses widely varied between individuals (Fig. 1D). The average reactivity to VP1_{WT3} did not differ between groups and was similar to responses against VP1_{MAD1}. In contrast, reduced serum responses against the PML-associated variant VP1_{L55F} were observed in NAT-PML, indicating compromised immune recognition. Both serum antibody responses to variants L55F and S269F were lower in JCPyV-seropositive NAT-treated MS patients (Fig. 1D). The JCPyV variant VP1_{S267F} was poorly recognized by all groups, suggesting that the S267F mutation affects an immunodominant epitope that is targeted by a large fraction of the JCPyV VP1-specific antibody repertoire. Recognition patterns of individual NAT-PML patients varied from robust humoral responses against all variants (patient 4), to stronger recognition of one VP1 variant but lower response to others (patients 8 and 12), to low responses to all PML-associated mutations (patients 7 and 13) (Fig. 1E).

Immune reconstitution leads to broadened antibody responses against PML-causing JCPyV VP1 variants

It is important to note that the serum antibody responses against VP1_{L55F} and VP1_{S269F} increased during immune reconstitution (NAT-PML-IRIS) (Fig. 1D). Intrathecal JCPyV VP1-specific antibody titers also increased upon immune reconstitution from NAT-PML to NAT-PML-IRIS (Fig. 2A). Whereas intrathecal antibodies against VP1_{L55F} were lower compared to MAD1 in NAT-PML patients, similar to what we observed in the serum, these responses increased and were higher in NAT-PML-IRIS patients (Fig. 2B). Because the above analysis, which compares the antibody response against JCPyV VP1 variants to VP1_{MAD1}, does not allow us to judge whether antibodies are synthesized at higher levels in the CSF compared to serum, we assessed the intrathecal antibody production against different JCPyV VP1 proteins. In line with a previous report (28), about half of the NAT-PML patients (6 of 14) showed signs of intrathecal antibody production [JCPyV-specific CSF/serum antibody index (CAI_{JCPyV}) >1.5] against both JCPyV VP1_{MAD1} and VP1_{WT3} (Fig. 2C, first and second panels). We then extended this analysis to the JCPyV PML variants, and again, a substantial fraction of NAT-PML patients showed intrathecal antibody responses against all three mutant VP1 proteins (Fig. 2C, third to fifth panels). Most important in the context of this study, the intrathecal antibody responses against all VP1 proteins (MAD1, WT3, and mutant proteins) increased 10-fold or more during NAT-PML-IRIS in seven of eight patients, indicating a high-titer and broad antibody response directed against multiple variants in the CNS compartment upon immune reconstitution.

Generation and characterization of human monoclonal antibodies against JCPyV reveal different binding properties

The above data indicate that our original assumptions were likely correct in that patients who eliminated JCPyV from the CNS after PML-IRIS mount robust and broad antibody responses against several JCPyV VP1 proteins including nonpathogenic JCPyV archetype (WT3), prototypic PML variant MAD1, and frequent VP1 PML-causing mutations. The observation that the antibody responses are even stronger in the CNS compartment than in

the serum further hints at their biological relevance. We therefore selected a NAT-PML-IRIS patient (for patient information, see Supplementary Materials and Methods) who had successfully controlled PML and showed strong antibody responses to JCPyV VP1. From this individual and selected HDs, we isolated memory B cells expressing VP1-specific antibodies to identify broadly neutralizing antibody lead candidates for passive immunization.

As expected, on the basis of seroprevalence in the general population, we could identify B cells expressing antibodies binding to JCPyV VP1 VLPs from most of the HDs (27 of 40). The frequency of JCPyV VP1-reactive memory B cells increased more than 10-fold in the NAT-PML-IRIS patient, suggesting an efficient antibody response. We cloned and recombinantly expressed 30 human-derived monoclonal antibodies (10 from HDs and 20 from the NAT-PML-IRIS patient) and characterized their binding affinities toward JCPyV and BKPyV VP1 VLPs and their specificity to intact or denatured VLPs.

Most of the antibodies from the NAT-PML-IRIS patient showed extremely high affinity for the JCPyV VP1 VLPs with a half-maximal binding (EC_{50}) down to the femtomolar range (Fig. 3A) and weak or no binding to BKPyV VP1 VLPs. These antibodies were only able to recognize intact VLPs and did not show binding to denatured VLPs on ELISA or Western blot after denaturation with SDS-PAGE, suggesting the targeting of conformational epitopes specific to the JCPyV VP1 capsid (Fig. 3A).

A second class of antibodies showed similarly high affinity not only to JCPyV but also to BKPyV VP1 VLPs with EC_{50} in the low picomolar range. These antibodies also bound only to intact VLPs and did not recognize any linear epitope. Thus, we classified this second category as antibodies that specifically recognize JCPyV and BKPyV VP1 VLPs with high affinity and target a conformational epitope that is shared by the capsids of both viruses (Fig. 3B).

In contrast to the antibodies from the NAT-PML-IRIS patient, antibodies from HDs displayed a wide range of binding affinities with EC_{50} values in the nanomolar range. They did not discriminate between intact and denatured VLPs by ELISA and recognized JCPyV as well as BKPyV VP1 proteins. These data suggest a profound difference in the memory B cell repertoires in HDs versus NAT-PML-IRIS patients, with most of the antibodies generated by HDs targeting linear epitopes that are mostly shared between JCPyV and BKPyV (Fig. 3C).

Human JCPyV-specific monoclonal antibodies inhibit JCPyV infection in vitro

To assess the neutralizing ability of the recombinant human-derived monoclonal antibodies, we established an infection assay using JCPyV_{MAD4} strain infection of the human fetal astrocyte-derived glial cell line SVG-A (Fig. 4A, top middle panel). Antibodies that were previously determined to target a conformational VP1 capsid epitope completely abolished JCPyV infection, whereas antibodies recognizing linear epitopes did not, comparable to an unrelated isotype control antibody (Fig. 4A). Whereas only one of the antibodies cloned from HDs was capable of blocking JCPyV infection, most of the antibodies (18 of 20)

cloned from the NAT-PML-IRIS patient showed complete neutralization of the viral infection (Fig. 4B, right panel).

Human monoclonal antibodies show cross-reactivity against multiple JCPyV VP1 variants

The structure of JCPyV VP1 highlights three exterior loops at the outer surface of the JCPyV capsid, which are in principle accessible as epitopes for VP1-specific antibodies (fig. S1). The most common PML-associated mutations, such as L55F, S267F, and S269F, are located in these loops (fig. S1), which are involved in host receptor binding (36), explaining why these mutations from an aliphatic to an aromatic (L55F) or a relatively small polar (S) to a large aromatic amino acid (F) in positions 267 and 269 have a strong impact on antibody recognition. We therefore tested the monoclonal antibodies for binding to the respective VP1 mutants by ELISA. In line with the above data on serum and CSF antibodies, we confirmed at the level of the individual monoclonal antibody that most of the VP1-specific antibodies bound with reduced affinity or even completely failed to recognize some of the VP1 variants in comparison to VP1_{MAD1} (Fig. 5A). The observed reduction in antibody binding was independent of the affinity established for JCPyV_{MAD1} VLP. Consistent with the much weaker recognition of VP1_{S267F} by serum and CSF antibodies in HDs and patients, binding to S267F seemed to be affected in most of the antibodies derived from the NAT-PML-IRIS patient; however, the absence of antibody recognition was also observed for other VP1 variants in some monoclonal antibodies.

The observation that the mean response of the NAT-PML-IRIS patient-derived antibodies closely matches the autologous serum response underscores that memory B cells, which had been picked for the generation of the monoclonal antibodies, mirrored the humoral immune response in vivo (Fig. 5B). During NAT-PML-IRIS, several antibodies developed with increased binding to some VP1 variants compared to MAD1, in particular to VP1_{S269F} (Fig. 5B). Reduced antibody binding to some of the VP1 variants was also observed in HD-derived monoclonal antibodies but was less pronounced than in the NAT-PML-IRIS patient-derived monoclonal antibodies (Fig. 5C). Nonneutralizing antibodies (for example, 72F7, 43E8, and eight of nine HD-derived antibodies) were less affected in their recognition of VP1 variants, indicating that the epitope of these antibodies is probably located outside of the mutation-associated exterior loops of VP1.

Regarding the overall goal of our study, it is most important that we identified five monoclonal antibody candidates (27C11, 47B11, 26A3, 50H4, and 98H1) derived from the NAT-PML-IRIS patient, which revealed not only high affinity to JCPyV VLP and potent neutralization capacity but also very efficient recognition of all five VP1 variants. Furthermore, we could demonstrate this broad binding also to additional VP1 variants with mutations located within the exterior loops (for example, VP1_{N74S}, VP1_{R75K}, and VP1_{T117S}) by using a complementary approach with pCAG-JCPyV-transfected cells (38) combined with intracellular staining and flow cytometry (fig. S2).

On the basis of the established binding profiles of the monoclonal antibodies, we tested whether the broadly recognizing antibodies target shared or independent binding regions. Toward that end, we performed competition experiments assessing whether selected antibodies could recognize JCPyV VLPs after saturation with the antibody 98D3. Although

antibody 72F7 displayed an unaltered binding profile, antibodies 27C11, 47B11, 26A3, 50H4, and 98H1 were unable to bind to the 98D3-bound VLPs, suggesting that they target the same binding pocket in JCPyV VP1, whereas the nonneutralizing 72F7 antibody binds to a distinct epitope (fig. S3). However, it is unlikely that these antibodies bind to the exact same amino acid residues as they display different recognition profiles of PML-associated VP1 mutants.

Further characterization of the antibodies with respect to their sequence revealed that at least 10 of 20 immunoglobulin heavy chain (IGH) clones isolated from the NAT-PML-IRIS patient originated from different germline sequences (Table 2). This indicates that a broad spectrum of B cell clones is involved in mounting a JCPyV-specific humoral immune response with a variable degree of affinity to JCPyV VP1 variants and in neutralization and cross-recognition of JCPyV VP1 variants and wild-type BKPyV VP1 (Fig. 6). Among all these recombinant antibodies, a subgroup of evolutionary convergent antibodies (98H1, 50H4, and 27C11) represent highly promising candidates for the development of a broadly neutralizing passive immunotherapy against JCPyV.

DISCUSSION

The occurrence of PML in diseases such as systemic lupus erythematosus (SLE) during immunomodulatory treatments, which affect B cells (5, 6), and in immunodeficiencies that primarily involve antibodies [such as Franklin disease (39)] or B cells/antibodies and T cells [such as Good's syndrome (40) and hyper IgE syndrome (41)] indicates that not only T cells but also B cells are possibly involved in the development of PML. NAT not only compromises CNS immune surveillance by reducing the migration of T cells, CD19⁺ B cells, and CD138⁺ plasma cells through the blood-brain barrier (42) but also perturbs peripheral B cell niches by increasing the number of memory- and marginal zone-like B cells (43). The release of CD34⁺ progenitor cells into the peripheral blood may favor the evolution and reactivation of neurotropic JCPyV variants (44, 45). Rituximab depletes CD20⁺ B cells and perturbs B cell homeostasis (5, 46); however, it is currently unknown whether the B cell depletion by rituximab results in PML because of reduced antibody responses against JCPyV, which is less likely, or perturbation of other B cell functions such as antigen presentation to T cells (47). Hence, B cells could be involved in multiple steps of PML development from viral mutation to PML variants, to the transport of glial/neurotropic JCPyV variants into the CNS, or both. Further, the underlying diseases (for example, HIV/AIDS or idiopathic CD4 lymphopenia), or specific immune interventions such as NAT or chemotherapy probably play a role as well.

Regarding their protective function, several, albeit mostly indirect, lines of evidence indicate that B cells and JCPyV-specific antibodies play a role. These include the observations that IgG responses and CD4⁺ T cell counts positively correlate with survival in HIV/AIDS patients (7) and that CD19⁺ B cells, CD38⁺ plasmablasts, and CD138⁺ plasma cells are found in the immune cell infiltrate in the brains of PML-IRIS and GCN-IRIS patients (17, 18, 48). Further support stems from the analysis of the T cell infiltrate in the brains of PML-IRIS and GCN-IRIS patients (17, 48). During IRIS, CD4⁺ T cells with a T helper 1/2 phenotype (that is, cells that secrete both interferon- γ and IL-4, which is important to mount

B cell responses) are particularly prevalent, and it is of interest to note that most of these cells are directed against JCPyV VP1 epitopes (17, 49). Consistent with previous data (26, 28), we found an increase of JCPyV-specific antibodies in the serum when PML occurs. Probably more importantly, intrathecal JCPyV-specific antibody titers strongly rise and are considered as diagnostic evidence of ongoing PML in patients with clinical findings compatible with PML and, at the same time, with low or absent JCPyV DNA in the CSF, which is usually the case when IRIS has begun (12, 17, 28). Finally, the rise in CSF JCPyV antibodies with specificity against several PML variants including the one found in the CSF in a patient after vaccination with JCPyV VP1/VLP (35) argues that a B cell/antibody response in the CNS compartment plays an active role in containing and/or eliminating the virus.

JCPyV VP1-specific antibody responses in the serum are used for risk stratification of patients who are treated with NAT (25). Patients with intermediate antibody indices have a much lower risk of developing PML, whereas those with high indices show an elevated risk (26). It is currently not clear whether this unexpectedly higher PML risk in the presence of higher JCPyV VP1-specific antibody titers indicates that antibodies are functionally deficient (that is, not capable of neutralizing the virus) or whether they are directed against the VP1 that is used in the assay (MAD1 strain) but fail to bind other relevant mutant strains. Regarding risk stratification, JCPyV viremia has been observed in patients seronegative for anti-JCPyV VP1 antibodies, indicating that testing JCPyV-specific antibodies might not be sufficient to assess the status of JCPyV infection in NAT-treated MS patients (50). Our data show that VP1 mutations of JCPyV probably play a critical role in determining whether a humoral response is protective or not. JCPyV VP1 mutations are frequently found in addition to prototype MAD1 sequences in the CNS of PML patients (32, 33). In contrast to archetypal JCPyV strains that are excreted in the urinary tract, PML-associated JCPyV genotypes present characteristic mutations of VP1 and rearrangements of the noncoding regulatory region, which probably allow entry to and propagation in the CNS as well as preferential tropism for glial cells or neurons (3, 31, 32). To date, it is not clear whether PML-associated alterations of the JCPyV genome occur inside or outside of the CNS and which mechanisms contribute to their occurrence or selection (32, 33, 45). Besides influencing cellular tropism and CNS invasion, the individual heterogeneity in serum and CSF antibody responses against the most frequently found JCPyV VP1 mutations at positions 55, 267, and 269 indicates that antibody “recognition holes” likely play a role in PML. This notion is supported by the data of Ray *et al.* (35) showing that PML patients fail to produce antibodies recognizing their “own” PML mutant. Particularly, JCPyV VP1_{L55F} and VP1_{S267F} are recognized less well by the sera and CSF in NAT-PML, and whereas the responses to JCPyV VP1_{L55F} rise in NAT-PML-IRIS, the recognition of VP1_{S267F} remains lower, even despite the strong intrathecal antibody responses during NAT-PML and a further increase in NAT-PML-IRIS against all tested JCPyV VP1 variants.

The above data indicate that JCPyV-specific antibodies play an important role in controlling JCPyV infection and probably also in containing and eliminating virus from the brain once PML has developed. In MS patients, a recent proof-of-concept study demonstrated that 29% of sera from MS patients lack anti-JCPyV neutralizing activity despite the presence of anti-JCPyV VP1 antibodies (51). Hence, the functional properties of JCPyV antibodies (that is,

their neutralizing activities) and their breadth regarding recognition of multiple JCPyV PML variants may determine how well JCPyV infection is controlled.

NAT remains one of the most effective and generally well-tolerated therapies, but the impaired immune surveillance of the CNS leads to a PML risk between 1/1000 to 1/100 or even higher for patients who have been treated for longer than 2 years and after prior immunosuppression (25). Once PML occurs, immune surveillance is restored after antibody washout by plasmapheresis, and when NAT-PML-IRIS begins, JCPyV-infected cells are recognized by virus-specific CD4⁺ and CD8⁺ T cells with preferential specificity for JCPyV VP1 and a strong intrathecal JCPyV VP1-specific antibody response (17). NAT-PML-IRIS carries the risk of too vigorous immune responses with subsequent brain damage and clinical worsening and death from acute brain swelling. For these reasons and different from patients with underlying immunodeficiencies or compromised immune function, active immunization with JCPyV VP1, for which we recently provided promising preliminary data in immunocompromised patients (14), carries risks and would not be recommendable. However, a treatment with JCPyV VP1-specific antibodies could be applicable to all patients with PML immediately after their diagnosis, with a significantly lower expected risk of clinical liabilities including IRIS, although high doses of antibody may be needed because of the limited access of antibodies through the blood-brain and blood-CSF barriers. Furthermore, because almost all NAT-PML-IRIS patients are capable of eliminating JCPyV from the CNS and recover from PML, we assumed that they would represent an optimal resource for the generation of recombinant therapeutic antibodies with ideal drug-like properties combining high-affinity targeting of the VP1 capsid, biological neutralization activity, and broad cross-recognition of clinically relevant JCPyV PML variants. The molecular engineering technology applied to generate the human-derived antibodies preserves the favorable immunobiological features of the human antibody response, including epitope selection, affinity maturation, and tolerance mechanisms, creating a set of drug candidates with optimal biophysical, pharmacological, and safety properties. When we compared the memory B cell repertoires from the HDs and the NAT-PML-IRIS patient using JCPyV and BKPyV VLPs, we were surprised to find that the human antibodies cloned from HDs (that is, during the state of persistent JCPyV infection of the kidney) were of relatively low affinity and cross-reactive with BKPyV and that only one of the antibodies had neutralizing activity. In contrast, the antibodies cloned from the NAT-PML-IRIS patient showed, with few exceptions, high affinity; many showed broadly neutralizing activity and were highly specific for JCPyV VP1 but failed to recognize BKPyV VP1. This is in agreement with the sequence differences between the BKPyV and JCPyV VP1 pentamers, which mostly map to surface-exposed loops that display mutations in PML and would be likely targets for antibody neutralization (fig. S4).

Regarding antibody function, few data are currently available; however, the IgG subclass distribution in the CSF of two patients with NAT-PML-IRIS, who recovered well from the infection (17), showed that IgG1 and IgG3 were most prominent, which may indicate, in addition to direct virus neutralization, effector functions including opsonization, complement activation, phagocytosis, and/or antibody-dependent cellular cytotoxicity. It is also not clear whether JCPyV-specific antibodies act primarily by neutralizing extracellular or intracellular virions in infected cells or by inhibiting the binding of the mutant viruses to a

yet unknown receptor, which is different from the glycan receptor LSTc (sialylneolacto-N-tetraose c), particularly to the sialic acid moiety of that receptor (52). The latter point is supported by the fact that infection of oligodendrocytes and astrocytes during PML has been proposed to be LSTc-independent (53). Antibodies against intracellular and nuclear epitopes are, however, well documented in autoimmune diseases (54), and a recent study showed that the intracellular antibody receptor tripartite motif-containing protein 21 (TRIM21), a ubiquitously expressed E3 ubiquitin ligase, provides a mechanism to protect mice from lethal adenoviral infection through intracellular antibody recognition (55). Hence, JCPyV-specific antibodies may contribute to both extracellular and intracellular mechanisms in PML.

Consistent with our above assumptions, a biologically different antibody repertoire had been generated during NAT-PML and subsequent NAT-PML-IRIS. Considering the reduced recognition of JCPyV PML variants VP1_{L55F} and VP1_{S267F} and the general lack of recognition of different JCPyV VP1 variants (Fig. 1E) by serum antibodies from HDs and NAT-PML patients, we defined a set of criteria for the selection of lead antibody candidates for future therapeutic development: (i) recognition of conformational capsid epitopes of a broad spectrum of JCPyV VP1 PML variants, (ii) high binding affinity, and (iii) biological activity in neutralization of JCPyV infectivity. 27C11 represents the most promising human-derived monoclonal antibody that was identified in the present study. It shows low picomolar affinity, neutralizing activity, and equivalent recognition of all JCPyV VP1 variants examined (Fig. 5A), and beyond these, it also recognizes cells transfected with the pseudoviral constructs (pCAG-VP1) of VP1 variants, VP1_{N74S}, VP1_{R75K}, VP1_{T117S}, and VPN_{GcN} (fig. S2).

We did not find other examples of CNS infections where broadly neutralizing antibodies developed during the course of infection; however, such antibodies arise in HIV-infected individuals independent of their germline repertoire (56) and are characterized by long heavy-chain complementarity-determining region 3 (CDR3), polyreactivity, and high levels of somatic mutations (57), which is also the case for the antibodies described here.

Limitations of the present study include the moderate number of PML and PML-IRIS patients studied; that neutralization was only shown against infection with a prototypic JCPyV PML strain, although broad recognition of the most prevalent JCPyV PML strains has been tested with different methods; and the limited understanding of the contribution of the humoral immune response and JCPyV-specific antibodies during PML and the exact mechanism of action—that is, whether they act primarily extracellularly and inhibit propagation of infection or also have antiviral effects intracellularly in infected cells. Proof-of-concept clinical testing will shed light on this point. However, together with our data on active vaccination with JCPyV VP1 (14, 35), the above findings provide insight about the role of the humoral immune responses during PML and lay the foundation for the development of targeted immunotherapy for PML with a newly identified class of broadly neutralizing human recombinant monoclonal antibodies.

MATERIALS AND METHODS

Study design

This study was a nonrandomized laboratory study designed to investigate the antibody responses in the sera and CSF of HDs and patients with MS, NAT-PML, or NAT-PML-IRIS and to recombinantly clone and characterize human-derived antibodies targeting JCPyV VP1 for further development toward a treatment of PML. A newly developed JCPyV VP1 pentamer ELISA was used to evaluate humoral responses against PML-associated JCPyV variants in serum and CSF samples. JCPyV-specific human monoclonal antibodies were then cloned from memory B cells of selected HDs and a NAT-PML-IRIS patient, who had recovered from PML, and further analyzed for affinity, specificity, and functional properties by BkPyV/JCPyV VLP ELISA, Western blot, JCPyV VP1 pentamer ELISA, fluorescence-activated cell sorting of transfected VP1 variants, and a JCPyV neutralization assay. The sample size was dictated by the rate of sample collection [generally, 2 to 5 ml of serum, 5 to 10 ml of CSF, and 50 to 80 ml of peripheral blood for isolation of peripheral blood mononuclear cells (PBMCs)], and blinding was not used.

Study subjects

Peripheral blood from MS patients under NAT treatment, NAT-PML patients, and NAT-PML-IRIS patients and matched HDs, as well as CSF samples from NAT-PML and NAT-PML-IRIS patients, were obtained after written informed consent of patients and approval by the Cantonal Ethical Committee of Zürich, Switzerland (EC-ZH no. 2013–0001). MS patients under NAT therapy were all seropositive for JCPyV antibodies (Unilabs). The demographic data of all donors are shown in Table 1. The samples at onset of NAT-PML-IRIS were obtained from the corresponding NAT-PML cases after washout of NAT as well as clinical and radiological signs of IRIS. Peripheral blood was used to collect the serum and PBMCs for the isolation of memory B cells.

Cells and virus

SVG-A (transformed human fetal astrocytes; provided by R. Girones, University of Barcelona, Spain) and HEK 293TT [human embryonal kidney cell line; provided by C. Buck, Center for Cancer Research, National Institutes of Health (NIH), USA] cell lines were cultured in suitable culture medium according to the recommendations of American Type Culture Collection (ATCC). The JCPyV_{MAD4} strain (VR-1583) was obtained from ATCC and propagated upon infection in SVG-A cells. Infectious supernatants were collected from productively infected SVG-A cultures with late-stage cytopathic effects, separated from cell debris by centrifugation, and stored at –80°C. This strain is neurooncogenic and differs from the MAD1 strain only by a 19–base pair deletion in the regulatory region.

JCPyV VP1 variant pentamer ELISA

Purified recombinant JCPyV VP1 variant pentamers (provided by T. Stehle, University of Tübingen, Germany) (36) were preincubated with reassociation buffer [1 mM CaCl₂ in tris-buffered saline (pH 7.6)] before coating 100 ng of each VP1 variant pentamer per well of a 96-well microplate (Costar, Corning). After coating overnight at 4°C, the plates were

saturated with a casein-based blocking solution containing 1 mM CaCl₂ at 37°C. After washing, serial dilutions of standard serum, serum (1:404 and 1:2020 dilutions), CSF (1:2, 1:10, 1:100, and/or 1:1000 dilutions), or human monoclonal antibodies (100 pM, 1 nM, and 10 nM dilutions) were added for 2 hours at 37°C. Human IgG was detected using biotin-conjugated anti-human Fc antibody and horseradish peroxidase (HRP)-conjugated avidin (eBioscience). Trimethylboron single solution (Life Technologies) was used as colorimetric substrate for HRP, and optical density at 450 nm (OD₄₅₀) was measured with a Synergy H1 microplate reader (BioTek). Antibody reactivity was assessed in AUs using a standard curve obtained from a serial dilution of the respective standard serum for interpolating ODs by four-parameter logistic curve fit. AUs within standard curve were multiplied by the corresponding dilution factor to obtain absolute AU. The JCPyV variant VP1-specific CSF/serum antibody index (CAI VP1_{JCPyV}) was calculated according to Reiber (58). Briefly, CAI VP1_{JCPyV} was assessed as $CAI\ VP1_{JCPyV} = Q_{spec}/Q_{IgG}$, if $Q_{Lim} > IgG$, or $CAI\ VP1_{JCPyV} = Q_{spec}/Q_{Lim}$ (IgG), if $Q_{Lim} < IgG$. The variables were calculated as follows: $Q_{spec} = JCPyV\ variant-specific\ IgG_{CSF}\ [AU]/JCPyV\ variant-specific\ IgG_{serum}\ [AU]$; $Q_{IgG} = IgG_{CSF}/IgG_{serum}$; $Q_{Lim}\ (IgG) = 0.93 \times [Q_{alb}^2 + (6 \times 10^{-6})]^{0.5} - (1.7 \times 10^{-3})$; $Q_{alb} = alb_{CSF}/alb_{serum}$ (alb, albumin). $Q_{Lim}\ (IgG)$ refers to the upper discrimination line of the hyperbolic reference range for the blood-derived IgG in CSF as zero intrathecal IgG synthesis. Intrathecal antigen-specific antibody synthesis was defined as CAI VP1_{JCPyV}.

1.5. Data were analyzed using Microsoft Excel and GraphPad Prism 6. Heat maps were generated using Spice (National Institute of Allergy and Infectious Diseases, NIH) software.

Memory B cell screening and molecular cloning of human antibodies

Memory B cells were isolated from peripheral blood lymphocyte preparations and screened for the ability to bind to JCPyV VP1 VLPs. Microplates (96-well, Costar) were coated overnight at 4°C with VP1 or bovine serum albumin (BSA) (Sigma-Aldrich) diluted to a concentration of 1 µg/ml in reassociation buffer. Plates were washed in phosphate-buffered saline with Tween 20 (PBS-T) (pH 7.6), and nonspecific binding sites were blocked for 1 hour at room temperature with PBS/0.1% Tween 20 containing 2% BSA. The plates were incubated with B cell-conditioned medium for 1 hour at room temperature, then washed in PBS-T, and the binding was determined using HRP-conjugated anti-human immunoglobulin polyclonal antibodies (Jackson Immuno-Research) followed by measurement of HRP activity in a standard colorimetric assay. Selected reactive B cell cultures were subjected to cDNA cloning of IgG heavy chain and κ or λ light chain variable region sequences, and subcloned in expression constructs using the immunoglobulin framework-specific primers for human variable heavy and light chain families in combination with human J_H segment-specific primers. Functional recombinant monoclonal antibodies were obtained upon cotransfection into HEK 293 or CHO (Chinese hamster ovary) cells of an immunoglobulin heavy chain expression vector and a κ or λ immunoglobulin light chain expression vector. Recombinant human monoclonal antibodies were subsequently purified from the conditioned medium using a standard protein A column purification. VP1 antigens for recombinant full-length JCPyV VP1 (strain MAD1) and BKPyV VP1 (strain AS) were purchased from Abcam. The antigen was incubated for 48 hours at 24°C with shaking in reassociation buffer to form the VLPs.

JCPyV and BKPyV VLP ELISA

ELISA assays were performed with varying antibody concentrations to validate the binding of the antibodies to JCPyV- or BKPyV-derived VP1 (Abcam) and to determine their EC₅₀. The ELISA was performed in 96-well microplates (Costar) coated overnight at 4°C with JCPyV or BKPyV VP1 VLP solutions or BSA diluted to a concentration of 5 µg/ml in reassociation buffer. Nonspecific binding sites were blocked with PBS containing 2% BSA and 0.5% Tween 20. Binding of human antibodies was determined using a donkey anti-human IgG antibody conjugated to HRP (Jackson ImmunoResearch), followed by measurement of HRP activity in a standard colorimetric assay. EC₅₀ values were estimated by a nonlinear regression using GraphPad Prism. To determine the binding of the antibodies to the denatured VLPs, the 96-well microplates were coated overnight at 4°C with VP1 proteins diluted to a concentration of 5 µg/ml in carbonate buffer [15 mM Na₂CO₃, 35 mM NaHCO₃ (pH 9.42)], and the ELISA was performed as previously described.

Western blot

Recombinant VP1 proteins (1 µg) from JCPyV and BKPyV were separated on an SDS-PAGE gel and then transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with the human recombinant antibodies diluted to 1 µg/ml in blocking buffer. After multiple washes, the secondary antibody (HRP-conjugated donkey anti-human IgG Fcγ only) was added, and the membrane was then developed with enhanced chemiluminescence. The original blots are shown in fig. S5.

JCPyV neutralization assay

To test the ability of the VP1-specific antibodies to block the infection by JCPyV, we established an infection assay using the JCPyV_{MAD4} strain and the cell line SVG-A. Cells (20,000) were seeded on a coverslip placed in a well of a 24-well tissue culture plate. After 15 to 24 hours of incubation at 37°C to allow cells to adhere to the surface of the coverslip, the JCPyV_{MAD4} strain was added to the well at the concentration necessary to achieve up to 10% infection. The viruses were allowed to adhere to the cells for 1 hour at 37°C, and then the cells were washed with PBS and cultured in fresh medium at 37 °C to allow the infection to take hold. Infection of cells was determined 72 hours after infection by fixation, permeabilization, and labeling with a mouse JCPyV VP1-specific antibody (Abcam) and 4', 6-diamidino-2-phenylindole (DAPI). Analysis of virus-containing cells was performed with a Zeiss Axiovert 200M fluorescence microscope. Neutralization tests were performed by preincubating human-derived JCPyV VP1-specific monoclonal antibodies (50 µg/ml) with the virus for 1 hour at 37°C and adding the mixture to the cells for 1 hour at 37°C during viral attachment period. Infection of cells was determined in the presence and absence of human-derived VP1-specific antibodies as well as an isotype control, as described above.

Sequence analysis and inference of germline ancestors

IGH sequences isolated from monoclonal antibodies from the NAT-PML-IRIS patient were identified using IMGT/V-QUEST 3.3.2 (59). In particular, the most closely related germline sequences of *V_H*, *D_H*, and *J_H* genes as well as the numbers of nonsilent mutations were assigned to each clone by using IMGT/V-QUEST (Table 2). NAT-PML-IRIS *IGH* sequences

were aligned by using ClustalW 2.0.12 (60). Maximum parsimony lineage was inferred with the dnaphars application online tool of PHYLIP version 3.67, and an unrooted phylogenetic tree was constructed using Archaeopteryx 0.962 β 2N on the Pasteur Mobyle platform (61).

Statistical analysis

Statistical analyses were performed with GraphPad Prism. Data on antibody responses against the different VP1 pentamers were checked for normality (D'Agostino and Pearson omnibus normality test, $\alpha = 0.05$). Data on serum responses against the JCPyV VP1 variants (Fig. 1, C and D) were tested with a nonparametric one-way ANOVA (analysis of variance) with Kruskal-Wallis test and Dunn multiple comparisons test ($\alpha = 0.05$). Two-tailed Mann-Whitney *U* test was performed on data with CSF responses of NAT-PML and NAT-PML-IRIS patients (Fig.2, A to C). *P* values are reported in the figures and figure legends where significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. VP1 mutations compromise serum antibody responses in NAT-PML

(A) SDS–polyacrylamide gel electrophoresis (PAGE) of monomeric recombinant VP1 variants that were used as pentamers in ELISA. (B) Serial dilutions of a human standard serum with a high concentration of JCPyV VP1–specific antibodies and equal recognition of all VP1 variants used as a reference standard to estimate antibody binding expressed as arbitrary units (AU). (C) Serum antibody responses of HDs and NAT-treated MS, NAT-PML, and NAT-PML-IRIS patients to VP1_{MAD1} pentamers, depicted in AUs (mean ± SEM). Statistically significant P values are shown for HD versus NAT-PML-IRIS ($n = 38$; $P = 0.0027$, Kruskal-Wallis), HD versus NAT-PML ($n = 45$; $P = 0.0206$, Kruskal-Wallis), NAT versus NAT-PML-IRIS ($n = 36$; $P = 0.0037$, Kruskal-Wallis), and NAT versus NAT-PML ($n = 43$; $P = 0.0283$, Kruskal-Wallis). (D) Relative binding of individual sera of the different donor populations to VP1 variants in comparison to VP1_{MAD1} (mean ± SEM). Data from each donor were normalized to its corresponding recognition to VP1_{MAD1} set as 100% binding (red dotted line). Statistically significant P values are shown for HD versus NAT-PML ($n = 45$; $P = 0.0002$, Kruskal-Wallis), and NAT versus NAT-PML-IRIS ($n = 35$; $P = 0.0055$, Kruskal-Wallis), and NAT-PML versus NAT-PML-IRIS ($n = 23$; $P < 0.0001$, Kruskal-Wallis) for binding to VP1_{L55F}, and NAT versus NAT-PML-IRIS ($n = 35$; $P = 0.0034$, Kruskal-Wallis) for binding to VP1_{S269F}. (E) Relative binding of serum from

patients with NAT PML to VP1 variants in comparison to VP1_{MAD1}, represented as a heat map. Binding efficiency to VP1 variants is illustrated by color gradient. Data were normalized as in (D).

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Fig. 2. Immune reconstitution in NAT-PML enhances intrathecal antibody response against VP1 variants

(A) CSF antibody responses of NAT-PML and NAT-PML-IRIS patients to VP1_{MAD1} pentamers, depicted in AUs (mean $\pm$ SEM). Statistically significant *P* value is shown ($n = 22$; $P = 0.0022$, Mann-Whitney *U* test). (B) Relative binding of CSF from each individual at diagnosis of PML or onset of PML-IRIS to VP1 variants in comparison to VP1_{MAD1} (mean $\pm$ SEM). Data from each patient were normalized to its corresponding recognition to VP1_{MAD1} set as 100% binding (red dotted line). Statistically significant *P* value is shown for binding to VP1_{L55F} ($n = 22$; $P = 0.0106$, Mann-Whitney *U* test). (C) Intrathecal antibody production against VP1 variants in PML and PML-IRIS was determined by calculation of CAI VP1_{JCPyV}, which accounts for VP1-specific antibody levels in the serum and CSF normalized to total albumin and total IgG CSF/serum ratio. A CAI VP1_{JCPyV} >1.5 (red dotted line) was considered as evidence for intrathecal antibody synthesis. Statistically significant *P* values are shown for CAI VP1_{MAD1} ($n = 22$; $P = 0.0081$, Mann-Whitney *U* test), for CAI VP1_{WT3} ($n = 22$; $P = 0.0061$, Mann-Whitney *U* test), for CAI VP1_{L55F} ($n = 22$; $P = 0.0103$, Mann-Whitney *U* test), for CAI VP1_{S267F} ($n = 22$; $P = 0.0159$, Mann-Whitney *U* test), and for CAI VP1_{S269F} ($n = 22$; $P = 0.024$, Mann-Whitney *U* test).

Fig. 3. Human-derived monoclonal antibodies against JCPyV VP1 show distinct binding characteristics with respect to their affinity and cross-reactivity against BKV VP1

Human-derived JCPyV VP1-specific monoclonal antibodies were recombinantly cloned from HDs and a NAT-PML-IRIS patient and characterized with respect to their binding properties. (A to C) Examples of high-affinity JC VLP-specific antibody (98D3) targeting a conformational epitope, (B) JC/BK VLP cross-reactive antibody (44F6B) targeting a conformational epitope, and (C) JC/BK VLP cross-reactive antibody (11G6) targeting a linear epitope. Affinity and BKPyV cross-reactivity were determined by EC_{50} in a serial dilution of JC/BK VLP ELISA. ELISA of native and denatured JCPyV VLP and Western blot of JCPyV VP1 and BKPyV VP1 were used to characterize recognition of linear or conformational epitopes. mAbs, monoclonal antibodies.

Fig. 4. Identification of neutralizing JCPyV-specific human monoclonal antibodies
(A) Neutralization assay using JCPyV_{MAD4} on SVG-A cells. An appropriate dilution of the JCPyV_{MAD4} strain was incubated for 1 hour in the absence or presence of human monoclonal antibodies or isotype control at 50 µg/ml. Infected cells were detected by staining with a mouse anti-JCPyV VP1 (green) 72 hours after infection. The nuclei were counter-stained with DAPI (blue). Scale bars, 50 µm. Representative images illustrating the neutralization capacity of antibodies belonging to the different categories are shown. **(B)** Binding affinities represented as EC₅₀ value of HD-derived (left graph) and NAT-PML-IRIS patient-derived (right graph) JCPyV VP1-specific monoclonal antibodies against JCPyV and BKPyV VLP. Antibodies were classified as non-neutralizing (blue) or neutralizing (green).

Fig. 5. Human monoclonal antibodies display diverse binding profiles to JCV VP1 variants (A and B) Relative binding of NAT-PML-IRIS patient-derived monoclonal antibodies to VP1 variants in comparison to VP1_{MAD1} depicted as a heat map (A) or scatter plot (mean $\pm$ SEM) (B). Data for each antibody were normalized to its corresponding recognition to VP1_{MAD1} set as 100% binding (red dotted line in B). The antibodies were arranged according to their affinities for JCPyV VLP, and JCPyV neutralization capacity as well as BKPyV cross-reactivity are indicated. (B) Gray-filled circles represent individual monoclonal antibodies and red filled circles reflect serum antibody responses of the NAT-PML-IRIS patient, from whom the antibodies were cloned. (C) Relative binding of different HD-derived monoclonal antibodies to VP1 variants in comparison to VP1_{MAD1}, represented as a heat map. Binding efficiency to VP1 variants is illustrated with a color gradient. nd, not determined.

Fig. 6. Phylogeny of *IGH* sequences of NAT-PML-IRIS patient-derived monoclonal antibodies as inferred by maximum parsimony
The branch length represents the amount of evolutionary divergence.

Table 1
Demographic data of patients and controls

Age and duration of NAT treatment are shown for NAT-treated MS, NAT-PML, and NAT-PML-IRIS patients and HDs. No statistically significant differences in age, female to male ratio, and duration of NAT treatment were detected between the groups.

	NAT	NAT-PML	NAT-PML-IRIS	HD
Number (<i>n</i>)	28	15	8	30
Mean age (years $\pm$ SD)	37.6 $\pm$ 9.2	38.1 $\pm$ 8.3	42.0 $\pm$ 6.6	33.9 $\pm$ 7.5
Range of age (years)	19–53	23–55	32–55	25–49
Female to male ratio	1.8	2.0	1.7	1.5
Mean duration of NAT treatment (months $\pm$ SD)	42.9 $\pm$ 22.0	51.1 $\pm$ 16.3	49.8 $\pm$ 11.3	—
Range of treatment duration (months)	3–86	14–81	35–72	—

Table 2
***IGH* gene usage of NAT-PML-IRIS patient-derived monoclonal antibodies**

The germline usage for heavy-chain variable region (V_H), heavy-chain diversity region (D_H), and heavy-chain joining region (J_H) segments of *IGH* genes defined using the IMGT database and the number of amino acids in the CDR3 region as well as amino acid mutations as compared to the germline sequence are shown. aa, amino acids.

mAb	V_H	D_H	J_H	CDR3 length	aa mutations
98D3	3-30*14	5-5*01	4-1*02	16	13/96
43E8	3-30*04	3-22*01	4-1*02	14	19/95
105C7	3-23*04	2-15*01	4-1*02	19	14/96
45E10	3-23*04	2-15*01	4-1*02	19	13/96
29B1	3-23*04	3-22*01	4-1*02	17	17/96
26A3	3-23*04	3-3*02	6-1*02	17	13/96
58C7	3-11*01	6-13*01	4-1*02	16	10/96
47B11	3-11*01	6-13*01	4-1*02	16	5/96
56A8	3-21*01	1-1*01	4-1*02	11	3/96
27C2	3-21*01	1-1*01	4-1*02	11	8/96
72F7	3-48*03	3-3*01	6-1*02	21	5/96
59A7	3-48*03	5-5*01	4-1*01	16	4/96
57D4	3-9*01	4-17*01	6-1*02	14	7/96
105A6	3-9*01	5-5*01	6-1*02	14	2/96
72F10	3-9*01	5-12*01	4-1*02	14	10/96
27C11	4-4*02	1-14*01	6-1*02	20	5/96
50H4	4-4*02	1-14*01	6-1*02	20	6/96
7J3	4-30.4*01	3-10*01	6-1*02	20	3/97
98H1	4-31*03	3-22*01	6-1*02	21	11/97
53B11	5-51*01	1-26*01	4-1*02	13	3/96