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 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.

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Broadly Neutralizing Human Monoclonal JC Polyomavirus VP1-Specific Antibodies for the Treatment of Progressive Multifocal Leukoencephalopathy

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One Sentence Summary: Cloning, recombinant expression and characterization of human monoclonal antibodies that broadly neutralize multiple PML-causing JC polyomavirus variants.
ABSTRACT

In immunocompromised individuals, JC polyomavirus (JCPyV) may mutate and gain access to the central nervous system (CNS) 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. Here, we examined antibody responses against JCPyV major capsid protein VP1 variants in serum and cerebrospinal fluid (CSF) of healthy donors (HD), JCPyV-positive multiple sclerosis (MS) patients treated with the anti-VLA-4 monoclonal antibody natalizumab, and patients with natalizumab-associated PML. Prior to and during PML, CSF antibody responses against JCPyV VP1 variants show "recognition holes"; however, upon immune reconstitution, CSF antibody titers rise, then recognize PML-associated JCPyV VP1 variants and eliminate 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 HD and a NAT-PML-IRIS patient. These antibodies exhibited diverse binding affinity, cross-reactivity with the closely related BK polyomavirus, recognition of PML-causing VP1 variants, and JCPyV neutralization. Interestingly, 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 ideal candidates for the treatment of PML.
INTRODUCTION

JCPyV establishes life-long persistent infection of the kidney in a large fraction of the healthy population without known clinical consequences (1). During acquired or hereditary immunodeficiency such as AIDS, cancer, CD4 lymphopenia or monoclonal antibody therapy, archetypic JCPyV may acquire mutations. Whether these mutations are a prerequisite for CNS entry or occur in the CNS is not clear. Mutated JCPyV variants (JCPyV<sub>PML/GCN</sub>) either cause a 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 -suppressive treatments with specific monoclonal antibodies including efalizumab, rituximab and particularly natalizumab have been shown to increase 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 IL-2 (9) and IL-7 (10, 11), administration of polyvalent intravenous immunoglobulins (12) as well as the 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 shown promise. These data together with the abovementioned studies indicate that specific immune recognition by T cells and antibodies are critical for terminating PML. In patients with AIDS and also in natalizumab-associated PML restored CD4<sup>+</sup> T cell function and recovering immune surveillance of the CNS after natalizumab washout 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 often also results in acute neurological deterioration, additional brain damage, and can even lead to death of the patient due to tissue swelling (15-18).

Attempts to treat PML with 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 IgG titers rise under antiretroviral therapy (5, 7) and also data from PML in natalizumab (NAT)-treated MS patients, who eliminate JCPyV once the anti-VLA4 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+, 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 natalizumab-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). Both in AIDS patients and in NAT-associated PML, the major capsid protein of JCPyV VP1 has been identified as most important target antigen for CD4+ and CD8+ T cells in several studies (17, 22-24). While 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 in AIDS patients (7). In NAT-treated MS patients risk stratification strategies have been based on prior exposure to immunosuppressive drugs, length of NAT treatment as well as JCPyV-specific antibody indices (25, 26). Approximately 54% of MS patients are JCPyV-seropositive (26-28). Both serum
JCPyV-specific antibody levels and intrathecal JCPyV-specific antibody production are increased in patients at onset of NAT-associated PML (28, 29). The latter finding and the fact that pre-PML samples revealed sustained higher anti-JCPyV antibody indices over time (26) questions the role of JCPyV-specific antibodies in the prevention of PML development and clearance of JCPyV infection from the brain. However, no data are available on functional aspects of JCPyV-specific antibodies regarding neutralization, affinity and target epitopes, and neither for healthy subjects, MS patients nor in PML patients.

Besides the quality and quantity of the adaptive immune response of the infected host, characteristics of the virus play an important role in PML. Archetypic or wild type (WT) virus, which causes life-long, clinically inapparent infection of the epithelial cells in the kidney and urinary tract, has to undergo mutations in order to be able to cause PML (30). PML-associated JCPyV genotype variants show characteristic mutations of VP1 and rearrangements of the non-coding 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 of VP1 of PML-causing JCPyV variants influence the immune surveillance of JCPyV VP1-specific antibodies.

Based on the above considerations, it is highly desirable to establish prophylactic and therapeutic immunization regimens in order 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 globally immune reconstituting agent combined with active immunization using JCPyV VP1 and a toll-like receptor 7/9 agonist as adjuvant (imiquimod) led to clearance of JCPyV from the CNS and recovery from PML (14). However, active immunization with JCPyV VP1 may only be effective if the patient either has residual immune function or if the latter is restored by cytokines like 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, i.e. 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 third patient with idiopathic CD4 lymphopenia, whom we successfully vaccinated with JCPyV VP1/VLP, imiquimod and recombinant IL-7 as previously shown (14). In this patient, the JCPyV-specific antibody response not only rapidly increased following vaccination and broadened with respect to recognition of PML variants including the one infecting the patient, but in parallel to the increasing antibody response the patient's CSF JCPyV viral load declined to zero (35).

The present study aimed at developing 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 following IRIS eliminated the virus, should harbor antibodies that are suitable for passive vaccination. For this purpose we evaluated the antibody response in serum and CSF of HD, NAT-treated MS patients, NAT-PML and NAT-PML-IRIS patients against various JCPyV VP1 variants, in order to identify individuals 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
functionality and specificity profile and selected antibody candidates for the treatment/passive immunization 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, 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\text{MAD1} (36, 37). Equivalence in purity and quantity of the recombinant VP1 proteins was demonstrated by gel electrophoresis (Fig. 1A). A HD serum was selected as reference standard with equivalent concentration-dependent binding to all VP1 variants (Fig. 1B). Screening using VP1\text{MAD1} revealed an increase of anti-JCPyV VP1 antibodies in serum of NAT-PML patients and even higher levels in NAT-PML-IRIS patients, compared to healthy subjects (HD) and JCPyV-seropositive MS patients under natalizumab treatment (NAT)(Fig. 1C; demographics in Table 1), consistent with a previous report (28). Sera were further analyzed for binding activity to VP1 variants following normalization to their response against prototypic MAD1. Responses varied widely between individuals (Fig. 1D). The average reactivity to VP1\text{WT3} did not differ between groups and was similar to responses against VP1\text{MAD1}. In contrast, reduced serum responses against the PML-associated variant VP1\text{L55F} were observed in NAT-PML, indicating compromised immune recognition. Interestingly, both serum antibody responses to variants L55F and S269F are lower in JCPyV-seropositive NAT MS patients (Fig. 1D). The JCPyV variant VP1\text{S267F} is 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 (e.g. patient 4), patients with stronger recognition against one VP1 variant but lower response to others (e.g. patients 8 and 12) to patients with 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 VP1L55F and VP1S269F 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). While intrathecal antibodies against VP1L55F are lower compared to MAD1 in NAT-PML patients, similar to what we observed in serum, these responses increased and were higher in NAT-PML-IRIS patients (Fig. 2B). Since the above analysis, which compares the antibody response against JCPyV VP1 variants to VP1MAD1, does not allow us to judge whether antibodies are synthesized at higher levels within the CSF compared to serum, we assessed the intrathecal antibody production against different JCPyV VP1 proteins. In line with a previous report (28), approximately half of the NAT-PML patients (6 out of 14) showed signs of intrathecal antibody production (CAI\(_{JCPyV}>1.5\)) against both JCPyV VP1MAD1 and VP1WT3 (Fig. 2C, left and second panel from left). 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 middle, second from right and right panels). Most important in the context of this study, the intrathecal antibody responses against all VP1 proteins (MAD1, WT3 and mutant proteins) increased by tenfold or more during NAT-PML-IRIS in 7 of 8 patients indicating a high-titer and broad antibody response directed against multiple variants within the CNS compartment upon immune reconstitution.

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

The above data indicate that our original assumptions were likely correct in that patients who eliminated JCPyV from the CNS compartment following PML-IRIS mount robust and broad antibody responses against several JCPyV VP1 proteins including non-pathogenic 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), who had successfully controlled PML and showed strong antibody responses to JCPyV VP1. From this individual and selected HD we isolated memory B cells expressing VP1-specific antibodies with the aim to identify broadly neutralizing antibody lead candidates for passive immunization.

As expected based on seroprevalence in the general population we could identify B cells expressing antibodies binding to JCPyV VP1 VLPs from most of the healthy donors (HD) (27 out of 40). The frequency of JCPyV VP1-reactive memory B-cells was increased by 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 HD and 20 from the NAT-PML-IRIS patient) and characterized their binding affinities towards JCPyV and BKPyV VP1 VLPs and their specificity to intact or denatured VLPs.

The majority of the antibodies from the NAT-PML-IRIS patient showed extremely high affinity for the JCPyV VP1 VLPs with a half-maximal binding (EC50) down to the femtomolar range (Fig. 3A) and weak or no binding to BKPyV VP1 VLPs. Interestingly, those antibodies were only able to recognize intact VLPs and did not show binding to denatured VLPs on ELISA or on Western blot following denaturing 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 to JCPyV but also to BKPyV VP1 VLPs with EC\textsubscript{50} in the low picomolar range. These antibodies also only bound 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 HD displayed a wide range of binding affinities with EC\textsubscript{50} values to the low picomolar 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 HD versus NAT-PML-IRIS with the majority of antibodies generated by HD 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 monoclonals, we established an infection assay using JCPyV\textsubscript{MAD4} strain infection of the human fetal astrocyte-derived glial cell line SVG-A (Fig. 4, 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). While only one of the antibodies cloned from HD was capable of blocking JCPyV infection, the majority of antibodies (18/20) cloned from the PML-IRIS patient showed complete neutralization of the viral infection (Fig. 4B, right graph).

**Human monoclonal antibodies show crossreactivity 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). Interestingly, the most common PML-associated mutations such as L55F, S267F and S269F are located in several of 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 such an 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 HD and patients, binding to S267F seemed to be affected in the majority of antibodies derived from the NAT-PML-IRIS patient, however, absence of antibody recognition was observed also for other VP1 variants in some monoclonals. The observation that the mean responses of the NAT-PML-IRIS patient-derived antibody responses closely match with the autologous serum response underscores that the memory B cells, which had been picked for generation of the monoclonal antibodies, reflected the humoral immune response in vivo (Fig. 5B). Interestingly, 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 less pronounced than in the NAT-PML-IRIS patient (Fig. 5C). This is probably due to the much higher affinities of the NAT-PML-IRIS-derived antibodies. Non-neutralizing antibodies (e.g. 72F7, 43E8 and 8 of 9 HD-derived antibodies) were less
affected in their recognition of VP1 variants, indicating that epitope binding of these antibodies most probably occurs 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 not only revealed high affinity to JCPyV VLP and potent neutralization capacity, but also very efficient recognition all five VP1 variants. Furthermore, we could also demonstrate this broad binding to additional VP1 variants with mutations located within the exterior loops (e.g. VP1<sub>N74S</sub>, VP1<sub>R75K</sub>, VP1<sub>T117S</sub>) by using a complementary approach with pCAG-JCPyV - transfected cells (38) combined with intracellular staining (Fig. S2).

Based on the established binding profiles of the monoclonal antibodies, we tested whether the broadly recognizing antibodies target shared or independent binding regions. Towards that end, we performed competition experiments assessing whether selected antibodies could recognize JCPyV VLPs following saturation with the antibody 98D3. While 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 non-neutralizing 72F7 antibody binds to a distinct epitope (Fig. S3). It is, however, unlikely that these antibodies bind to the exact same amino acid residues since they are showing 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 IGH clones isolated from the 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 neutralization and cross-recognition of JCPyV VP1 variants and wild-type BKPyV VP1 (Fig. 6). Amongst 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 like SLE, during immunomodulatory treatments, which affect B cells (5, 6), and in immunodeficiencies that primarily involve antibodies, i.e. Franklin disease (39), or B cells/antibodies and T cells, i.e. 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. Natalizumab 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, if 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 at multiple steps of PML development from viral mutation to PML variants, and transport of glial/neurotropic JCPyV variants into the CNS, and both the underlying disease, e.g. HIV/AIDS or idiopathic CD4 lymphopenia, or specific immune interventions such as natalizumab or chemotherapy can probably contribute.

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 brain 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, i.e. cells that secrete both
interferon-γ and interleukin-4, which is important to mount B cell responses, are particularly prevalent, and it is of interest to note that the vast majority of these 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 low or absent JCPyV DNA in the CSF, which is not rarely the case, when IRIS has begun (12, 17, 28). Finally, the rise in CSF anti-JCPyV antibodies with specificity against several PML variants including the one found in the CSF in a patient following 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 that are treated with natalizumab (25). Interestingly, patients with intermediate antibody indices have a much lower risk to develop PML, while those with high indices show an elevated risk (26). It is currently not clear if this unexpectedly higher PML risk in the presence of higher anti-JCPyV VP1 antibody titers indicates that antibodies are functionally deficient, i.e. not capable of neutralizing the virus, or if 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 it has been shown that JCPyV viremia was 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 natalizumab-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 archetypic JCPyV strains that are excreted in the
urinary tract, PML-associated JCPyV genotypes present characteristic mutations of VP1 and rearrangements of the non-coding 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). Up 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, however, indicate that antibody “recognition holes” likely play a role in PML. This notion is supported by data of Ray et al. (35) showing that PML patients fail to produce antibodies recognizing their "own" PML mutant. Particularly JCPyV VP1L55F and VP1S267F are recognized less well by sera and CSF in NAT-PML patients, and, while the responses to JCPyV VP1L55F rise at NAT-PML-IRIS, recognition of VP1S267F remains lower. Despite strong intrathecal antibody responses already during NAT-PML and further increase in NAT-PML-IRIS against all tested JCPyV VP1 variants, the response to VP1S267F remains weaker compared to JCPyV VP1MAD1.

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

Natalizumab remains one of the most effective and generally very well tolerated therapies, but the impaired immune surveillance of the CNS leads to a PML risk between 1/100-1/1000 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 the 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 up to death from acute brain swelling. From 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), would not be indicated or at least carry substantial risk. However, a treatment with anti-JCPyV VP1-specific antibodies could be applicable to all patients with PML immediately following their diagnosis, with a significantly lower expected risk of clinical liabilities including IRIS, although high doses of antibody may be needed due to the limited access of antibodies through the blood-brain- and blood-CSF barriers. Furthermore, since 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 ideal 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 a 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 optimized drug candidates with optimal biophysical, pharmacological and safety properties. When we compared the memory B cell repertoires from both HD and a NAT-PML-IRIS patient using JCPyV and BKPyV VLPs we were surprised to find that the human antibodies cloned from HD, i.e. during
the state of persistent JCPyV infection of the kidney, are of relatively low affinity, are 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 were 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 sequence differences between the BKPyV and JCPyV VP1 pentamers, which mostly map to surface-exposed loops that display mutations in PML and that 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 natalizumab-associated 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 a role also of effector functions including opsonization, complement activation, phagocytosis, and/or antibody-dependent cellular cytotoxicity. It is also not clear if anti-JCPyV antibodies act primarily by neutralizing extracellular virions or also intracellularly in infected cells or by inhibiting binding of the mutant viruses to an as yet unknown receptor, which is different from the glycan receptor LSTc and in particular 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 even nuclear epitopes are, however, well documented in autoimmune diseases (54), and a recent study showed that the intracellular antibody receptor, tripartite-motif protein 21 (TRIM21), a ubiquitously expressed E3 ubiquitin ligase provides a mechanism to protect mice from lethal adenoviral infection via 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 PML and subsequent PML-IRIS. Considering the reduced recognition of JCPyV PML variants VP1_{L55F} and VP1_{S267F} and generally the lack of recognition of different JCPyV VP1 variants (Fig. 1E) by serum antibodies of HD and NAT-PML patients, we defined a set of criteria for the selection of lead antibody candidates for future therapeutic development as a) recognition of conformational capsid epitopes of a broad spectrum of JCPyV VP1 PML variants, b) high binding affinity, and c) biological activity in neutralization of JCPyV infectivity. 27C11 represents the most promising human-derived monoclonal that was identified in the present study. It shows low picomolar affinity, neutralizing activity, equivalent recognition of all JCPyV VP1 variants examined in Fig. 5A, and beyond these also recognizes cells transfected with the pseudoviral constructs (pCAG-VP1) of VP1 variants VP1_{N74S}, VP1_{R75K}, VP1_{T117S}, 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 regions 3, 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 anti-JCPyV antibodies during PML and the exact mechanism of action, i.e. 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 hopefully shed light
on this point. However, together with our promising 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 non-randomized laboratory study designed to investigate the antibody responses in serum and CSF in healthy subjects and patients with MS, PML or PML-IRIS and to recombinantly clone and characterize human-derived antibodies targeting JCPyV VP1 for the further development towards 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 HD and a NAT-PML-IRIS patient, who had recovered from PML, and further investigated regarding their affinities, specificities and functional properties using BKPyV/JCPyV VLP ELISA, Western Blot, JCPyV VP1 pentamer ELISA, FACS analysis of transfected VP1 variants as well as a JCPyV neutralization assay. Sample size was dictated by rate of sample collection (generally 2-5 ml serum, 5-10 ml CSF, 50-80 ml of peripheral blood for isolation of PBMCs), and blinding was not used.

Study subjects

Peripheral blood of patients with MS under natalizumab, natalizumab-associated PML, and PML-IRIS and matched healthy controls, as well as CSF samples from natalizumab-associated PML/PML-IRIS 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 natalizumab therapy were all seropositive for JCPyV antibodies (Unilabs). Demographic data of all donors are shown in table 1. Samples at onset of PML-IRIS were obtained from the corresponding PML cases after washout of natalizumab and clinical as well as radiological signs of IRIS. Peripheral blood was used to collect serum and peripheral blood mononuclear cells.
Cells and virus

SVG-A (transformed human fetal astrocytes, kindly provided by R. Girones, University of Barcelona, Spain) and HEK 293TT (human embryonal kidney cell line, kindly provided by C. Buck, Center for Cancer Research, NIH, USA) cell lines were cultured in suitable culture medium according to recommendations of ATCC. The JCPyV\textsubscript{MAD4} strain (VR-1583\textsuperscript{TM}) 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 MAD1 strain only by a 19 bp 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 CaCl\textsubscript{2} in TBS, 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, plates saturated with a Casein-based blocking solution containing 1 mM CaCl\textsubscript{2} at 37°C. Following wash steps, 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 h at 37°C. Human IgG was detected using biotin-conjugated anti-human Fc antibody and horseradish peroxidase (HRP)-conjugated avidin (eBioscience). TMB single solution (Lifetechnologies) was used as colorimetric substrate for HRP and optical density at 450nm (OD\textsubscript{450}) was measured with a Synergy H1 microplate reader (BioTek). Antibody reactivity was assessed in arbitrary units.
(AU) 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 = \( Q_{\text{spec}} / Q_{\text{IgG}} \), if \( Q_{\text{Lim}} > \text{IgG} \), or \( \text{CAI VP1 JCPyV} = Q_{\text{spec}} / Q_{\text{Lim}} (\text{IgG}) \), if \( Q_{\text{Lim}} < \text{IgG} \). The variables were calculated as follows: \( Q_{\text{spec}} = \) JCPyV variant-specific IgGCSF [AU]/JCPyV variant-specific IgGserum [AU]; \( Q_{\text{IgG}} = \) IgGCSF/IgGserum; \( Q_{\text{Lim}} (\text{IgG}) = 0.93x (Q_{\text{alb}}^2 + 6x10^{-6})^{0.5} -1.7x10^{-3} \). \( Q_{\text{alb}} = \text{albCSF}/\text{albserum} \) (with alb = albumin). \( Q_{\text{Lim}} (\text{IgG}) \) 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 \( \geq 1.5 \). Data were analyzed using Microsoft Excel and GraphPad Prism 6. Heat maps were generated using Spice (NIH, NIAID) 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. 96-well microplates (Costar) were coated overnight at 4°C with VP1 or BSA (Sigma-Aldrich) diluted to a concentration of 1 \( \mu \text{g/ml} \) in reassociation buffer. Plates were washed in PBS-T pH 7.6 and non-specific binding sites were blocked for 1 h at RT with PBS/0.1% Tween-20 containing 2% BSA. Plates were incubated with B cell conditioned medium for one hour at RT, washed in PBS-T and binding was determined using HRP-conjugated anti-human immunoglobulins polyclonal antibodies (Jackson ImmunoResearch) followed by measurement of HRP activity in a standard colorimetric assay. Selected reactive B-cell cultures were subjected to cDNA cloning of IgG heavy and kappa or lambda light chains variable region sequences, and sub-cloned in expression constructs using Ig-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 co-transfection into HEK 293 or CHO cells of an Ig-heavy-chain expression vector and a kappa or lambda Ig-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.

**JC and BK 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 be able to determine their half maximal effective concentration (EC$_{50}$). The ELISA was performed in 96-well microplates (Costar) coated overnight at 4°C with JCPyV or BKPyV VP1 VLPs solutions or BSA diluted to a concentration of 5 µg/ml in reassociation buffer. Non-specific binding sites were blocked PBS containing 2% BSA and 0.5% Tween20. Binding of human antibodies of the present invention was determined using a donkey anti-human IgG antibody conjugated with HRP (Jackson ImmunoResearch), followed by measurement of HRP activity in a standard colorimetric assay. EC$_{50}$ values were estimated by a non-linear 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 Na$_2$CO$_3$, 35 mM NaHCO$_3$, pH 9.42) and the ELISA was performed as previously described.

**Western Blot**
Recombinant VP1 proteins (1µg) from JCPyV and BKPyV were separated on a SDS-PAGE gel and then transferred onto nitrocellulose membranes. After blocking, the membrane was incubated with the human recombinant antibodies diluted to 1ug/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 ECL. The original blots are shown in Fig.S5.

**JCPyV neutralization assay**

To test the ability of the anti-VP1 antibodies to block the infection by JCPyV, we established an infection assay using the JCPyV\textsubscript{MAD4} strain and the cell line SVG-A. 20,000 cells were seeded on a cover slip placed in a well of a 24-well tissue culture plate. After 15-24 h of incubation at 37°C to allow cells to adhere to the surface of the cover slip, the JCPyV\textsubscript{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 h at 37°C, and then cells were washed with PBS and cultured in fresh media at 37 °C to allow the infection to take hold. Infection of cells was determined 72 h post-infection by fixation, permeabilization and labeling with a mouse JCPyV VP1-specific antibody (Abcam) and DAPI. Analysis of virus-containing cells was performed with a Zeiss Axiovert 200M fluorescence microscope. Neutralization tests were performed by pre-incubating human-derived JCPyV VP1-specific monoclonal antibodies (50 µg/ml) with the virus for 1 h at 37°C adding the mixture to the cells for 1 h at 37°C during viral attachment period. Infection of cells was determined in the presence and absence of human-derived anti-VP1 antibodies as well as an isotype control as described above.

**Sequence analysis and inference of germline ancestors**
Immunoglobulin heavy chain (IGH) sequences isolated from monoclonal antibodies from the NAT-PML-IRIS patient were identified by using IMGT/V-QUEST 3.3.2 (59). In particular, the most closely related germline sequences of variable (VH), diversity (DH) and joining (JH) heavy chain genes as well as the numbers of non-silent mutations was assigned to each clone by using IMGT/V-QUEST (Table 2). PML-IRIS IGH sequences were aligned by using ClustalW 2.0.12 (60). Maximum parsimony lineage was inferred with the dnapars application online tool of PHYLIP v3.67 and an unrooted phylogenetic tree was constructed using Archaeopteryx 0.962 beta 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. 1C and 1D) were tested with a nonparametric one-way ANOVA 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 (Fig.2A-C). \( P \) values are reported in the figure and figure legend where significant.
SUPPLEMENTARY MATERIALS

Supplementary materials and methods: Patient information

Supplementary materials and methods: Transfection and flow cytometry analysis

Supplementary materials and methods: Cross-competition assay

Fig. S1. Mutations in JCPyV viral capsid protein VP1.

Fig. S2. Recognition of JCPyV VP1/VLP variants by human monoclonal antibodies.

Fig. S3. Cross-competition assay of VP1-specific monoclonal antibodies.

Fig. S4. Differences between JCPyV and BKPyV VP1.

Fig. S5. Recognition of denatured JCPyV VP1 and BKPyV VP1 (Western Blot).

Source data in tabular form (separate excel file)
REFERENCES AND NOTES


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Fig. 1. VP1 mutations compromise serum antibody responses in natalizumab-associated PML. (A) SDS-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 was used as a reference standard to estimate antibody binding expressed as arbitrary units (AU). (C) Serum antibody responses of healthy donors (HD), natalizumab-treated MS patients (NAT), natalizumab-associated PML patients (NAT-PML) and PML-IRIS patients (NAT-PML-IRIS) to VP1_{MAD1} pentamers, depicted
in AU (Mean ± SEM). Statistically significant $P$ values are shown for HD vs. NAT-PML-IRIS ($n = 38; P = 0.0027$, Kruskal-Wallis), HD vs. NAT-PML ($n = 45; P = 0.0206$, Kruskal-Wallis), NAT vs. NAT-PML-IRIS ($n = 36; P = 0.0037$, Kruskal-Wallis) and NAT vs. 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 was normalized to its corresponding recognition to VP1$_{MAD1}$ set as 100% binding (red dotted line). Statistically significant $P$ values are shown for HD vs. NAT-PML ($n = 45; P = 0.0002$, Kruskal-Wallis), NAT vs. NAT-PML-IRIS ($n = 35; P = 0.0055$, Kruskal-Wallis), NAT-PML vs. NAT-PML-IRIS ($n = 23; P < 0.0001$, Kruskal-Wallis) for binding to VP1$_{L55F}$ and NAT vs. NAT-PML-IRIS ($n = 35; P = 0.0034$, Kruskal-Wallis) for binding to VP1$_{S269F}$. (E) Relative binding of serum from patients with natalizumab-associated PML to VP1 variants in comparison to VP1$_{MAD1}$ represented as heat map. Binding efficiency to VP1 variants is illustrated by color gradient. Data were normalized as in (D).
Fig. 2. Immune reconstitution in natalizumab-associated PML enhances intrathecal antibody response against VP1 variants. (A) CSF antibody responses of natalizumab-associated PML patients (NAT-PML) and PML-IRIS patients (NAT-PML-IRIS) to VP1$_{MAD1}$ pentamers, depicted in AU (Mean ± 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 ± SEM). Data from each patient was 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 a JCPyV variant-specific CSF-serum antibody index (CAI VP1$_{JCPyV}$), which accounts for VP1-specific antibody levels in serum and
CSF normalized to total albumin and total IgG CSF/serum ratio. A CSF/serum JCPyV variant-specific antibody index >1.5 (red dotted line) was considered as evidence for intrathecal antibody synthesis. Statistically significant $P$ values are shown for CAI VP1$_{\text{MAD1}}$ (n = 22; $P = 0.0081$, Mann-Whitney U test), for CAI VP1$_{\text{WT3}}$ (n = 22; $P = 0.0061$, Mann-Whitney U test), for CAI VP1$_{\text{L55F}}$ (n = 22; $P = 0.0103$, Mann-Whitney U test), for CAI VP1$_{\text{S267F}}$ (n = 22; $P = 0.0159$, Mann-Whitney U test) and for CAI VP1$_{\text{S269F}}$ (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 HD and NAT-PML-IRIS and characterized with respect to their binding properties. (A) Example of a high affinity JC VLP-specific antibody (98D3) targeting a conformational epitope, (B) example of a JC/BK VLP-cross-reactive antibody (44F6B) targeting a conformational epitope, and (C) example of a JC/BK VLP-cross-reactive antibody (11G6) targeting a linear epitope. Affinity and BKPyV-cross-reactivity was determined by EC$_{50}$ in a serial dilution of a JC/BK VLP ELISA. ELISA of native and denatured JC VLP as well as Western Blot of JCPyV VP1 and BKPyV VP1 were used to characterize recognition of linear or conformational epitope.
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 h in the absence or presence of the human monoclonal antibodies or isotype control at 50 µg/ml. Infected cells were detected by staining with a mouse anti-JCPyV VP1 (green) 72 h after infection. Nuclei were counterstained with DAPI (blue). Scale bars, 50 µm. Representative images illustrating neutralization capacity of antibodies belonging to the different categories are shown. (B) Binding affinities represented as EC$_{50}$ value of HD- (left graph) and NAT-PML-IRIS-derived (right graph) JCPyV VP1-specific monoclonal antibodies against JC and BK VLP. Antibodies were classified as non-neutralizing (blue) or neutralizing (green).
Fig. 5. Human monoclonal antibodies display diverse binding profiles to JCV VP1 variants.

Relative binding of NAT-PML-IRIS-derived monoclonal antibodies to VP1 variants in comparison to VP1\textsubscript{MAD1} depicted as heat map (A) or scatter plot (Mean ± SEM) (B). Data of each antibody was normalized to its corresponding recognition to VP1\textsubscript{MAD1} set as 100% binding (red dotted line in B). The antibodies were arranged according to their affinities for JC VLP and JCPyV neutralization capacity as well as BKPyV cross-reactivity are indicated. In (B) grey 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\textsubscript{MAD1} represented as heat map. Binding efficiency to VP1 variants is illustrated with a color gradient. \textit{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 natalizumab treatment are shown for natalizumab-treated multiple sclerosis patients (NAT), natalizumab-associated PML patients (NAT-PML), natalizumab-associated PML-IRIS patients (NAT-PML-IRIS) and healthy donors (HD). No statistically significant differences in age, female to male ratio and duration of natalizumab treatment were detected between the groups. SD = standard deviation.

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Table 2. *IGH* gene usage of NAT-PML-IRIS patient-derived monoclonal antibodies. The germline usage for V-,D- and J-segment 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. IGH = immunoglobulin heavy chain; mAb = monoclonal antibody; VH = V-segment heavy chain; DH = D-segment heavy chain; JH = J-segment heavy chain; CDR3 = complementarity determining region 3; aa = amino acids.

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