UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Institut für Neuroimmunologie und Klinische Multiple Sklerose Forschung Hamburg

Direktor
Prof. Dr. Roland Martin

Characterization of JC virus-specific $CD4^+$ T cell epitopes in healthy individuals

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg

Vorgelegt von:

Lilian Marlene Aly
aus Anaheim, USA

Hamburg 2013

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 31.01.2014

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der/die Vorsitzende: Prof. Dr. R. Martin

Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. G. Tiegs

Prüfungsausschuss, dritte/r Gutachter/in: Prof. Dr. T. Berger

JVI
Journals.ASM.org

T Cell Epitope Mapping of JC Polyoma Virus-Encoded Proteome Reveals Reduced T Cell Responses in HLA-DRB1*04:01$^+$ Donors

Ilijas Jelčić,[a,b] Lilian Aly,[a] Thomas M. C. Binder,[c] Ivan Jelčić,[b] Sílvia Bofill-Mas,[d] Raquel Planas,[a,b] Victoria Demina,[e] Thomas H. Eiermann,[c] Thomas Weber,[f] Rosina Girones,[d] Mireia Sospedra,[a,b] Roland Martin[a,b]

Institute for Neuroimmunology and Clinical MS Research (inims), Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, Hamburg, Germany[a]; Department of Neuroimmunology and MS Research, Neurology Clinic, University Hospital Zürich, Zurich, Switzerland[b]; Department of Transfusion Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany[c]; Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain[d]; Life Science Inkubator, Bonn, Germany[e]; Department of Neurology, Marienkrankenhaus, Hamburg, Germany[f]

JC polyomavirus (JCV) infection is highly prevalent and usually kept in a persistent state without clinical signs and symptoms. It is only during immunocompromise and especially impaired CD4$^+$ T cell function in the brain, as seen in AIDS patients or natalizumab-treated multiple sclerosis patients, that JCV may cause progressive multifocal leukoencephalopathy (PML), an often life-threatening brain disease. Since CD4$^+$ T cells likely play an important role in controlling JCV infection, we here describe the T cell response to JCV in a group of predominantly HLA-DR-heterozygotic healthy donors (HD) by using a series of overlapping 15-mer peptides spanning all JCV-encoded open reading frames. We identified immunodominant epitopes and compared T cell responses with anti-JCV VP1 antibody production and with the presence of urinary viral shedding. We observed positive JCV-specific T cell responses in 28.6% to 77.6%, humoral immune response in 42.6% to 89.4%, and urinary viral shedding in 36.4% to 45.5% of HD depending on the threshold. Four immunodominant peptides were mapped, and at least one immunogenic peptide per HLA-DRB1 allele was detected in DRB1*01$^+$, DRB1*07$^+$, DRB1*11$^+$, DRB1*13$^+$, DRB1*15$^+$, and DRB1*03$^+$ individuals. We show for the first time that JCV-specific T cell responses may be directed not only against JCV VP1 and large T antigen but also against all other JCV-encoded proteins. Heterozygotic DRB1*04:01$^+$ individuals showed very low T cell responses to JCV together with normal anti-VP1 antibody levels and no urinary viral shedding, indicating a dominant-negative effect of this allele on global JCV-directed T cell responses. Our data are potentially relevant for the development of vaccines against JCV.

JC virus (JCV) is a member of the family of *Polyomaviridae* (PyVs) (1), a small DNA virus family, which encompasses eight more human viruses: BK PyV (BKV) (2), KI PyV (KIV) (3), WU PyV (WUV) (4), Merkel cell PyV (MCV) (5), human PYV6 (HPyV6) and HPyV7 (6), trichodysplasia spinulosa-associated PyV (TSV) (7), and HPyV9 (8). JCV consists of a circular double-stranded DNA genome of 5,130 bp length and three capsid proteins, namely, VP1, VP2, and VP3, with the VP1 major capsid protein being able to self-assemble into virus-like particles (VLP) (9), and a few additional proteins, agnoprotein, large T (LT) antigen (LTAg), small T (ST) antigen (STAg), and three T′ antigens (T′135, T′136, and T′165) (10).

Infection with JCV is common in healthy individuals with IgG seroprevalence rates between 58% and 84% (11, 12). JCV entry into the organism might occur via infection of tonsillar tissue after inhalation or via a fecal-oral route but also by vertical transmission (13, 14). Usually, JCV infection remains clinically unapparent, and the virus persists in tonsils and hematopoietic precursor cells in the bone marrow. JCV also infects kidney epithelial cells in a large fraction of infected individuals and is associated with viral shedding in the urine in approximately 50% of individuals (15). The reasons for urinary viral shedding in only a fraction of infected individuals are not clear. Under circumstances of immunocompromise and especially impaired CD4$^+$ T cell function such as late-stage HIV infection, hematological malignancies, and organ transplantation, but also in clinically inconspicuous idiopathic CD4$^+$ lymphopenia, JCV is able to cause an opportunistic infection of the brain, progressive multifocal leukoencephalopathy (PML) (15, 16). PML is caused by infection of oligodendrocytes and astrocytes by neurotropic JCV strains with altered regulatory regions and often certain amino acid exchanges in the JCV major capsid protein VP1 compared to archetypic strains (17, 18). Cell lysis of oligodendrocytes leads to widespread demyelination, a serious neurological impairment with fatal outcome in 30% to 50% of cases (15, 16). Besides the above-mentioned causes, PML has become a serious concern during therapy with a few monoclonal antibodies, e.g., in multiple sclerosis (MS) patients receiving natalizumab, the highly effective and usually well-tolerated antibody against α-4-integrin (VLA-4; CD49d), but also in systemic lupus erythematosus and psoriasis patients receiving anti-CD20 and anti-LFA-1 antibodies, respectively (15, 16). Until now, 285 of 104,400 natalizumab-treated MS patients have developed PML in the postmarketing setting worldwide, and approximately 22% have died from the complication (19). Current risk estimates range between 2.33:1,000 and 2.95:1,000 in MS patients on natalizumab therapy but rise to approximately to 9:1,000 or higher in JCV-seropositive individuals with more than 2 years treatment

Received 6 October 2012 Accepted 3 January 2013
Published ahead of print 9 January 2013
Address correspondence to Roland Martin, roland.martin@usz.ch.
Ilijas Jelčić and Lilian Aly contributed equally to this article as first authors.
Mireia Sospedra and Roland Martin contributed equally to this article as last authors.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.02803-12.

doi:10.1128/JVI.02803-12

and prior immunosuppression (19). The treatment complication of PML therefore threatens to lead to serious regulatory restrictions or even market withdrawal.

JCV-specific immunity is probably important not only for containing JCV infection in healthy individuals but also for recovery from PML, since immune reconstitution is associated with the best clinical outcome among patients with PML (20–22). Serum antibodies against JCV are frequent in clinically healthy individuals, whereas intrathecal JCV-specific antibodies are found at high prevalence and high titers in PML patients (12). Furthermore, JCV-specific IgG levels increase during the PML disease course of PML survivors (23). However, as most PML patients harbor JCV-specific antibodies before or at the onset of the disease (24), the humoral immune response may not be sufficient to prevent the development of PML. JCV-specific cellular immune responses have been investigated as well, particularly the role of $CD8^+$ cytotoxic T cells (25, 26). In individuals expressing the common human leukocyte antigen (HLA) class I molecule, (HLA-) A*02:01, two nonamer peptides have been identified as immunodominant JCV peptides: VP1 (100–109) peptide (ILMWEAVTL) and VP1 (36–44) peptide (SITEVECFL) (25, 26). The frequency of $CD8^+$ T cells specific for these epitopes ranges between 1:100,000 and 1:2,494 in peripheral blood mononuclear cells (PBMC), and 73% of immunocompetent individuals possess such cells (27). JCV-specific $CD8^+$ T cells have been shown in 91% of PML survivors, while they are detectable in only 9% of PML progressors (26). Furthermore, the number of JCV-specific $CD8^+$ T cells at the onset of the disease can predict subsequent disease progression. Based on these data, it has been concluded that the JCV-specific $CD8^+$ cytotoxic T cell response is essential for the prevention of and resolution from PML.

Despite the previously cited experience of an increased risk of PML in AIDS patients with low $CD4^+$ T levels, and in patients with idiopathic $CD4^+$ lymphocytopenia, both suggesting an important role of JCV-specific $CD4^+$ T cells in the control of JCV infection, $CD4^+$ T cell function has been examined in fewer studies. Peripheral $CD4^+$ T cell counts below 200 cells/μl at the time of PML diagnosis were found associated with disease progression and a survival rate at 1 year that is lower than the rate seen with $CD4^+$ T cell counts above 200 cells/μl (22). $CD4^+$ T cells reactive to JCV-like particles have been detected in healthy and HIV-infected non-PML individuals with urinary excretion of JCV (28). Furthermore, HIV-infected PML survivors harbor $CD4^+$ T cells reactive to JCV-like particles, while the cells are absent in HIV-infected individuals with active PML (28). With 80% of PML patients suffering from AIDS as an underlying disease and, reciprocally, up to 5% of AIDS patients developing PML (15, 16), $CD4^+$ T cells are likely of particular importance in the control of JCV. Moreover, like antibody-producing B cells, $CD8^+$ T cells are at least in part dependent on functional help from $CD4^+$ T cells. Supporting this notion, we have recently demonstrated that JCV-specific $CD4^+$ T cells of T helper 1 (T_h1) or T_h1 and -2 phenotypes predominantly infiltrate the brain in PML-immune reconstitution inflammatory syndrome (PML-IRIS), a condition occurring, for example, upon washout of natalizumab in MS or restoration of $CD4^+$ numbers in AIDS patients (29). While PML-IRIS and the accompanying inflammation and swelling of the brain are also life-threatening, they lead to the elimination of JCV infection and PML and underscore the role of $CD4^+$ T cell responses.

In the present study, we used a series of overlapping peptides that span all six open reading frames of JCV, including the 35 most frequent variants of JCV, and characterized the proliferative response in 49 healthy donors (HD). We subsequently identified immunodominant epitopes of the four most immunogenic pools. As expected, and consistent with the fact that our strategy primarily tested $CD4^+$ T cells, we found that the responses depended on the HLA class II haplotype with respect to peptide specificity and the magnitude of the response. Of particular note, HLA-DRB1*04:01$^+$ donors showed low to absent proliferative responses to JCV peptides and were negative for urinary JCV viral DNA but produced large amounts of anti-JCV VP1 antibodies. JCV-specific T cell responses were also reduced in individuals expressing DRB1*01:01. Our data are of interest in the context of vaccine development and studies of the cellular immune responses to JCV under physiological and pathological conditions.

TABLE 1 Demographic data of HD and experiments performed in the study

Parameter	Value(s)	
	HD cohort 1 ($n = 49$)	HD cohort 2 ($n = 22$)
Male/female ratio (no. of males:no. of females)	1:1.33 (21:28)	1:2.14 (7:15)
Mean age at date of collection, yr (range)	46.5 (23–72)	35.4 (26–46)
No. of Caucasians	49	22
No. HLA typed	49	22
No. with cellular response to peptide pools tested	49	0
No. with cellular response to single peptides and virus-like particles tested	20	0
No. with humoral response tested	25	22
No. with urinary viral shedding tested	0	22

MATERIALS AND METHODS

Donors. 49 HD were recruited from the Blood Bank at the University Medical Center Hamburg-Eppendorf, Hamburg, Germany, forming cohort 1 (Table 1). The mean age of the donors was 46.5 years (range, 23 to 72 years), and the gender distribution was 28 females and 21 males. Cohort 1 was used to determine the peripheral cellular response to JCV peptide pools, individual peptides, and JCV VP1 virus-like particles (VLP). The humoral response to JCV VLP was assessed by testing the supernatant fluids obtained after Ficoll density centrifugation in this cohort, since we did not have access to serum samples from these donors. Cohort 2 contained 22 HD recruited from our institute staff, with a mean age of 35.4 years (range, 26 to 46 years) (Table 1). The gender distribution in cohort 2 was 15 females and 5 males. Cohort 2 was employed to investigate urinary viral shedding and the relation between T cell responses and the presence of JCV-specific IgG antibodies in serum. The study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg, no. 2758), and informed consent was obtained from all study subjects.

HLA typing. All individuals in cohort 1 and 2 were typed for HLA class II molecules at the HLA laboratory, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Isolation of DNA from whole blood, with a final concentration of 30 ng/μl, was performed with the help of QiaCube (Qiagen GmbH, Hilden, Germany). The samples were high resolution typed for HLA class II (DRB1*, DRB3*, DRB4*, DRB5*, DQA1*, and DQB1*) with a sequence-specific oligonucleotide (SSO)-based test, Dynal Reli SSO (Invitrogen GmbH, Karlsruhe, Germany), and with a sequencing-based typing kit, AlleleSEQR HLA-SBT (Abbott Molecular, Wiesbaden, Germany). HLA class II types are listed in Table S1 (cohort 1)

and Table S2 (cohort 2) in the supplemental material. Frequencies of HLA class II alleles among cohorts 1 and 2 are summarized in Table S3 in the supplemental material.

JCV sequence analyses. JCV sequences available in all databanks were retrieved and analyzed by using the HUSAR software package (30). The amino acid sequences of all proteins (VP1, VP2, VP3, agnoprotein, LTAg, and STAg) from all nine human polyomaviruses were extracted from reference genomes available in GenBank, i.e., BKV (accession number NC_001538), JCV (NC_001699), KIV (NC_009238), WUV (NC_009539), MCV (NC_010277), TSV (NC_014361), HPyV6 (NC_014406), HPyV7 (NC_014407), and HPyV9 (NC_015150). Each of the corresponding proteins derived from all human polyomavirus reference genomes was aligned with the ClustalW program (31). Furthermore, 479 VP1 amino acid (aa) sequences were aligned. Conservation of the sequence alignments was plotted with the Plotcon program from the EMBOSS software package (32) by using a window size of 25. All multiple sequence alignments can be provided by us upon request. Maximum sequence similarity of immunodominant JCV peptides was calculated by using epitope conservancy analysis from the Immune Epitope Database (IEDB) and Analysis Resource (www.immuneepitope.org) (33).

Proteins and peptides. For the identification of JCV-specific $CD4^+$ T cells, 204 (13- to 16-mer) peptides covering the entire JC viral proteome (Fig. 1A) were applied. Peptides were synthesized and provided by pe (peptides&elephants GmbH, Potsdam, Germany). The peptides overlap by 5 amino acids and include 35 peptides with common single amino acid mutations (see Table S4 in the supplemental material). To account for amino acid variations (Fig. 1B) that occur among the different JCV genotypes and strains, amino acid sequences of each JCV-encoded protein, including agnoprotein (Agno), VP1, VP2, VP3, large T antigen (LTAg), small T antigen (STAg), and three T′ antigens (T′135, T′136, and T′165) from all 479 JCV genomic sequences available in GenBank (by March 2008) were aligned with the ClustalW program (31), and those polymorphisms which were prevalent in more than 1% of all retrieved sequences were defined as common mutations. Peptides were arranged in a set of 42 pools, each pool containing 5 different peptides (Fig. 1C; see also Table S5 in the supplemental material). Variations of the same peptide were not arranged in the same pool.

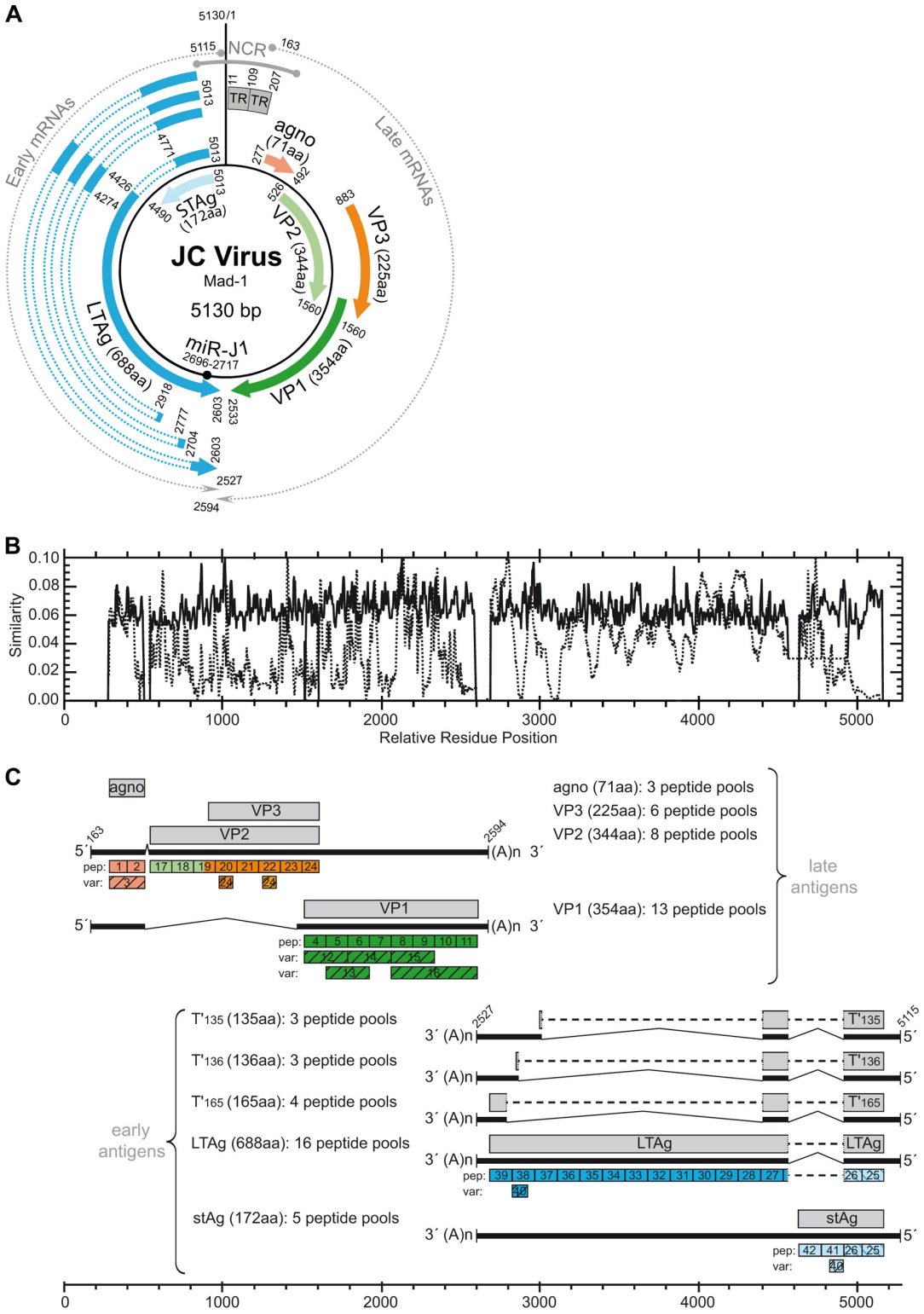
JCV VP1 protein forms virus-like particles (VLP), and VP1 and VLP are therefore used as interchangeable terms. VP1 protein-forming VLP (VP1/VLP) were generated by the Life Science Inkubator, Bonn, Germany, as previously described (34). Tetanus toxoid (TTxd) (Novartis Behring, Marburg, Germany) was used as a positive control.

Proliferation and activation assays. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll density gradient centrifugation (PAA, Pasching, Austria), always using the same volume/volume ratio of blood/Ficoll. Proliferation against peptide pools was performed with fresh PBMC and proliferative assays with individual peptides with frozen cells from the same individuals from the same date of sampling. In both assays, T cell responses to VLP, TTxd, and phytohemagglutinin (PHA) were also analyzed. A total of 2×10^5 PBMC per well were seeded in 96-well U-bottom microtiter plates in RPMI medium (Invitrogen) containing 2 mM glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin-streptomycin (all from Whittaker Bioproducts), and 5% pooled human AB serum. JCV peptide pools, JCV individual peptides, VLP, or TTxd was added to the cultures, and cells were cultured for 7 days. PHA-L (Sigma, St. Louis, MO) (1 μg/ml) was added to some wells on day 4. Wells containing only cells and medium served as negative controls. All JCV peptides were tested either in pools at a final concentration of 2 μM per peptide or as individual peptides at a concentration of 10 μM. TTxd was tested at 5 μg/ml. To detect frequencies of JCV-specific cells lower than 1 in 2×10^5 PBMC, 8 replicate wells were seeded for each peptide pool. Individual peptides were tested by seeding 10 wells per peptide. To control for variability between 96-well U-bottom microtiter plates in samples from each HD, each plate contained wells stimulated with JCV peptides, PHA, TTxd, and VLP, as well as unstimulated wells.

After 7 days of incubation, proliferation was measured by ^{3}H-thymidine (Hartmann Analytic, Braunschweig, Germany) incorporation in a scintillation beta counter (Wallac 1450; PerkinElmer, Rodgau-Jürgesheim, Germany). The stimulation index (SI) was calculated as SI = counts per minute (cpm) of an individual well with antigen/mean cpm of 72 negative-control wells. Individual wells with peptide pools were considered positive if the SI was >3 (Fig. 2A). In order to detect reactivity of thawed cells to single peptides, we considered positive individual wells, with SI > 2.

To determine whether $CD4^+$ or $CD8^+$ T cells or both of these T cell subsets were equally or preferentially stimulated by the above-described JCV-derived 13- to 16-mer peptides, representative blood samples from cohort 1 were analyzed by using carboxyfluorescein succinimidyl ester (CFSE; Sigma, St. Louis, MO) dye dilution for proliferation and by intracellular cytokine staining (ICS) using gamma interferon (IFN-γ) for activation. Briefly, 10^7 PBMC/ml were incubated with 0.5 μM CFSE for 8 min at room temperature, labeling was stopped using 33.3% fetal calf serum, and unbound CFSE was washed out. A total of 2×10^5 CFSE-labeled PBMC per well were seeded in 96-well U-bottom plates in the above-mentioned medium. For each donor, two JCV peptide pools, which had been identified as immunodominant in the respective donor by ^{3}H-thymidine incorporation assays, were selected for stimulation and, for each peptide pool, 20 wells were stimulated at a final concentration of 2 μM per peptide. Six wells were plated without antigen to serve as a negative control. TTxd at 5 μg/ml served as a positive control. After 10 to 14 days, five JCV peptide-stimulated wells were pooled and processed for IFN-γ ICS. Therefore, the respective wells were restimulated with the corresponding JCV peptide pool at a final concentration of 2 μM for each peptide, and after 1 h of incubation at 37°C, Golgistop (BD Biosciences, Heidelberg, Germany) was added, followed by incubation for 5 h at 37°C. After two washes with phosphate-buffered saline (PBS), cells were labeled with a Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen GmbH, Karlsruhe, Germany). After cellular Fc receptors were blocked with human IgG (Sigma, St. Louis, MO), the following surface markers were stained with antibodies: CD3 (phycoerythrin [PE], clone SK7; Biolegend, London, United Kingdom), CD4 (allophycocyanin [APC], clone RPA-T4; eBioscience, San Diego, CA), and CD8 (Pacific Blue [PB], clone DK25; Dako, Glostrup, Denmark). Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's protocol, washed, and stained with an anti-IFN-γ antibody (PE-cy7, clone 4S.B3; BD Biosciences). Cells were washed twice with 200 μl fluorescence-activated cell sorter (FACS) buffer (0.01% sodium azide–1% bovine serum albumin–PBS) before samples were analyzed with a LSR-II flow cytometer (BD Biosciences) and FACS Diva Software (BD Biosciences), gating on lymphocytes, singlet cells, live cells, $CD3^+$ cells, $CD4^+$ and $CD8^+$ cells, and $CFSE^{dim}$ and IFN-γ^+ cells (see Fig. S1A in the supplemental material). Approximately 30,000 singlet, live, $CD3^+$ lymphocytes were recorded per sample. The stimulation index for CFSE-labeled cells was calculated as SI [CFSE] = percentage of $CFSE^{dim}$ T cells among a pooled set of five wells with antigens/percentage of $CFSE^{dim}$ T cells among a pool of six unstimulated control wells (Fig. 2B; see also Fig. S1B in the supplemental material). The stimulation index for IFN-γ ICS was calculated as SI [IFN-γ] = percentage of IFN-γ^+ T cells among a pooled set of five wells with antigens/percentage of IFN-γ^+ T cells among a pool of six unstimulated control wells (Fig. 2B; see also Fig. S1B in the supplemental material).

ELISA for VP1/VLP-specific antibodies. The level of JCV VP1/VLP-specific IgG antibodies was determined as described previously (12). Briefly, enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μl JCV VLP (1 mg/ml)/well and incubated with serum samples or Ficoll supernatant, diluted 1:5,000 in blocking buffer (5% nonfat powdered milk–25 mM Tris–150 mM NaCl), and serial dilutions of a positive control. Human IgG was captured by a biotin-conjugated anti-human Fc antibody (eBioscience, Frankfurt, Germany) and detected by an avidin horseradish peroxidase (eBioscience, Frankfurt, Germany). Extinction



was measured as the optical density at 450 nm (OD_{450}) using a μQuant microplate reader (PerkinElmer, Waltham, MA). The measured extinctions were normalized to the OD_{450} of the positive control on the plate and reported as normalized OD_{450} (nOD_{450} = mean OD_{450} of sample replicates/mean OD_{450} of positive-control replicates on the plate). The same human serum was always used as the positive control, and its reactivity approximated an OD_{450} of 1. To check comparability between Ficoll supernatants and sera, JCV-specific antibody levels were measured in sera and Ficoll supernatants of three healthy donors, from whom sera and whole blood for Ficoll gradient centrifugation were collected at the same time. The Ficoll supernatant reproducibly showed a dilution factor of 1.2 in comparison to the serum sample from the same donor.

In order to determine serological cross-reactivity between JCV and BKV VP1 proteins (kindly provided by Natalie Meinerz and Robert Garcea, Department of Molecular, Cellular, and Developmental Biology, University of Colorado Boulder), representative serum samples underwent competitive inhibition ("preadsorption") assays. Specifically, serial dilutions of BKV VP1 and JCV VP1 proteins were added to a 1:5,000 dilution of serum in blocking buffer and incubated for 1 h at 4°C prior to incubating samples with immobilized JCV VP1 and completing the ELISA as described previously (12). The concentration needed to sufficiently and reliably compete VP1-specific antibodies was 4.86 μg/ml. Percent inhibition (PI) was calculated as PI = 100 × [1 − (average nOD_{450} of VP1 preincubated samples/average nOD_{450} of buffer-incubated samples)].

Quantitative PCR assay for JCV detection. The JCV DNA load was quantified in urine by applying a quantitative PCR (qPCR) with a set of primers and probe located at the T antigen of JCV (35). Viral DNA was extracted from 750 μl of urine by applying a QIAampUltraSens virus kit (Qiagen, Hilden, Germany). Nucleic acids were recovered in 60 μl of elution buffer. Thus, in a qPCR, 125 μl of urine was analyzed. The assay was specific for JCV detection, and the lower limit of JCV DNA detection was 1 to 10 copies per reaction, with a linear range from 10^2 to 10^7 genome copies per reaction. Amplifications were performed in a 25-μl reaction mixture containing 10 μl of sample and 12.5 μl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Madrid, Spain), which offers accurate, real-time PCR-based pathogen detection and is highly sensitive even in the presence of inhibitors. The concentration of each primer (JE3R and JE3F) was 0.9 μM and that of the fluorogenic probe (JE3P) 0.225 μM. This primer/probe set is specific for JCV and does not detect human polyomavirus BK PyV or simian virus 40 (SV40) or bovine polyomavirus (35). Following activation of the AmpliTaq Gold for 10 min at 95°C, 40 cycles (15 s at 95°C and 1 min at 60°C) were performed in a Mx3000P detection system (Stratagene, Santa Clara, CA). The samples were run neat and as a 10-fold dilution in duplicate (4 runs/sample), whereas each dilution of standard DNA suspension (from 10^2 to 10^7) was run in triplicate. In all qPCR assays, the amount of DNA was defined as the mean of the data obtained. A nontemplate control (NTC) was added to each run in addition to the negative control of the nucleic acid extraction. The possible presence of inhibitors, potentially leading to false-negative results, has been tested, adding low copy numbers of viral DNA to urine samples. JCV Mad1 cloned into the EcoRI site of a pBR322 vector was used for the standard curve. All qPCRs were done in different rooms isolated from post-PCR samples to prevent contamination.

RESULTS

JCV-specific immune response in healthy donors. In order to study the $CD4^+$ T cellular immune response to JCV, 204 13- to 16-mer peptides overlapping by 5 amino acids and spanning all JCV proteins (Agno, VP1, VP2, VP3, LTAg, STAg, and T′ antigens T′135, T′136, and T′165) were synthesized and arranged in 42 pools (Fig. 1; see also Table S5 in the supplemental material). PBMC from 49 HD (cohort 1) were tested for antigen-specific proliferation against the 42 pools. T cell responses showed a high degree of interindividual variability with respect to recognition of different peptide pools, with respect to the magnitude of the proliferative response, i.e., the stimulation indices of individual proliferating wells, and also with respect to the number of positive wells responding to a single pool (Fig. 2A). Each of the measures described above contains different information about JCV-specific T cell responses. First, the number of different pools that elicit a proliferative response in a given donor expresses whether the JCV-specific T cell response is broad, when many peptide pools are recognized, or focused, if only a few or one pool is positive. Second, the magnitude of the response (i.e., stimulation index) expresses the amount of incorporated thymidine and hence, indirectly, the number of cell divisions. And third, since a defined number of PBMC is seeded per well, the number of positive wells per pool serves as a measure of the presence of the response in a predefined amount of cells, serving as a very rough approximation of the precursor frequency of antigen-specific T cells. These 3 measures together allow assessing the JCV-specific immune response within a single individual. Regarding our data, PBMC from some of the HD failed to proliferate against any of the peptide pools (Fig. 2A, upper graph), while others showed a strong proliferative response to multiple peptide pools (Fig. 2A, lower graph). The mean SI values for negative-control wells and tetanus toxoid (TTxd)-stimulated wells of the same individuals are shown

FIG 1 JCV genome organization, protein sequence variability, and mapping of peptide pools. (A) The genomic organization of the JCV reference genome (NC_001699), consisting of 5,130 bp, is shown with the nucleotide positions of all open reading frames indicated by colored arrows. Nucleotides are numbered relative to the JCV reference genome (NC_001699). Large T antigen (LTAg, dark blue arrow), small T antigen (STAg, light blue arrow), three additional alternative splice variants of the large T antigen (T′135, T′136, and T′165, dark blue arrows), agnoprotein (agno, red arrow), VP1 (dark green arrow), VP2 (light green arrow), and VP3 (orange arrow) are shown, as well as early and late mRNAs (gray, dotted arrows), the tandem repeats (TR, gray boxes) in the noncoding region (NCR, gray line), and JCV microRNA (miRNA) (miR-J1, black dot). Introns within the respective open reading frames are depicted as boxes with dashed lines. (B) Sequence variability of amino acid sequences among different JCV strains (continuous line) and among nine human PyVs (dashed line) comprised of BKV (accession number NC_001538), JCV (NC_001699), KIV (NC_009238), WUV (NC_009539), MCV (NC_010277), HPyV6 (NC_014406), HPyV7 (NC_014407), TSV (NC_014361), and HPyV9 (NC_015150). Similarity scores were plotted against the respective amino acid of each protein and matched to the relative nucleotide position of the JCV reference genome (NC_001699). (C) Mapping of 42 peptide pools (colored boxes) to the relative nucleotide positions of the JCV reference genome (NC_001699). LTAg, STAg, T′135, T′136, and T′165, agno, VP1, VP2, and VP3 (gray-shaded boxes) are shown, as well as early and late mRNAs (black lines) including introns (dashed lines) and polyadenylated 3′ ends [3′ (A)n], in relation to the JCV reference genome (NC_001699). Each pool consists of 5 different peptides. A total of 169 peptides overlap by 5 amino acids (pep; nonhatched boxes), and 35 peptides contain single amino acid substitutions derived from common JCV variants (var; hatched boxes). Agno is covered by 3 peptide pools (pools 1 to 3, red), where pool 3 contains variants of peptides 5 (pool 1) and 7, 9, and 12 (pool 2), all matching agno. VP1 is covered by 13 peptide pools (pools 4 to 16, dark green), where pools 12 to 16 contain variants of peptides included in VP1 pools 4 to 11. VP2 is covered by 8 peptide pools (pools 17 to 24, light green), where pool 24 contains variants of peptides 96 (pool 20) and 106 (pool 22). VP3, the sequence of which is identical to the N-terminal part of VP2, is covered by 6 peptide pools (peptides 90 to 92 from pool 19 and pools 20 to 24, orange), where pool 24 contains variants of peptides 96 (pool 20) and 106 (pool 22). LTAg is covered by 16 peptide pools (pools 25 to 40, dark blue), and STAg is covered by 5 peptide pools (pools 25, 26, and 40 to 42, light blue), where pool 40 contains variants of peptides 185, 187, and 189 included in pool 39 (LTAg) and variants of peptides 193 and 195 included in pool 41 (STAg).

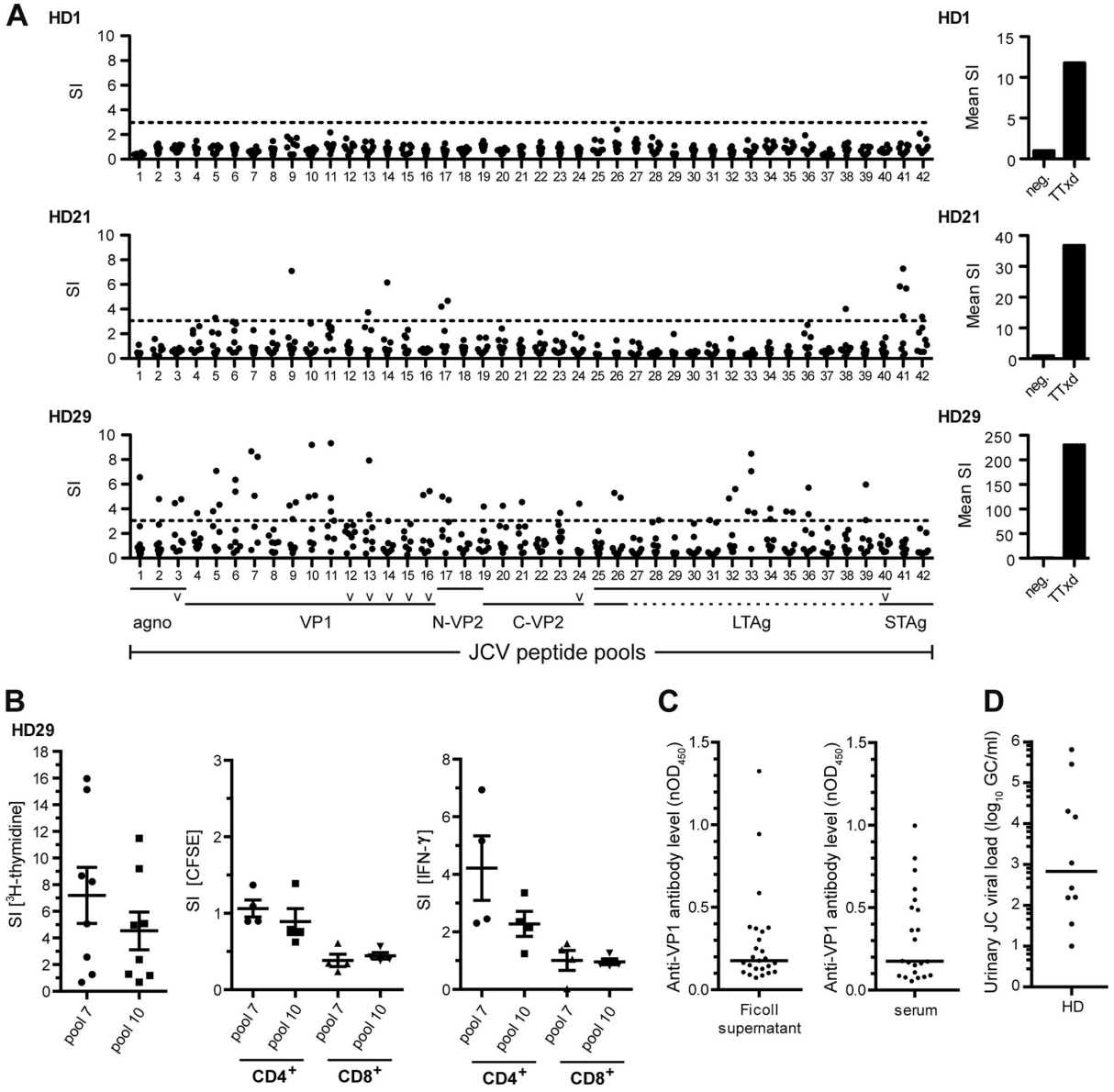


FIG 2 Interindividual variability in JCV-specific immune response in healthy donors. (A, left graphs) Proliferation of PBMC from three representative HD to 42 JCV-derived peptide pools as measured by ^{3}H-thymidine incorporation. PBMC were stimulated with JCV peptides assembled in 42 peptide pools (*x* axis) and eight wells per pool. Pools containing peptide variants are marked (v). Pool 3 contains variants of peptides included in pool 1 and 2 (agno). Pools 12 to 16 contain variants of peptides included in pools 4 to 11 (VP1). Pool 40 contains variants of peptides included in pools 39 (LTAg) and 41 (STAg). Dots represent single stimulation indices (SI) (*y* axis) and were considered positive when SI $\geq$ 3 (horizontal line). (A, right graphs) Histograms representing the mean SI of unstimulated and TTxd-stimulated wells. (B) A representative sample shows cell proliferation by ^{3}H-thymidine incorporation (left graph; mean SI [^{3}H-thymidine] $\pm$ standard error of the mean [SEM]; one dot corresponds to 2×10^5 stimulated PBMC) and preferential proliferation and activation of CD4$^+$ T cells over CD8$^+$ T cells after stimulation by JCV peptide pools 7 and 10 as measured by CFSE dilution (central graph; mean SI [CFSE] $\pm$ SEM; one dot corresponds to 10^6 stimulated PBMC) and intracellular cytokine (IFN-γ) staining (right graph; mean SI [IFN-γ] $\pm$ SEM; one dot corresponds to 10^6 stimulated PBMC) in a FACS analysis. Peptide pools 7 and 10 were selected because HD29 showed pool 7- and pool 10-reactive wells as measured by ^{3}H-thymidine incorporation. (C) Spectrum of anti-JCV humoral responses measured by ELISA using normalized optical density at 450 nm (nOD$_{450}$) in Ficoll supernatants of cohort 1 (left graph) and sera of cohort 2 (right graph). Ficoll supernatants were diluted by the factor 1.2 in relation to sera. (D) Urinary JC viral shedding expressed as log$_{10}$ GC/ml.

in the right graphs. CFSE labeling and IFN-γ ICS of PBMC from representative donors confirmed that CD4$^+$ T cells, and not CD8$^+$ T cells, preferentially responded to stimulation with donor-specific immunodominant JCV peptide pools as identified by ^{3}H-thymidine incorporation assays (Fig. 2B; see also Fig. S1B in the supplemental material). CFSE^{dim} cells representing proliferated cells were predominantly activated upon restimulation with JCV peptides as shown by IFN-γ secretion in the ICS, and IFN-γ-

secreting cells were predominantly $CFSE^{dim}$. Since ^{3}H-thymidine incorporation assays are more convenient for the analysis of such a high number of peptide pools and donors, we decided to analyze the fine specificity of JCV-specific $CD4^+$ T cells in depth by using ^{3}H-thymidine incorporation assays.

We also analyzed the JCV-specific humoral response and shedding of JCV DNA in urine. VLP-specific antibodies were quantified by ELISA in 44 HD. JCV DNA was determined by PCR in urine from 22 HD. JCV-specific humoral responses and shedding of JCV DNA in urine also showed a high degree of interindividual variability (Fig. 2C and D). Considering a dilution factor of 1.2 for Ficoll supernatant related to serum, nOD_{450} values among Ficoll supernatants of cohort 1 and sera of cohort 2 showed similar variabilities (Fig. 2C). Displayed are the urinary JCV DNA copy numbers of all 10 JCV-excreting individuals (Fig. 2D).

Next, in order to examine whether JCV-specific T cellular immune responses are indicative of JC virus infection, we calculated the percentage of HD with a positive T cell response to JCV and compared this value with the percentage of HD presenting VLP-specific antibodies and with JCV DNA in urine. The latter two parameters are currently used to identify JCV-infected individuals. To calculate the percentage of HD with positive JCV-specific T cell responses, we used two different arbitrary thresholds for positivity. The low threshold considers that a HD has a positive JCV T cell response if she or he shows 3 or more wells with SI $\geq$ 3, while the high threshold considers 10 or more wells with SI $\geq$ 3. Analogous thresholds were used to determine the percentage of HD with VLP-specific antibodies (low threshold at $nOD_{450} \geq 0.08$; high threshold at $nOD_{450} \geq 0.2$) and with JCV DNA in urine (low threshold at copy number $\geq$ 10 GC/ml; high threshold at copy number $\geq$ 100 GC/ml). Using the low thresholds for positivity, 77.6% of the HD were positive for JCV-specific T cell response compared to 89.4% for anti-VLP antibodies and only 45.5% of HD shedding JCV DNA in urine. Using the high thresholds, 28.6% of HD were positive for JCV-specific T cell response, 42.6% for anti-VLP antibodies, and 36.4% for JCV DNA in urine. Considering a low threshold of $nOD_{450} \geq 0.08$ for the definition of seropositive donors, 33.3% of seropositive individuals showed no T cell proliferation to JCV peptides (defined as fewer than 3 wells with SI $\geq$ 3). A total of 45.5% of seropositive donors showed no T cell proliferation to JCV peptides when a high threshold of $nOD_{450} \geq 0.2$ was applied. The association between JCV-specific antibody level and urinary JC viral load was weak (correlation coefficient $r = 0.27$), similar to the results published by Gorelik et al. (36). As a result of the use of two cohorts, measures of all the three parameters in the same HD that would allow us to study correlations are not available. This was due to using healthy blood bank donors, from whom serum and urine could not be obtained.

Identification of JCV immunodominant epitopes. First, we analyzed T cell responses from the 49 HD of cohort 1 against the 42 peptide pools. Individuals responded to peptide pools from all JCV proteins (see Fig. S2 in the supplemental material). The preference and strength of reactivity to peptide pools from a specific JCV protein varied between HD (see Fig. S3 in the supplemental material). Most donor samples recognized pools derived from four different JCV proteins (36.7%), some donor samples recognized three different proteins (20.4%), and only 12.2% of all HD samples recognized peptide pools derived from all JCV-encoded proteins.

To determine the JCV peptide pools that likely contained immunodominant peptides, we performed a detailed analysis of T cell responses of cohort 1. Responses of individual wells were considered positive when the SI was higher than 3. First, the sum of SIs [$\sum$(SI $>$ 3)] from positive wells was calculated to account for the strength and proliferative capacity of each donor with respect to each peptide pool (Fig. 3A, upper graph). To correct for background proliferation, we calculated the $\sum$(SI $>$ 3) also for unstimulated wells, normalized the value for the number of wells, and subtracted it from the $\sum$(SI $>$ 3) corresponding to each pool and donor. Next, in order to integrate an estimation of the precursor frequency of JCV-peptide-specific T cells, we calculated the fraction of positive wells (total number of positive peptide wells [SI $>$ 3] per number of seeded wells) (Fig. 3A, middle graph). Again, with the purpose of excluding background proliferation, the fraction of unstimulated wells with SI $>$ 3 was calculated, normalized, and subtracted from the fraction of positive peptide-stimulated wells. Finally, we determined as a third indicator of immunogenicity the frequency of donors with at least one positive well for each pool (Fig. 3A, lower graph). Using these three parameters, we created a reactivity score [RS = $\sum$(SI $>$ 3) $\times$ percentage of reactive wells $\times$ percentage of reactive individuals] for each pool that takes into account the stimulatory capacity of the pool, the frequency of peptide-specific cells in the pool, and the frequency of donors with at least one well positive for the pool (Fig. 3B). Using this approach, we identified pools 14 (containing VP1 peptides), 17 (containing VP2 peptides), and 32 and 33 (containing LTAg peptides) as the pools showing a higher RS and in consequence to be the best candidates to contain immunodominant peptides. These pools had a $\sum$(SI $>$ 3) higher than 125, with a proportion of reactive wells of at least 5.0% and recognition by more than 30% of HD.

Although the use of 15-mer peptides in combination with the settings of the proliferative assay favors detection of $CD4^+$ T cell responses according to current knowledge, we cannot exclude the contribution of $CD8^+$ T cells in the proliferative response to pools 14, 17, 32, and 33 since 15-mer peptides can be shortened by proteolytic cleavage and presented on HLA class I molecules.

To identify immunodominant JCV peptides, we tested PBMC from 20 HD (cohort 2) with the 20 individual peptides contained in pools 14, 17, 32, and 33. Reactivity scores for each individual peptide were calculated as mentioned above, but considering positive a response with SI $>$ 2, since we used fewer cells per plate (100,000/well) due to a limited amount of cells and cells had been frozen before their use. Accordingly, a lower proliferative capacity of the cells enrolled in these assays along with correspondingly less background proliferation appeared. Results are summarized in Fig. 3C. We identified peptide 41 [VP1 (123–137) variant 3; V3] as the immunodominant peptide contained in pool 14, and peptides 81 [VP2 (30–43)] and 82 [VP2 (39–51)] as the immunodominant peptides contained in pool 17. Pool 33 contained the immunodominant peptide 162 [LTAg (415–429)]. We were not able to identify an immunodominant peptide in pool 32.

HLA class II restriction of JCV-specific cellular immune responses. $CD4^+$ T cells recognize antigenic peptides in the context of HLA class II molecules. Table S1 in the supplemental material shows the HLA class II alleles of the 49 HD from cohort 1. HD were divided into 12 groups depending on HLA-DR expression (see Table S3 in the supplemental material). Group DR1 included all donors expressing the HLA-DRB1*01 alleles ($n = 8$), and we similarly grouped donors expressing HLA class II molecules of the

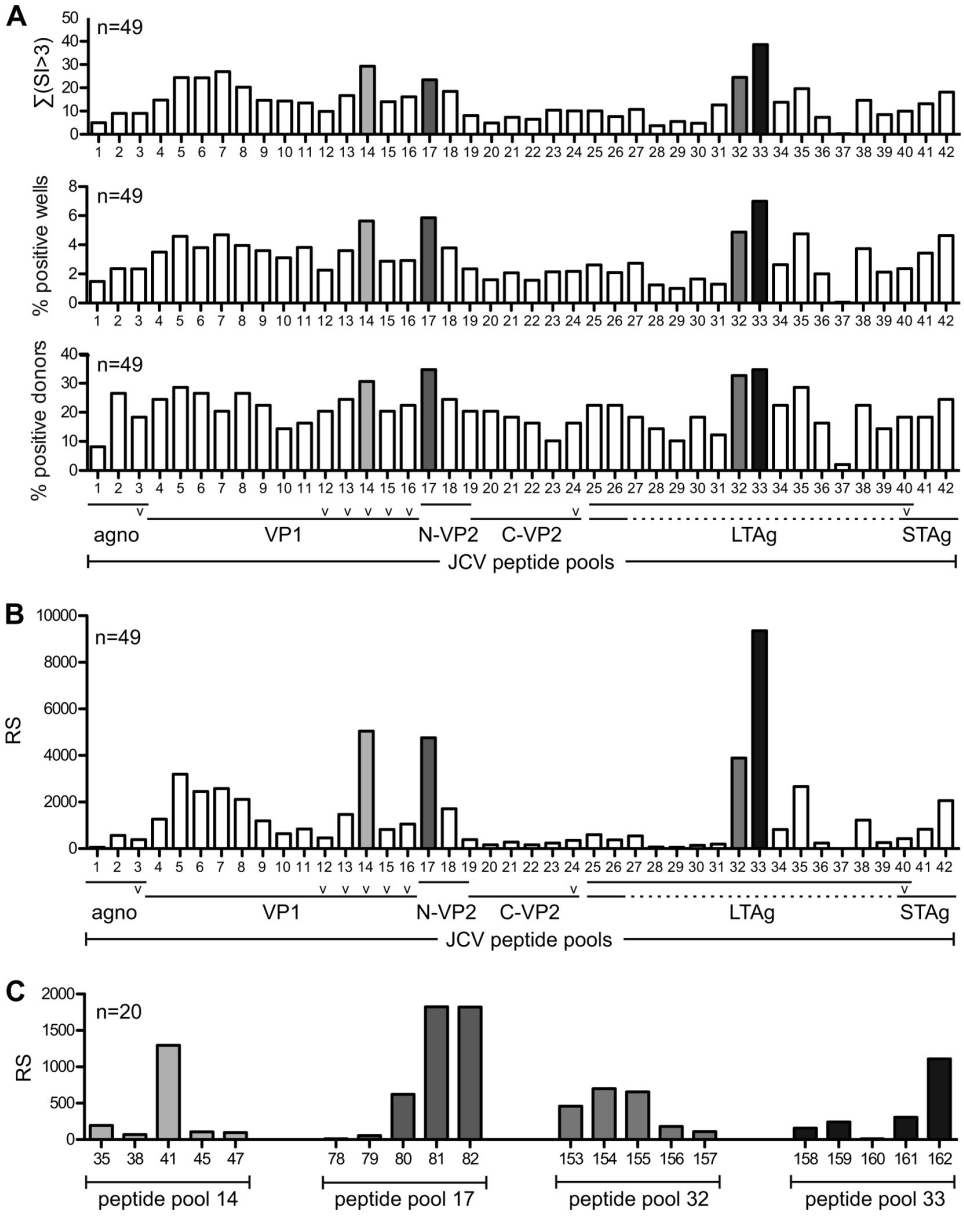


FIG 3 Proliferation of PBMC to peptide pools and individual peptides. (A) Immunogenicity of 42 pools was assessed with regard to stimulatory capacity (sum of SI > 3) (upper graph), estimated precursor frequency (percentage of positive wells) (middle graph), and prevalence in tested cohort 1 (percentage of positive donors) (lower graph). (B) Graph showing the reactivity score (RS) of the same cohort corresponding to each pool [Σ(SI > 3) × percentage of reactive wells × percentage of reactive individuals], which accounts for all three aspects of immunogenicity. Pools containing peptide variants are marked (v). Pool 3 contains variants of peptides included in pool 1 and 2 (agno). Pools 12 to 16 contain variants of peptides included in pools 4 to 11 (VP1). Pool 40 contains variants of peptides included in pools 39 (LTAg) and 41 (STAg). Pools 14, 17, 32, and 33 are most immunogenic in all three aspects (columns marked in light or dark grey). (C) Graphs showing the RS of cohort 2 corresponding to each of the 20 single peptides from pools 14, 17, 32, and 33. The higher proliferations were evoked by peptides 41 [VP1 (123-137) V3], 81 [VP2 (30-43)], 82 [VP2 (39-51)], and 162 [(LTAg (415-429)].

major subtypes DRB1*04 (n = 15, DR4), DRB1*07 (n = 13, DR7), DRB1*08 (n = 2, DR8), DRB1*10 (n = 1, DR10), DRB1*11 (n = 9, DR11), DRB1*12 (n = 4, DR12), DRB1*13 (n = 12, DR13), DRB1*14 (n = 1, DR14), DRB1*15 (n = 13, DR15), DRB1*16 (n = 7, DR16), and DRB1*03 (n = 10, referred to as DR17). The circle diagram in Fig. 4A represents the frequencies of the most common DR types within this HD cohort. Note that nearly all donors were heterozygotic for HLA-DRB1*alleles and are therefore contained in two different HLA-DR groups. Proliferative responses were analyzed for HLA-DRB1* cohorts, which were rep-

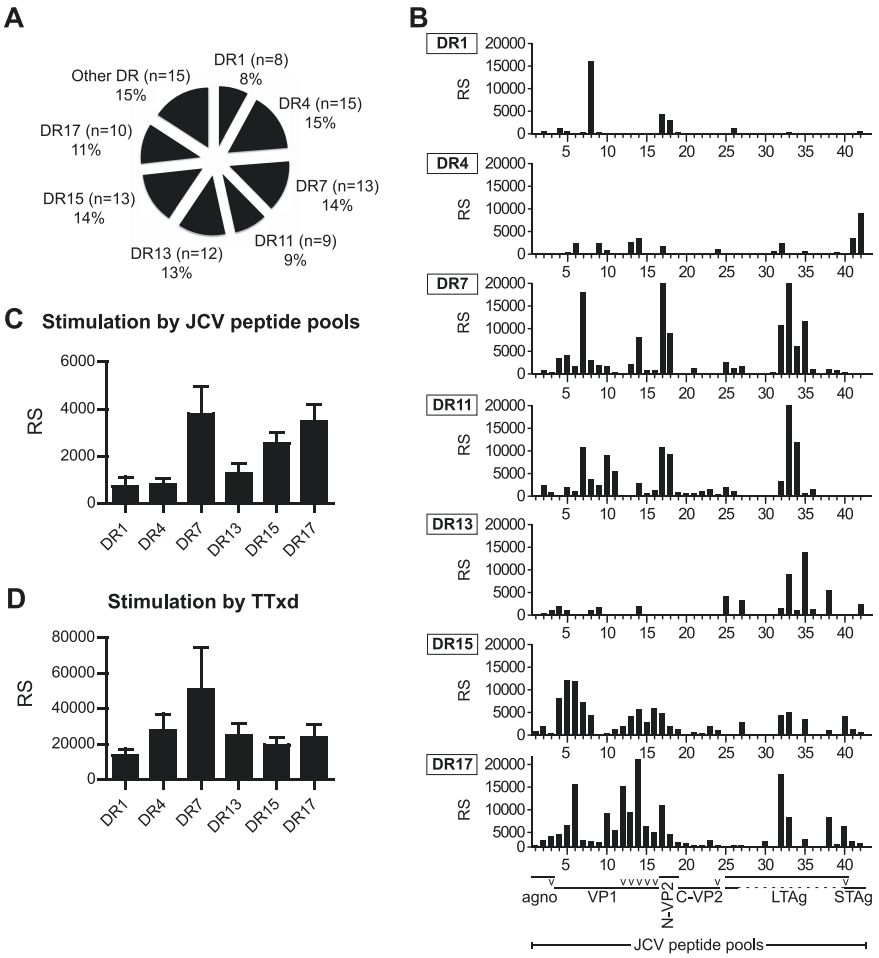


FIG 4 HLA-restricted proliferation to JCV peptide pools. (A) Frequency of DRB1 expression among 49 HD from cohort 1. (B) Proliferation measured as RS of PBMC from DR1$^+$, DR4$^+$, DR7$^+$, DR11$^+$, DR13$^+$, DR15$^+$, and DR17$^+$ HD to 42 peptide pools. Because most donors are heterozygotic with respect to DRB1* alleles, the same donor might appear in two different HLA cohorts. For quantitative comparisons of proliferation, RS values in each HLA group were normalized to a group of 10 HD. Pools containing peptide variants are marked (v). Pool 3 contains variants of peptides included in pool 1 and 2 (agno). Pools 12 to 16 contain variants of peptides included in pools 4 to 11 (VP1). Pool 40 contains variants of peptides included in pools 39 (LTAg) and 41 (STAg). (C) Proliferation of PBMC from DR1$^+$, DR4$^+$, DR7$^+$, DR11$^+$, DR13$^+$, DR15$^+$, and DR17$^+$ HD measured as the sum of RS from all peptide pools. (D) Proliferation measured as RS of PBMC from DR1$^+$, DR4$^+$, DR7$^+$, DR11$^+$, DR13$^+$, DR15$^+$, and DR17$^+$ HD to TTxd. Values shown represent mean RS ± standard error of the mean (SEM).

resented by at least 8 individuals. To analyze the T cell response of each HD group against the 42 pools, we calculated the RS as described above. Because each group was made up by different numbers of HD, we normalized each group for the number of donors; therefore, values are directly comparable and adjusted to $n = 10$. PBMC from HD expressing different HLA class II molecules responded to different peptide pools (Fig. 4B); also, the RS varied considerably among groups. HD from groups DR7 and DR17 showed significantly higher RS to JCV peptides than HD from groups DR1 and DR4, while no differences were observed in RS to TTxd (Fig. 4C and D). The preference for specific JCV proteins also varied among HLA-DR groups (see Fig. S2 and Fig. S3 in the supplemental material). All HD except the DR13$^+$ and DR16$^+$ HD recognized peptides from the amino-terminal region of VP2 more often than expected (see Fig. S2 in the supplemental material). Peptides from the carboxy-terminal region of VP2 were recognized less frequently than expected (see Fig. S2 in the supplemental material). DR8$^+$, DR12$^+$, and DR13$^+$ HD recognized peptides from the LTAg more often than expected (see Fig. S2 in the supplemental material). Importantly, the preference for specific JCV proteins was also variable within a group expressing certain HLA alleles (see Fig. S3 in the supplemental material), which is most likely due to the fact that there is variability with respect to the second HLA-DR allele expressed by the heterozygotic donors. The pools inducing the strongest cellular response in the entire cohort (pools 14, 17, 32, and 33) were pools that were stimulatory in the context of several class II alleles, including the two groups showing the strongest responses (group DR7 and group DR17) (Fig. 4B).

Next, we examined whether the peptides identified as immunodominant after analyzing the whole HD cohort were also immunodominant for all HLA-DR haplotypes. PBMC from cohort 2

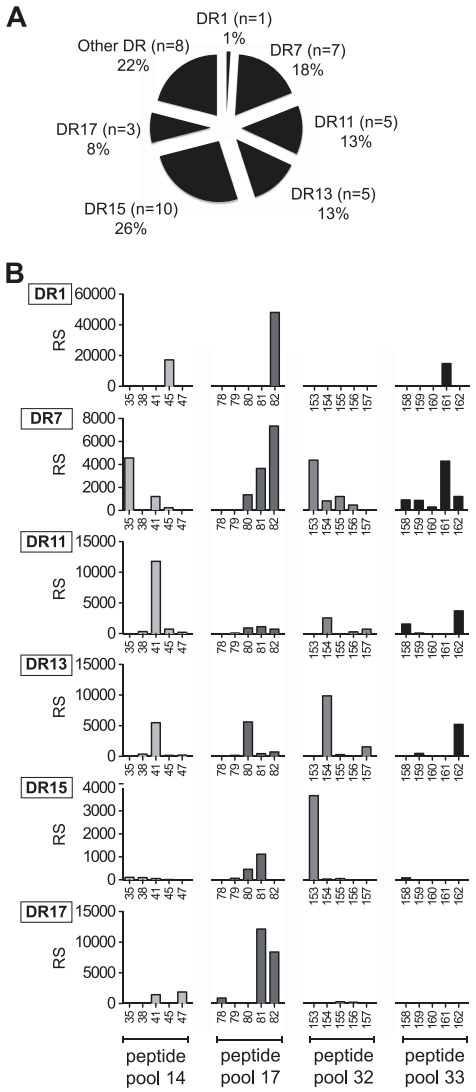


FIG 5 HLA-restricted proliferation to individual JCV peptides. (A) Frequency of DRB1 expression among 20 HD from cohort 2. (B) Proliferation measured as RS of PBMC from DR1$^+$, DR7$^+$, DR11$^+$, DR13$^+$, DR15$^+$, and DR17$^+$ HD to JCV individual peptides, which were contained in the most immunogenic peptide pools (pools 14, 17, 32, and 33). For quantitative comparisons of proliferation, RS values in each HLA group were normalized to a group of 10 HD.

expressing different HLA class II alleles (Fig. 5A) were tested against the 20 peptides contained in the pools inducing the strongest cellular response in the entire cohort (pools 14, 17, 32, and 33). The reactivity scores induced by these 20 peptides are summarized in Fig. 5B. The peptides inducing the highest RS clearly differed between HLA subgroups, showing that HLA class II alleles contribute to the fine specificity of CD4$^+$ T cells against JCV peptides. An important factor in determining immunodominance is binding to the respective HLA class alleles, which can be predicted *in silico* by a number of publicly accessible databases. When we calculated the *in silico*-predicted binding scores by using the consensus method from the IEDB Analysis Resource (33), the binding scores of immunodominant JCV peptides varied broadly depending on the HLA allele (see Table S6 in the supplemental material). Peptide 41 [VP1 (123–137) V3] was identified as one of the most immunodominant peptides when analyzing the whole cohort, probably because it was strongly stimulatory in DR11 and DR13 donors, who represented 50% of all tested individuals. Similarly, peptide 82 [VP2 (39–51)] was identified as immunodominant, most likely because it induced prominent responses in individuals expressing DR7 and DR17. Sequences for all immunodominant JCV peptides are summarized in Table 2. All peptides identified as immunodominant except for the two JCV VP1-derived peptides showed highest sequence similarity with BKV-encoded protein sequences (mean similarity, 78.4%; range, 53.3% to 100%), compared to all human PyV (Table 2). BKV-derived peptides previously described as immunodominant and/or cross-reactive to JCV were found to match to peptides from immunodominant pools 32 and 33 (LTAg) and peptides from substantially recognized pools 4, 5, 6, 35, and 38 of the present study (see Table S7 and Table S8 in the supplemental material).

Reduced cellular JCV-specific T cell immune response in DRB1*04:01$^+$ donors. A more detailed analysis of HD from the DRB1*04$^+$ group revealed marked differences in JCV-specific T cell responses. The RS of HD expressing the DRB1*04:01 allele were significantly lower than those of HD expressing other DRB1*04 alleles (Fig. 6A and B) or non-DR4 alleles (Fig. 4C). Interestingly, this reduced T cell responsiveness was "specific" for JCV, since the response to the recall antigen TTxd in these donors was comparable to those of HD expressing other DRB1*04 molecules (Fig. 6C).

In order to examine further the reduced JCV T cell response in DRB1*04:01$^+$ donors, we determined the presence of JCV DNA in urine samples from 8 DRB1*04:01$^+$ donors, from 7 donors expressing other DR4 alleles, and from 10 HD not expressing any DR4 allele. Results are summarized in Fig. 6D. Remarkably, none of the urine samples from HD expressing the DRB1*04:01 allele tested positive for JCV DNA. In contrast, JCV DNA was detected in 4 of 5 (80.0%) urine samples from HD expressing DR4 alleles other than DRB1*04:01 and in 6 of 10 (60.0%) from donors not expressing DR4 alleles (Fig. 6D). These results, together with the low frequency of JCV-specific T cells in DRB1*04:01$^+$ HD, suggested that these individuals might not have been exposed to the virus. To examine this issue further, we then tested the humoral response to JCV VP1 by ELISA to assess their infectious state in serum samples from 7 DRB1*04:01$^+$ donors, from 5 HD expressing DR4 alleles other than DRB1*04:01, and from 10 HD not expressing any DR4 allele. DRB1*04:01$^+$ donors showed levels of anti-JCV antibodies similar to or even higher than those seen with the two other groups (Fig. 6E). Serological cross-reactivity to BKV VP1 was excluded by competitive preadsorption assays (Fig. 6F). Reactivity of serum samples from five DRB1*04:01$^+$ donors was efficiently inhibited by preincubation with 4.86 μg/ml JCV VP1 (median PI, 64.9%; interquartile range [IQR], 58.5% to 68.6%; minimum inhibition, 52.1%) and was minimally inhibited by 4.86 μg/ml BKV VP1 (median PI, 1.4%; IQR, 0.0% to 2.2%; maximum inhibition, 3.0%). Similarly, serum samples from seven DRB1*04:01$^-$ donors, including three HD expressing DR4 alleles other than DRB1*04:01, showed strong inhibition after preincubation with

TABLE 2 Immunodominant JCV peptides and their maximum sequence identity with the proteome of eight other human PyVs[a]

JCV peptide no.	Immunodominant JCV epitope				Maximum identity with PyV proteome (%)	PyV protein and position	PyV peptide sequence
	Pool no.	Protein and position	Peptide sequence	Amino acid			
35	14	VP1 (108–122) V3	LKTEVIGVTALMNVH	15	66.7	TSV VP1 (119–133)	**V**KTEV**V**GV**SS**L**V**NVH
41	14	VP1 (123–137) V3	SNGQASHDNGAGKPV	15	46.7	MCV VP1 (131–145)	**WDMKRV**HD**Y**GAG**I**PV
80	17	VP2 (20–34)	AATGFSVAEIAAGEA	15	100.0	BKV VP2 (20–34)	AATGFSVAEIAAGEA
81	17	VP2 (30–43)	AAGEAAATIEVEIA	14	85.7	BKV VP2 (30–43)	AAGEAAA**A**IEV**Q**IA
82	17	VP2 (39–51)	EVEIASLATVEGI	13	92.3	BKV VP2 (39–51)	EV**Q**IASLATVEGI
153	32	LTAg (328–342)	FADSKNQKSICQQAV	15	93.3	BKV LTAg (329–343)	FA**E**SKNQKSICQQAV
154	32	LTAg (338–351)	CQQAVDTVAAKQRV	14	85.7	BKV LTAg (339–352)	CQQAVDTV**L**AK**K**RV
155	32	LTAg (347–361)	AKQRVDSIHMTREEM	15	80.0	BKV LTAg (348–362)	AK**K**RVD**TL**HMTREEM
161	33	LTAg (405–419)	VIYDFLKCIVLNIPK	15	73.3	BKV LTAg (406–420)	VI**F**DFL**H**CIV**F**N**V**PK
162	33	LTAg (415–429)	LNIPKKRYWLFKGPI	15	80.0	BKV LTAg (416–430)	**F**N**V**PK**R**RYWLFKGPI

[a] All protein sequences from the reference genomes of BKV (accession number NC_001538), KIV (NC_009238), WUV (NC_009539), MCV (NC_010277), HPyV6 (NC_014406), HPyV7 (NC_014407), TSV (NC_014361), and HPyV9 (NC_015150) were used for epitope conservancy analysis (www.immuneepitope.org). Nonmatching amino acids are indicated in bold and underlined.

4.86 μg/ml JCV VP1 (median PI, 83.7%; interquartile range [IQR], 68.3% to 88.2%; minimum inhibition, 52.2%), but minimal inhibition after preadsorption with 4.86 μg/ml BKV VP1 (median PI, 1.5%; IQR, 0.0% to 6.9%; maximum inhibition, 12.3%). Using a threshold of $nOD_{450} \geq 0.08$ for the definition of seropositive HD, 27.8% of DRB1*04:01$^-$ donors and 44.4% of DRB1*04:01$^+$ individuals showed no T cell proliferation to JCV peptides (fewer than 3 wells with SI $\geq$ 3 per donor). Using the higher threshold for seropositivity ($nOD_{450} \geq 0.2$), even 60% of DRB1*04:01$^+$, but only 33.3% of DRB1*04:01$^-$ donors, showed no T cell proliferation. The anti-JCV VP1 seropositivity strongly argues against the hypothesis that these donors were not exposed to JCV but, despite the weak CD4$^+$ T cell responses to JCV peptide pools, showed a robust antibody response to the major capsid protein from as-yet-unknown mechanisms.

Reduced cellular JCV-specific immune responses in DRB1*01:01$^+$ healthy donors. Similar to DRB1*04:01-heterozygous donors, HD positive for DRB1*01$^+$ mounted significantly weaker JCV-specific cellular responses than DR7$^+$, DR15$^+$, and DR17$^+$ HD (Fig. 4B and 5B), although cellular reactivities to TTxd did not differ among these groups (Fig. 4D). Unfortunately, we could not analyze urinary shedding or JCV-specific humoral response in DR1$^+$ HD because of a lack of samples.

DISCUSSION

In the present study, we examined the cellular immune response to JCV with respect to prevalence of positive CD4$^+$ T cell responses, specificity for JCV proteins/peptides, and relation to HLA class II haplotype of the donor to develop a better understanding of JCV-specific immune control, to identify potential risk factors for PML and as a basis for future vaccination approaches. We identified several immunodominant peptides within the most prominent peptide pools, peptide 41 (pool 14), peptides 81 and 82 (pool 17), peptide 155 (pool 32), and peptide 162 in pool 33 (Fig. 3C). However, after stratification for HLA class II haplotypes, differences in the responses to peptide pools as well as to individual peptides within these pools became visible (Fig. 4). While the magnitudes of the response to the pools differed, for example, between individuals carrying DR15 and DR17 alleles and those carrying DR1 and, even more so, DR4 alleles, the focus of the response remained on VP1 across different HLA-DR haplotypes (Fig. 4). At the level of individual peptides, HLA stratification revealed that the immunodominant single peptides within the immunodominant pools differed according to the donors' HLA-DR haplotypes. This is not too surprising, considering that the peptide binding grooves of the main alleles show considerable differences with respect to their binding motifs. In this context, it has to be mentioned that we did not formally establish HLA-restricted peptide recognition, which needs to be done with T cell lines or clones and antigen-presenting cells, e.g., HLA-DR transfectants, matched for each individual HLA-DR molecule. Such a detailed analysis is not possible at the level of the entire JCV proteome and across several HLA-DR haplotypes; however, our data should provide a basis for future studies in this direction.

Next we examined the issue of whether JCV-specific cellular immune responses are comparable to VLP seropositivity or whether there are substantial differences. Since there is at present no commonly established definition for what constitutes a positive T cell response to JCV and hence for what is indicative of whether the individual has been exposed to the virus, we arbitrarily defined thresholds for the cellular immune response to JCV peptides or JCV VLP, for urinary viral shedding, and for the humoral immune response. To determine if an individual shows a positive JCV-specific T cell response, we took into account the fraction of JCV peptide pool-reactive wells. We found a positive cellular response to JCV in 28.6% to 77.6%, depending on the threshold. Other ways to determine the status of exposure to or infection with JCV are analyses of urinary excretion and the presence of JCV-specific antibodies. Here we found positivity ranging from 36.4% to 45.5% (urinary excretion) and 42.6% to 89.4% (anti-VP1 antibody response), depending on the threshold and criterion. Since only approximately 50% of JCV-exposed individuals shed virus in the urine, ascertainment of JCV exposure cannot be based on JCV urinary excretion alone. On the other hand, an individual who sheds virus in the urine must be JCV infected even if JCV-specific T cell or antibody responses are low or borderline. Based on our thresholds for positive T cell and antibody responses, we assume that JCV exposure in our cohort ranged between 60% and 80%. However, to formally address this issue,

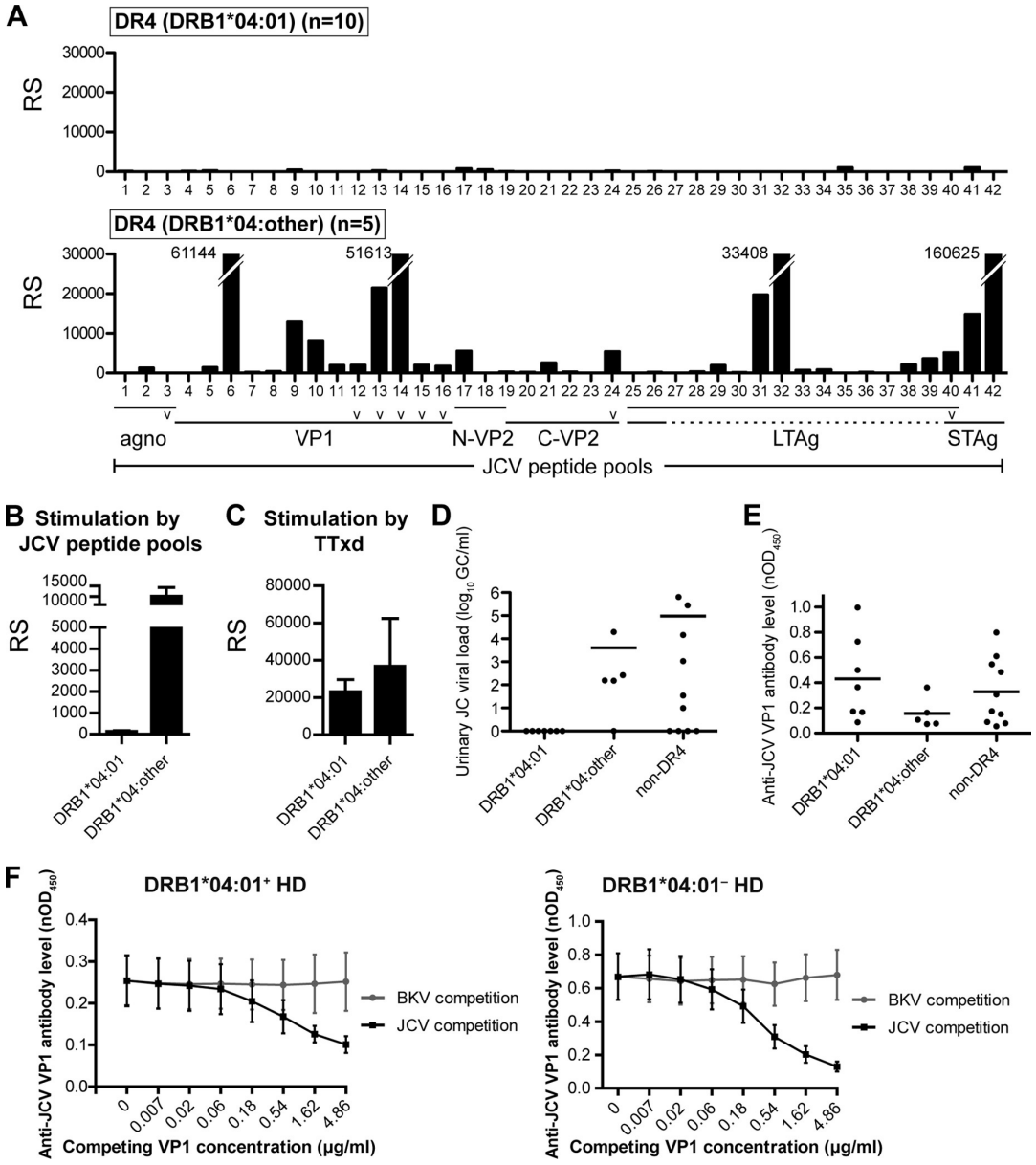


FIG 6 T cell proliferation to JCV, urinary viral shedding, and JCV-specific antibody levels in DRB1*04:01$^+$ healthy donors. (A) Proliferation of PBMC from DRB1*04:01$^+$ HD (n = 10) measured as RS to JCV peptide pools in comparison to PBMC from DRB1*04$^+$ other than DRB1*04:01$^+$ HD (n = 5). For quantitative comparisons of proliferation, RS values in each HLA group were normalized to a group of 10 HD. Pools containing peptide variants are marked (v). Pool 3 contains variants of peptides included in pool 1 and 2 (agno). Pools 12 to 16 contain variants of peptides included in pools 4 to 11 (VP1). Pool 40 contains variants of peptides included in pools 39 (LTAg) and 41 (STAg). (B) Proliferation of PBMC from DRB1*04:01$^+$ and DRB1*04$^+$ other than DRB1*04:01$^+$ HD measured as the sum of RS from all peptide pools. (C) Proliferation measured as RS of PBMC from DRB1*04:01$^+$ and DRB1*04$^+$ other than DRB1*04:01$^+$ HD to TTxd. Values shown represent mean RS ± SEM. (D) JCV urinary viral load expressed as $\log_{10}$ GC/ml of DRB1*04:01$^+$ (n = 7), DRB1*04$^+$ other than DRB1*04:01$^+$ (n = 5), and non-DR4$^+$ HD (n = 10). (E) VLP-specific antibody levels measured by ELISA using normalized optical density at 450 nm (nOD_{450}) in sera from DRB1*04:01$^+$ HD (n = 7), DRB1*04$^+$ HD other than DRB1*04:01$^+$ (n = 5), and non-DR4$^+$ HD (n = 10). (F) Antibody reactivity (mean nOD_{450} ± standard error of mean) to JCV VP1 proteins after competition with soluble BKV VP1 protein (BKV competition) or soluble JCV VP1 protein (JCV competition) in sera of DRB1*04:01$^+$ HD (n = 5, left graph) and DRB1*04:01$^-$ HD (n = 7, right graph).

much larger cohorts need to be examined and compared systematically.

The proteins triggering the highest overall proliferation are the major capsid protein VP1 and the amino-terminal end of the minor capsid protein VP2 (Fig. 3A and B). However, most donors also recognized other JCV proteins (see Fig. S3 in the supplemental material), and the preference for a JCV protein varied not only among individual donors, but also among groups that had been stratified according to HLA class II haplotype (see Fig. S2 and Fig. S3 in the supplemental material).

As already mentioned above, when assessing immunodominance we considered the strength of the proliferative responses and the precursor frequency of antigen-specific T cells. The latter measure expresses the fraction of T cells of an individual that is specific for JCV, while the prevalence of immunity in a population provides information as to what fraction of a population responds to a specific antigen. All three measures provide distinct information and can be used to determine immunodominance. Only peptide pools fulfilling all three criteria for immunodominance were considered here, and the peptides within these pools were investigated individually. With this strategy, we found peptides 41 [VP1 (123–137); SNGQASHDNGAGKPV] and 82 [VP2 (39–51); EVE IASLATVEGI] causing strong proliferation in the entire cohort of HD. Peptides 81 and 82, as well as peptides 154 and 155, overlap by 5 aa, which may in part explain the proliferation to both. It is possible that the optimal and most stimulatory peptide is situated between peptides 81 and 82, or between other peptides that were not identified here, due to the overlap of only 5 aa. However, the broad specificity of the $CD4^+$ T cell responses indicates that multiple JCV peptides can be recognized by Caucasian individuals, and this is consistent with the fact that JCV is a highly prevalent virus to which the human immune system is well adapted.

Previously, two $CD8^+$ T cell epitopes, peptides VP1 (100–109) and VP1 (36–44), have been described as immunodominant in HLA-A*0201-restricted donors (25, 26). In our study, the VP1 (100–109) peptide (ILMWEAVTL) was represented by peptides 31 [VP1 (91–105); LNEDLTCGN*ILMWEA*] and 32 [VP1 (101–115); *LMWEAVTL*KTEVIGV] in peptide pool 6. The VP1 (36–44) peptide (SITEVECFL) is covered by peptide 20 [VP1 (34–48); VD*SITEVECFL*TPEM] in pool 4. Both pools 4 and 6 were among the 12 and 6 pools, respectively, that elicited the strongest responses (Fig. 3B), and $DR15^+$ and $DR17^+$ individuals recognized pool 6 as the second strongest and strongest pool, respectively (Fig. 4B). Further support for the relevance of peptide VP1 (36–44) and peptide 20 from pool 4 (together with only 7 other JCV peptides) comes from our recent data indicating that these peptides are relevant targets for brain-infiltrating $CD4^+$ T cells that had been isolated from a brain biopsy specimen of a patient suffering from PML-immune reconstitution inflammatory syndrome (29). We also demonstrated $CD8^+$ T cells recognizing JCV VP1 (36–44) in the brain biopsy specimen of this HLA-$A2^+$ MS patient, although the $CD4^+$ T cells were more prevalent in the biopsy specimen than $CD8^+$ T cells. Data from the latter study and work presented here indicate that $CD8^+$ T cells and $CD4^+$ T cells recognize related epitopes and that JCV peptide VP1 (36–44) is of particular relevance.

Regarding the influence of certain HLA-DR haplotypes on cellular immune responsiveness to JCV, the comparably very low or absent reactivity in DRB1*$04:01^+$ individuals was of particular interest. Since all individuals in the two DRB1*04^+ subgroups were heterozygotic for DRB1*04:01 or DRB1*04:other except for one, and the effect of low proliferative response was not more pronounced in the homozygous DRB1*$04:01^+$ individual, we conclude that the DRB1*04:01 haplotype has a dominant-negative influence on the JCV-specific proliferative response. It remains to be elucidated which mechanisms lead to this gross reduction of JCV-specific T cell responses in heterozygotic DRB1*$04:01^+$ HD. Several associations between HLA-DRB1*04:01 and specific infections have been described in epidemiological studies, in which a considerable number of heterozygotic donors were included: HLA-DRB1*04:01 was reported to be associated with slower progression of HIV-1 infection (37), clearance of chronic hepatitis C virus (HCV) infection (38), and low hepatitis C virus activity (39). Furthermore, DRB1*04:01 is associated with increased risk for rheumatoid arthritis (RA) (40), mixed connective tissue disease (41), and insulin-dependent diabetes mellitus (42), as well as with a reduced age at onset of multiple sclerosis (MS) (43). DRB1*$04:01^+$ MS patients show a restricted T cell receptor repertoire in the context of the T cell response to one immunodominant myelin basic protein (residues 111 to 129) peptide (44). Taken together, these reports appear to document dominant-positive effects of the HLA-DRB1*04:01 allele. However, no experimentally proven domination of the DRB1*04:01 allele leading to reduced or abrogated function of other HLA-DR alleles has been reported. Previously, an HLA-associated low responsiveness to streptococcal antigen in healthy donors has been described and linked to the DR2-DQA1*0102-DQB1*0602 (DQw6)-Dw2 haplotype and DR2-DQA1*0103-DQB1*0601 (DQw6)-Dw12 haplotype (45, 46). One possible mechanism for such low responsiveness inherited in a dominant fashion is clonal deletion. A second mechanism could involve the deletion of JCV-specific T cells by chronic antigenic restimulation *in situ*. A third possibility is the induction of a T-regulatory phenotype of JCV-specific T cells responding to HLA-DRB1*$04:01^+$ antigen-presenting cells. Whether the HLA-DRB1*04:01 allele itself or a immunosuppressive gene in strong linkage disequilibrium confers this effect is of further interest, but remains to be clarified.

Additionally, we observed a B cell-skewed JCV-specific immune response in DRB1*$04:01^+$ individuals. The mechanisms underlying a strong humoral anti-JCV response in HLA-DRB1*04:01-restricted donors and at the same time a low to absent $CD4^+$ T cell response remain to be elucidated. It could represent a T cell-independent JCV-specific B cell response with the second stimulation signal delivered by the JCV antigen itself, e.g., binding to Toll-like receptors or extensive cross-linking of the membrane. Such a T cell-independent virus-specific antibody response has been shown in mice lacking T cells and infected with mouse PyV (47). A second mechanism could involve a cross-reactive B-cell response, which recognizes not only JCV-encoded epitopes, but also similar epitopes encoded by other human polyomaviruses or infectious agents, which has been generated by $CD4^+$ T cells specific against other human polyomaviruses or infectious agents, but not JCV. This mechanism could operate in the absence of JCV-specific $CD4^+$ T cell responses and lead to JCV-specific B cell responses that are detectable only due to cross-reactivity between epitopes of JCV and other human polyomaviruses or other infectious agents.

Antibody cross-reactivity has been suggested between JCV and BKV VP1 proteins (36), BKV and S40 VP1 proteins (48, 49), and JCV and SV40 VP1 proteins (48, 49). However, little or no anti-

body cross-reactivity between BKV and JCV VP1 proteins or between SV40 and both JCV and BKV VP1 proteins has been reported in the majority of studies (11, 49–55), and no antibody cross-reactivity was found between VP1 proteins of other polyomaviruses, e.g., lymphotropic polyomavirus (LPV) versus MCV or KIV versus WUV (48). Since we also did not find serum cross-reactivity between JCV VP1 and its most closely related VP1 protein among human polyomaviruses (BKV VP1) in a representative set of sera from HLA-DRB1*04:01$^+$ as well as from HLA-DRB1*04:01$^-$ donors, we expect the latter scenario to be less likely.

A third mechanism could be that the JCV-specific B cell response and a long-lasting JCV-specific B cell memory had been generated with CD4$^+$ T cell help earlier in life and that JCV-specific T cells had been deleted by chronic antigenic restimulation *in situ*. It is well known that the so-called type 2 helper T cell (T_h2) subset of CD4$^+$ T cells activates B cells to provide neutralizing antibody production of various IgG isotypes by CD4$^+$ T cell-mediated production of interleukin (IL)-4 and IL-5 (56), whereas type 1 helper T cells (T_h1) cells producing IL-2 and gamma interferon (IFN-γ) favor clonal expansion of cytotoxic CD8$^+$ T cells and macrophage activation. There are only a few reports on an influence of major histocompatibility complex (MHC) class II genes in shifting the CD4$^+$ T cell phenotype in humans and thereby predetermining an immune response, which is skewed to be more cell or more antibody mediated. Agrewala and Wilkinson (57) showed that the p(91–110) epitope of the 16-kDa antigen of *Mycobacterium tuberculosis* is able to elicit a T_h1 or T_h2 response, depending on the HLA-DR haplotype of the donor. It was suggested that a high MHC-peptide affinity leads to a T_h1 response and lower binding affinities to a T_h2 response (58, 59). Wen et al. (60) showed in HLA-DR4 transgenic mice that DRB1*04:01-encoded molecules may promote a T_h2 immune response. Furthermore, HLA-DRB1*04:01 has been associated with the development of antibodies against beta interferon in MS patients and autoantibodies against insulin (61, 62).

The above previously described associations between higher or lower prevalence of a specific infection and variations in disease presentation as well as the association with certain autoimmune diseases are poorly understood at the functional level. They argue, however, that specific host-pathogen interactions may alter immune responsiveness in a way that impacts on the outcome of the infection. The fact that such associations have been observed even for much larger/more complex organisms than JCV, e.g., M. *tuberculosis*, argues that the reasons must be more complex than a lack of binding of peptides to the DRB1*04:01 allele. Given the molecular/cellular mechanisms governing a low/absent CD4$^+$ T cell response to JCV in DRB1*04:01$^+$ individuals, together with a very strong humoral response and lack of urinary shedding, its implications for JCV immune control clearly merit further study.

Secondary to those of HLA DRB1*04:01 subjects, cells from DRB1*01$^+$ individuals also demonstrated low proliferation on JCV peptide pools, but the response was less pronounced than in the DRB1*04:01 subgroup (Fig. 4C). Additionally, PBMC from DRB1*01$^+$ HD responded less to TTxd stimulation than those from other DR groups. However, these differences were not significant. Related to viral immunity, HLA-DRB1*01 has been associated with resistance to HIV infection (63), with clearance of HCV infection (64–66), and with a good responsiveness to HBsAG vaccination (67). More importantly, in analogy to HLA-DRB1-04:01, HLA-DRB1*01:01 is associated with increased risk for RA (40), and these commonalities with DRB1*04:01 and other RA-associated DR alleles have been attributed to a shared motif in the peptide binding grooves of these DR molecules. Whether reduced JCV-specific T cell responses in HLA-DRB1-04:01$^+$ and HLA-DRB1*01:01$^+$ individuals with RA contribute to the precipitation of PML, especially during immunomodulatory treatment with, e.g., rituximab (68), remains to be elucidated in larger populations. In comparison to RA patients, PML occurs even more frequently in patients with systemic lupus erythematosus (69) and natalizumab-treated MS patients (15, 16, 19). Whether HLA associations contribute to an elevated PML risk needs to be clarified, however, in larger population-based studies.

Our assay does not allow us to distinguish directly between CD4$^+$ T cell- and CD8$^+$ T cell-driven proliferation. We have chosen 15-mer peptides, which preferentially stimulate CD4$^+$ T cell proliferation, since this length can be accommodated by the open HLA class II binding groove, in contrast to the HLA class I binding pocket, which is closed and optimally accommodates peptides of 8 to 10 amino acids in length. In order to use the most sensitive assay with a broad dynamic range that allowed us to detect even small numbers of proliferating cells reliably, we used thymidine incorporation as a readout. Alternative approaches using CFSE or enzyme-linked immunosorbent spots (ELISPOT) are less sensitive, but the former can distinguish CD4$^+$ from CD8$^+$ T cell proliferation. When checking the composition of the proliferating T cell population after JCV peptide or protein stimulation by flow cytometry, we observed mainly CD4$^+$ T cells, supporting the above assumption. This does not verify exclusive CD4$^+$ T cell proliferation but indicates that strong immunogenicity of some of the peptides mainly stems from CD4$^+$ T cells with the possibility that CD8$^+$ T cells contribute as well. By choosing an overlap of only 5 aa between adjacent peptides, we may have missed potential epitopes not covered by our selection of peptides. However, a wide range of different epitopes scattered over the entire JCV proteome may be recognized by single healthy donors; as shown in Fig. 1 and 3, peptide pools from all JCV proteins were recognized (see Fig. S2 in the supplemental material), and there was high interindividual and inter-HLA-DR-group variability regarding preference and strength of reactivity to single JCV peptide pools (see Fig. S3 in the supplemental material), indicating that a set of 15-mer peptides overlapping by 5 aa and covering the whole JCV proteome is sufficient to uncover the intra- and interindividual variability in epitope specificity and immunodominant JCV CD4$^+$ T cell epitopes.

We are aware that our data give rise to a number of questions. They do, however, provide a good basis for future, more focused studies on specific aspects as well as examination of larger patient numbers, which may employ additional tools, including HLA class II tetramers loaded with JCV peptides and subsequent screening of populations of interest, such as MS patients receiving natalizumab and other populations at risk for PML. In our view, assessing the T cell reactivity will, besides serological anti-JCV response and JCV viral DNA testing, provide important additional information with respect to virus exposure of the individual and the robustness of her or his immune status. Studying the strength, the antigen fine specificity, and, in the future, also the functional phenotype of the T cell response to JCV is expected to be useful as a basis for subsequent studies on JCV immune con-

trol, for diagnostic purposes and also for the development of anti-JCV vaccination.

ACKNOWLEDGMENTS

This work was supported by an unrestricted grant from Biogen Idec, Inc. Ilijas Jelčić was supported by the Deutsche Forschungsgemeinschaft (JE 530/1-1). The Institute for Neuroimmunology and Clinical Multiple Sclerosis Research was supported by the Gemeinnützige Hertie Stiftung.

We thank Nathalie Meinerz and Robert Garcea, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, for kindly providing BKV VP1 proteins. We thank all of the HD who supported our work by donating PBMC, serum, and/or urine for our experiments.

REFERENCES

1. **Padgett BL, Walker DL, ZuRhein GM, Eckroade RJ, Dessel BH.** 1971. Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. Lancet **i**:1257–1260.
2. **Gardner SD, Field AM, Coleman DV, Hulme B.** 1971. New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet **i**:1253–1257.
3. **Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B.** 2007. Identification of a third human polyomavirus. J. Virol. **81**:4130–4136.
4. **Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D.** 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog. **3**:e64. doi:10.1371/journal.ppat.0030064.
5. **Feng H, Shuda M, Chang Y, Moore PS.** 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science **319**:1096–1100.
6. **Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB.** 2010. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe **7**:509–515.
7. **van der Meijden E, Janssens RW, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, Feltkamp MC.** 2010. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. PLoS Pathog. **6**:e1001024.
8. **Scuda N, Hofmann J, Calvignac-Spencer S, Ruprecht K, Liman P, Kuhn J, Hengel H, Ehlers B.** 2011. A novel human polyomavirus closely related to the African green monkey-derived lymphotropic polyomavirus. J. Virol. **85**:4586–4590.
9. **Ou WC, Wang M, Fung CY, Tsai RT, Chao PC, Hseu TH, Chang D.** 1999. The major capsid protein, VP1, of human JC virus expressed in Escherichia coli is able to self-assemble into a capsid-like particle and deliver exogenous DNA into human kidney cells. J. Gen. Virol. **80**(Pt 1):39–46.
10. **Ferenczy MW, Marshall LJ, Nelson CD, Atwood WJ, Nath A, Khalili K, Major EO.** 2012. Molecular biology, epidemiology, and pathogenesis of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain. Clin. Microbiol. Rev. **25**:471–506.
11. **Egli A, Infanti L, Dumoulin A, Buser A, Samaridis J, Stebler C, Gosert R, Hirsch HH.** 2009. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. J. Infect. Dis. **199**:837–846.
12. **Weber T, Trebst C, Frye S, Cinque P, Vago L, Sindic CJ, Schulz-Schaeffer WJ, Kretzschmar HA, Enzensberger W, Hunsmann G, Luke W.** 1997. Analysis of the systemic and intrathecal humoral immune response in progressive multifocal leukoencephalopathy. J. Infect. Dis. **176**:250–254.
13. **Bofill-Mas S, Clemente-Casares P, Major EO, Curfman B, Girones R.** 2003. Analysis of the excreted JC virus strains and their potential oral transmission. J. Neurovirol. **9**:498–507.
14. **Monaco MC, Atwood WJ, Gravell M, Tornatore CS, Major EO.** 1996. JC virus infection of hematopoietic progenitor cells, primary B lymphocytes, and tonsillar stromal cells: implications for viral latency. J. Virol. **70**:7004–7012.
15. **Major EO.** 2010. Progressive multifocal leukoencephalopathy in patients on immunomodulatory therapies. Annu. Rev. Med. **61**:35–47.
16. **Tan CS, Koralnik IJ.** 2010. Progressive multifocal leukoencephalopathy and other disorders caused by JC virus: clinical features and pathogenesis. Lancet Neurol. **9**:425–437.
17. **Gorelik L, Reid C, Testa M, Brickelmaier M, Bossolasco S, Pazzi A, Bestetti A, Carmillo P, Wilson E, McAuliffe M, Tonkin C, Carulli JP, Lugovskoy A, Lazzarin A, Sunyaev S, Simon K, Cinque P.** 2011. Progressive multifocal leukoencephalopathy (PML) development is associated with mutations in JC virus capsid protein VP1 that change its receptor specificity. J. Infect. Dis. **204**:103–114.
18. **Jensen PN, Major EO.** 2001. A classification scheme for human polyomavirus JCV variants based on the nucleotide sequence of the noncoding regulatory region. J. Neurovirol. **7**:280–287.
19. **Biogen Idec.** 2012. Tysabri safety update. Biogen Idec Switzerland AG, Zug, Switzerland. http://www.biogenidec.ch/medizinische_fachkreise.aspx?ID=10389. Accessed 3 October 2012.
20. **Clifford DB, Yiannoutsos C, Glicksman M, Simpson DM, Singer EJ, Piliero PJ, Marra CM, Francis GS, McArthur JC, Tyler KL, Tselis AC, Hyslop NE.** 1999. HAART improves prognosis in HIV-associated progressive multifocal leukoencephalopathy. Neurology **52**:623–625.
21. **Du Pasquier RA, Kuroda MJ, Zheng Y, Jean-Jacques J, Letvin NL, Koralnik IJ.** 2004. A prospective study demonstrates an association between JC virus-specific cytotoxic T lymphocytes and the early control of progressive multifocal leukoencephalopathy. Brain **127**:1970–1978.
22. **Marzocchetti A, Tompkins T, Clifford DB, Gandhi RT, Kesari S, Berger JR, Simpson DM, Prosperi M, De Luca A, Koralnik IJ.** 2009. Determinants of survival in progressive multifocal leukoencephalopathy. Neurology **73**:1551–1558.
23. **Khanna N, Wolbers M, Mueller NJ, Garzoni C, Du Pasquier RA, Fux CA, Vernazza P, Bernasconi E, Viscidi R, Battegay M, Hirsch HH.** 2009. JC virus-specific immune responses in human immunodeficiency virus type 1 patients with progressive multifocal leukoencephalopathy. J. Virol. **83**:4404–4411.
24. **Bloomgren G, Richman S, Hotermans C, Subramanyam M, Goelz S, Natarajan A, Lee S, Plavina T, Scanlon JV, Sandrock A, Bozic C.** 2012. Risk of natalizumab-associated progressive multifocal leukoencephalopathy. N. Engl. J. Med. **366**:1870–1880.
25. **Du Pasquier RA, Kuroda MJ, Schmitz JE, Zheng Y, Martin K, Peyerl FW, Lifton M, Gorgone D, Autissier P, Letvin NL, Koralnik IJ.** 2003. Low frequency of cytotoxic T lymphocytes against the novel HLA-A*0201-restricted JC virus epitope VP1(p36) in patients with proven or possible progressive multifocal leukoencephalopathy. J. Virol. **77**:11918–11926.
26. **Koralnik IJ, Du Pasquier RA, Kuroda MJ, Schmitz JE, Dang X, Zheng Y, Lifton M, Letvin NL.** 2002. Association of prolonged survival in HLA-A2+ progressive multifocal leukoencephalopathy patients with a CTL response specific for a commonly recognized JC virus epitope. J. Immunol. **168**:499–504.
27. **Du Pasquier RA, Schmitz JE, Jean-Jacques J, Zheng Y, Gordon J, Khalili K, Letvin NL, Koralnik IJ.** 2004. Detection of JC virus-specific cytotoxic T lymphocytes in healthy individuals. J. Virol. **78**:10206–10210.
28. **Gasnault J, Kahraman M, de Goer de Herve MG, Durali D, Delfraissy JF, Taoufik Y.** 2003. Critical role of JC virus-specific CD4 T-cell responses in preventing progressive multifocal leukoencephalopathy. AIDS **17**:1443–1449.
29. **Aly L, Yousef S, Schippling S, Jelcic I, Breiden P, Matschke J, Schulz R, Bofill-Mas S, Jones L, Demina V, Linnebank M, Ogg G, Girones R, Weber T, Sospedra M, Martin R.** 2011. Central role of JC virus-specific CD4+ lymphocytes in progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome. Brain **134**:2687–2702.
30. **Senger M, Flores T, Glatting K, Ernst P, Hotz-Wagenblatt A, Suhai S.** 1998. W2H: WWW interface to the GCG sequence analysis package. Bioinformatics **14**:452–457.
31. **Thompson JD, Higgins DG, Gibson TJ.** 1994. Improved sensitivity of profile searches through the use of sequence weights and gap excision. Comput. Appl. Biosci. **10**:19–29.
32. **Rice P, Longden I, Bleasby A.** 2000. EMBOSS: the European molecular biology open software suite. Trends Genet. **16**:276–277.
33. **Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, Damle R, Sette A, Peters B.** 2010. The immune epitope database 2.0. Nucleic Acids Res. **38**:D854–D862.
34. **Goldmann C, Petry H, Frye S, Ast O, Ebitsch S, Jentsch KD, Kaup FJ, Weber F, Trebst C, Nisslein T, Hunsmann G, Weber T, Luke W.** 1999. Molecular cloning and expression of major structural protein VP1 of the human polyomavirus JC virus: formation of virus-like particles useful for immunological and therapeutic studies. J. Virol. **73**:4465–4469.
35. **Pal A, Sirota L, Maudru T, Peden K, and Lewis AM, Jr.** 2006. Real-time,

quantitative PCR assays for the detection of virus-specific DNA in samples with mixed populations of polyomaviruses. J. Virol. Methods **135**:32–42.
36. **Gorelik L, Lerner M, Bixler S, Crossman M, Schlain B, Simon K, Pace A, Cheung A, Chen LL, Berman M, Zein F, Wilson E, Yednock T, Sandrock A, Goelz SE, Subramanyam M.** 2010. Anti-JC virus antibodies: implications for PML risk stratification. Ann. Neurol. **68**:295–303.
37. **Kroner BL, Goedert JJ, Blattner WA, Wilson SE, Carrington MN, Mann DL.** 1995. Concordance of human leukocyte antigen haplotype-sharing, CD4 decline and AIDS in hemophilic siblings. Multicenter Hemophilia Cohort and Hemophilia Growth and Development Studies. AIDS **9**:275–280.
38. **McKiernan SM, Hagan R, Curry M, McDonald GS, Kelly A, Nolan N, Walsh A, Hegarty J, Lawlor E, Kelleher D.** 2004. Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. Hepatology **40**:108–114.
39. **Yu RB, Hong X, Ding WL, Tan YF, Zhang YX, Sun NX, Wu GL, Zhan SW, Ge DF.** 2008. The association between the genetic polymorphism of HLA-DQA1, DQB1, and DRB1 and serum alanine aminotransferase levels in chronic hepatitis C in the Chinese population. J. Gastroenterol. Hepatol. **23**:1394–1402.
40. **Newton JL, Harney SM, Wordsworth BP, Brown MA.** 2004. A review of the MHC genetics of rheumatoid arthritis. Genes Immun. **5**:151–157.
41. **Dong RP, Kimura A, Hashimoto H, Akizuki M, Nishimura Y, Sasazuki T.** 1993. Difference in HLA-linked genetic background between mixed connective tissue disease and systemic lupus erythematosus. Tissue Antigens **41**:20–25.
42. **Tait BD, Drummond BP, Varney MD, Harrison LC.** 1995. HLA-DRB1*0401 is associated with susceptibility to insulin-dependent diabetes mellitus independently of the DQB1 locus. Eur. J. Immunogenet. **22**:289–297.
43. **Wu JS, James I, Qiu W, Castley A, Christiansen FT, Carroll WM, Mastaglia FL, Kermode AG.** 2010. HLA-DRB1 allele heterogeneity influences multiple sclerosis severity as well as risk in Western Australia. J. Neuroimmunol. **219**:109–113.
44. **Muraro PA, Vergelli M, Kalbus M, Banks DE, Nagle JW, Tranquill LR, Nepom GT, Biddison WE, McFarland HF, Martin R.** 1997. Immunodominance of a low-affinity major histocompatibility complex-binding myelin basic protein epitope (residues 111–129) in HLA-DR4 (B1*0401) subjects is associated with a restricted T cell receptor repertoire. J. Clin. Invest. **100**:339–349.
45. **Kamikawaji N, Fujisawa K, Yoshizumi H, Fukunaga M, Yasunami M, Kimura A, Nishimura Y, Sasazuki T.** 1991. HLA-DQ-restricted CD4+ T cells specific to streptococcal antigen present in low but not in high responders. J. Immunol. **146**:2560–2567.
46. **Nishimura Y, Sasazuki T.** 1983. Suppressor T cells control the HLA-linked low responsiveness to streptococcal antigen in man. Nature **302**:67–69.
47. **Szomolanyi-Tsuda E, Welsh RM.** 1996. T cell-independent antibody-mediated clearance of polyoma virus in T cell-deficient mice. J. Exp. Med. **183**:403–411.
48. **Kean JM, Rao S, Wang M, Garcea RL.** 2009. Seroepidemiology of human polyomaviruses. PLoS Pathog. **5**:e1000363. doi:10.1371/journal.ppat.1000363.
49. **Viscidi RP, Rollison DE, Viscidi E, Clayman B, Rubalcaba E, Daniel R, Major EO, Shah KV.** 2003. Serological cross-reactivities between antibodies to simian virus 40, BK virus, and JC virus assessed by virus-like-particle-based enzyme immunoassays. Clin. Diagn. Lab. Immunol. **10**:278–285.
50. **Aoki N, Mori M, Kato K, Sakamoto Y, Noda K, Tajima M, Shimada H.** 1996. Antibody against synthetic multiple antigen peptides (MAP) of JC virus capsid protein (VP1) without cross reaction to BK virus: a diagnostic tool for progressive multifocal leukoencephalopathy. Neurosci. Lett. **205**:111–114.
51. **Hamilton RS, Gravell M, Major EO.** 2000. Comparison of antibody titers determined by hemagglutination inhibition and enzyme immunoassay for JC virus and BK virus. J. Clin. Microbiol. **38**:105–109.
52. **Major EO, Neel JV.** 1998. The JC and BK human polyoma viruses appear to be recent introductions to some South American Indian tribes: there is no serological evidence of cross-reactivity with the simian polyoma virus SV40. Proc. Natl. Acad. Sci. U. S. A. **95**:15525–15530.
53. **Stolt A, Sasnauskas K, Koskela P, Lehtinen M, Dillner J.** 2003. Seroepidemiology of the human polyomaviruses. J. Gen. Virol. **84**:1499–1504.
54. **Sundsfjord A, Flaegstad T, Flo R, Spein AR, Pedersen M, Permin H, Julsrud J, Traavik T.** 1994. BK and JC viruses in human immunodeficiency virus type 1-infected persons: prevalence, excretion, viremia, and viral regulatory regions. J. Infect. Dis. **169**:485–490.
55. **Viscidi RP, Rollison DE, Sondak VK, Silver B, Messina JL, Giuliano AR, Fulp W, Ajidahun A, Rivanera D.** 2011. Age-specific seroprevalence of Merkel cell polyomavirus, BK virus, and JC virus. Clin. Vaccine Immunol. **18**:1737–1743. doi:10.1128/CVI.05175-11.
56. **Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL.** 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. **136**:2348–2357.
57. **Agrewala JN, Wilkinson RJ.** 1999. Influence of HLA-DR on the phenotype of CD4+ T lymphocytes specific for an epitope of the 16-kDa alpha-crystallin antigen of Mycobacterium tuberculosis. Eur. J. Immunol. **29**:1753–1761.
58. **Kumar V, Bhardwaj V, Soares L, Alexander J, Sette A, Sercarz E.** 1995. Major histocompatibility complex binding affinity of an antigenic determinant is crucial for the differential secretion of interleukin 4/5 or interferon gamma by T cells. Proc. Natl. Acad. Sci. U. S. A. **92**:9510–9514.
59. **Murray JS, Pfeiffer C, Madri J, Bottomly K.** 1992. Major histocompatibility complex (MHC) control of CD4 T cell subset activation. II. A single peptide induces either humoral or cell-mediated responses in mice of distinct MHC genotype. Eur. J. Immunol. **22**:559–565.
60. **Wen L, Chen NY, Tang J, Sherwin R, Wong FS.** 2001. The regulatory role of DR4 in a spontaneous diabetes DQ8 transgenic model. J. Clin. Invest. **107**:871–880.
61. **Congia M, Patel S, Cope AP, De Virgiliis S, Sonderstrup G.** 1998. T cell epitopes of insulin defined in HLA-DR4 transgenic mice are derived from preproinsulin and proinsulin. Proc. Natl. Acad. Sci. U. S. A. **95**:3833–3838.
62. **Hoffmann S, Cepok S, Grummel V, Lehmann-Horn K, Hackermuller J, Stadler PF, Hartung HP, Berthele A, Deisenhammer F, Wassmuth R, Hemmer B.** 2008. HLA-DRB1*0401 and HLA-DRB1*0408 are strongly associated with the development of antibodies against interferon-beta therapy in multiple sclerosis. Am. J. Hum. Genet. **83**:219–227.
63. **MacDonald KS, Fowke KR, Kimani J, Dunand VA, Nagelkerke NJ, Ball TB, Oyugi J, Njagi E, Gaur LK, Brunham RC, Wade J, Luscher MA, Krausa P, Rowland-Jones S, Ngugi E, Bwayo JJ, Plummer FA.** 2000. Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection. J. Infect. Dis. **181**:1581–1589.
64. **Fanning LJ, Levis J, Kenny-Walsh E, Wynne F, Whelton M, Shanahan F.** 2000. Viral clearance in hepatitis C (1b) infection: relationship with human leukocyte antigen class II in a homogeneous population. Hepatology **31**:1334–1337.
65. **McKiernan SM, Hagan R, Curry M, McDonald GS, Nolan N, Crowley J, Hegarty J, Lawlor E, Kelleher D.** 2000. The MHC is a major determinant of viral status, but not fibrotic stage, in individuals infected with hepatitis C. Gastroenterology **118**:1124–1130.
66. **Thio CL, Thomas DL, Goedert JJ, Vlahov D, Nelson KE, Hilgartner MW, O'Brien SJ, Karacki P, Marti D, Astemborski J, Carrington M.** 2001. Racial differences in HLA class II associations with hepatitis C virus outcomes. J. Infect. Dis. **184**:16–21.
67. **Desombere I, Willems A, Leroux-Roels G.** 1998. Response to hepatitis B vaccine: multiple HLA genes are involved. Tissue Antigens **51**:593–604.
68. **Clifford DB, Ances B, Costello C, Rosen-Schmidt S, Andersson M, Parks D, Perry A, Yerra R, Schmidt R, Alvarez E, Tyler KL.** 2011. Rituximab-associated progressive multifocal leukoencephalopathy in rheumatoid arthritis. Arch. Neurol. **68**:1156–1164.
69. **Molloy ES, Calabrese LH.** 2009. Progressive multifocal leukoencephalopathy: a national estimate of frequency in systemic lupus erythematosus and other rheumatic diseases. Arthritis Rheum. **60**:3761–3765.

Characterization of JC virus-specific $CD4^+$ T cell epitopes in healthy individuals

Infection with John Cunningham virus (JCV) is very common in humans and usually remains asymptomatic in healthy individuals. However, under circumstances of severe immunocompromise, the polyomavirus JC may cause progressive multifocal leukoencephalopathy (PML), a life-threatening demyelinating disease of the human brain. PML preferentially occurs in situations of compromised $CD4^+$ T cell function or number and little is known about the JCV epitopes recognized by $CD4^+$ T cells. In this study, we mapped the JCV-specific $CD4^+$ T cell response in healthy individuals using proliferation assays with peptides spanning the entire JCV proteome. Our aim was to obtain a better understanding of the mechanisms assuring immune control of JCV.

Introduction

History

PML was first described in 1958, in a report of three patients presenting with progressive weakness and gait disturbances among other symptoms. Upon autopsy, widespread lesions in the central nervous system (CNS) were detected, which could not be explained by the patients' underlying diagnoses of chronic lymphocytic leukemia or Hodgkin's disease (Astrom et al., 1958). Pathological examination of two of the three patients' brains revealed small focal lesions in the white matter and swollen basophilic nuclei of oligodendrocytes with intracellular granules. The causative agent of the disease was not defined, but viral infection was an early suspect (Cavanagh et al., 1959). Richardson discussed 22 cases of clinically and pathologically similar disease courses and suspected a viral etiology in 1961 (Richardson, 1961). This was confirmed by the demonstration of viral inclusion bodies in the formalin-fixed brain biopsy of a PML patient when electron microscopy became feasible a few years later (Figure 1) (ZuRhein & Chou, 1965). The virus gained it's name after the initials of the PML-patient John Cunningham, whose autopsy material was used for the first isolation and cultivation of the virus in 1971 (Padgett et al., 1971).

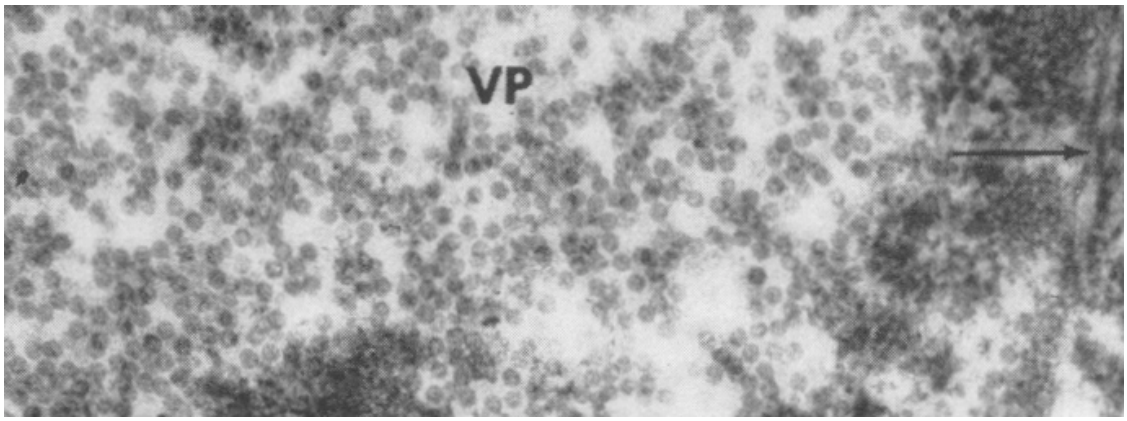


Figure 1: First illustration of virus-like particles (VP) in the nucleoplasm of a JCV infected glial cell by electron microscopy. Arrow points to nuclear membrane (ZuRhein & Chou, 1965).

The virus

JCV has an icosahedral shape with a capsid size of less than 40 nm in diameter. It is non-enveloped and contains 5.1kb doublestranded supercoiled DNA. Together with BK-, Merkel-cell-, murine-, WU polyomavirus, Simian virus 40, and a few others, it is classified as a polyomavirus. The virus has five open reading frames, subdivided into early and late genes. The early genes encode for the large T (LTAg) and small T (STAg) antigens, which are responsible for regulatory processes. The late genes encode for the structural agnoprotein, and the viral capsid proteins VP1, VP2, and VP3 (Tan & Koralnik, 2010). Among these, VP1 is considered the major capsid protein, and it is able to self assemble into virus-like particles (VLPs) in solution (Ou et al., 1999). The function of VP1 includes the mediation of cell attachment by interaction with an n-linked glycoprotein, which contains terminal α(2-6)-linked sialic acids (Liu et al., 1998). Furthermore, the genome contains a non-coding regulatory region that is important for the neurovirulence of the virus (Tan & Koralnik, 2010).

JCV infection

Infection with JCV occurs via tonsillar tissue through either inhalation or fecal-oral transmission (Bofill-Mas et al., 2003). Additionally, vertical transmission during pregnancy appears to be a possible mechanism of viral spreading (Monaco et al., 1996). JCV usually causes a latent infection, and can be detected in renal tissue,

lymphoid tissue and bone marrow of clinically asymptomatic individuals (Chesters et al., 1983; Tan et al., 2009; Monaco et al., 1996; Randhawa et al., 2005; Gallia et al., 1997).

PML is by far the most established disease caused by JCV, but the virus can also cause granule cell neuronopathy through lesions in the granule cell layer of the cerebellum (Koralnik et al., 2005) and potentially JC virus meningitis and JC virus encephalopathy (Blake et al., 1992; Tan & Koralnik, 2010).

Since PML occurs mostly in patients with human immunodeficiency virus (HIV) infections and along with other conditions of immunocompromise, the disease is considered an opportunistic infection. In addition to increased susceptibility of the patient, changes in the virus itself are required for the development of PML although the exact sequence of events is not fully understood. The regulatory region of the viral genome is hypervariable and determines the level of viral replication (Jensen & Major, 2001). Increased early gene expression was found in the CNS of PML patients (Gosert et al., 2010). Moreover, rearranged regulatory regions of JCV are associated with a poorer outcome of PML compared to wild-type regulatory regions (Pfister et al., 2001). These findings lead to the assumption that viral rearrangements contribute to viral pathogenicity.

Preconditions for PML

PML was mainly associated with lymphoproliferative disorders before the HIV/acquired immunodeficiency syndrome (AIDS) epidemic started (Richardson, 1974). Since then, it has appeared in about 4% of AIDS cases as a major opportunistic infection (Johnson, 1998), and more than 80% of PML patients were HIV positive in 1993 (Selik et al., 1997). Less frequently, the disease also appears along with hematological malignancies, chronic inflammatory disorders, $CD4^+$ lymphocytopenia and in organ transplant recipients (Tan & Koralnik, 2010; Gheuens et al., 2010)

With the introduction of anti-retroviral therapy, the incidence of PML in HIV-infected individuals dropped substantially, but in 2005 PML again gained major attention, when Natalizumab (Tysabri®), a monoclonal antibody used in the treatment of multiple sclerosis (MS) (Polman et al., 2006), was withdrawn from the market due to three events of PML during treatment. Since the U.S. Food and Drug

Administration (FDA) recommended the re-launch in 2006, Natalizumab has been used for the treatment of highly active relapsing-remitting MS (RRMS) after other treatments have failed to prevent relapses. As a result, 395 cases of Natalizumab-associated PML have been reported as of August 6, 2013 (Anon, 2013). In addition to compromised immune status and viral alterations, the risk for the development of PML depends on multiple other variables including the presence of anti-JCV antibodies, the duration of Natalizumab treatment, and prior use of immunosuppressants. Dependent on these factors, the incidence ranges from less than 0.1 to more than 10 PML cases per 1000 Natalizumab-treated MS patients (Bloomgren et al., 2012; Sorensen et al., 2012). The overall risk is estimated to be 2.2 cases per 1000 treated patients (Bloomgren et al., 2012). In addition, PML has also been observed during treatment with other monoclonal antibodies, e.g. the anti-CD20 antibody Rituximab (Freim Wahl et al., 2007; Molloy & Calabrese, 2012), or the anti-LFA-1 antibody Efalizumab (Molloy & Calabrese, 2009). The latter had been used for the treatment of severe plaque psoriasis, but was withdrawn from the market in 2009 because of PML (Major, 2010).

PML

PML is characterized by a lytic infection of oligodendroglial cells and non-productive infection of astrocytes (Koralnik, 2006). Clinical features include visual deficits, cognitive impairment, motor weakness or speech deficits, and seizures have also been reported (Clifford et al., 2010). The symptoms and signs correlate with the site of demyelination as well as the extent of the lesion, but the underlying disease leading to PML also influences the pattern of symptoms (Berger, 2011). Magnetic resonance imaging (MRI) in PML typically displays subcortical white matter lesions, appearing hyperintense in T2- and FLAIR images. Lesions can also be found in the grey matter, such as the basal ganglia or thalamus (Tan & Koralnik, 2010). In contrast to MS lesions or PML-immune reconstitution inflammatory syndrome (IRIS), there is usually no edema, mass effect or contrast enhancement due to the absence of inflammatory cells and opening of the blood-brain-barrier (Figure 2) (Whiteman et al., 1993).

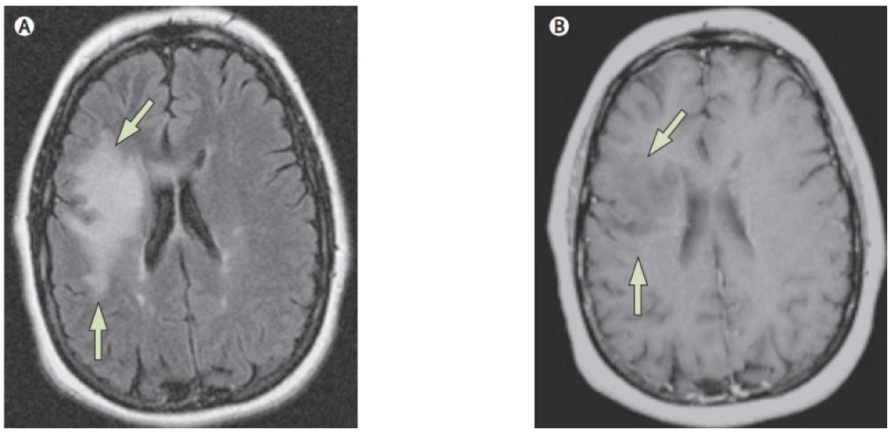


Figure 2: PML in an MS patient who had been treated with Natalizumab. Arrows indicate a frontal lobe white matter lesion, which appears hyperintense on FLAIR (A) and shows no contrast enhancement on the T1-weighted MRI (B) (Tan & Koralnik, 2010).

Despite these features, differentiating between an MS relapse and PML, or PML-IRIS, can be difficult (Boster et al., 2013). Diagnosing PML requires typical changes in MRI or a consistent clinical presentation. The diagnosis of definitive PML is made by detection of JCV in brain-derived material or evidence of JCV DNA in cerebrospinal fluid (CSF) (Cinque et al., 2009). All other PML-typical changes in disease course or imaging but without detection of JCV in either CSF or biopsy material are classified as possible PML (Cinque et al., 2009).

Prognosis and treatment of PML

The prognosis of the disease has changed substantially within the last few decades: in the early 1970s none of the 100 reported cases survived (Richardson, 1974), and before highly active antiretroviral therapy (HAART) was introduced in the 1990s, only 10% of HIV-PML patients were still alive after the first year of PML (Berger et al., 1998). Today, about 50% of HIV-PML patients survive the first year (Antinori et al., 2003), and with Natalizumab-associated PML, the mortality rate is around 22% (Bloomgren et al., 2012). However, it should be noted, that disability in survivors is often severe. Treatment mainly consists of restoration of the immune system, although this can promote the development of further complications, namely an IRIS and brain swelling. Formerly applied drugs like the antiviral Cidofovir or

malaria medication Mefloquine have shown to be ineffective in AIDS-related PML (De Luca et al., 2008; Friedman, 2011). Preventing viral entry into the host cells may be improved theoretically by the use of serotonin receptor agonists like Risperidone or Olanzapine (Altschuler & Kast, 2005); however, preliminary clinical testing has so far also been disappointing. There is currently no approved therapy for PML.

Role of B cells, $CD8^+$ T cells and $CD4^+$ T cells

B cell responses and antibodies appear incapable of preventing the onset of PML, because even though PML patients show normal to high antibody titers against JCV, they still develop the disease (Weber et al., 2001). Still, JCV antibody titers actually increase during the disease course in PML survivors (Khanna et al., 2009), and intrathecal antibody production is exclusively seen in PML patients (Weber et al., 1997).

$CD8^+$ T cells seem to play a more pronounced role in PML, since their presence correlates with recovery from PML: JCV-specific $CD8^+$ T cells are present in 91% of PML survivors, and in only 9% of PML progressors (Koralnik et al., 2002). Two human leukocyte antigen (HLA) A*0201-restricted immunodominant epitopes for $CD8^+$ T cells have been identified in VP1, namely the peptides $VP1_{(100\text{-}109)}$ (ILMWEAVTL) and $VP1_{(36\text{-}44)}$ (SITEVECFL) (Du Pasquier et al., 2003; Koralnik et al., 2002).

The importance of $CD4^+$ T cells becomes obvious when considering the characteristics of PML patients. 3-5% of HIV-1-infected individuals develop PML as their AIDS-defining illness (Major, 2010), with AIDS being mainly characterized by a reduction of $CD4^+$ T cells. At the same time the vast majority of PML patients suffer from AIDS as their underlying diagnosis (Tan & Koralnik, 2010). Besides other determinants, low $CD4^+$ T cell counts of less than 200 cells/µl at the time of PML diagnosis is associated with a worse outcome than $CD4^+$ T cell counts of more than 200 cells/µl (Marzocchetti et al., 2009). Also, $CD4^+$ lymphocytopenia (Stoner et al., 1986; Zonios et al., 2008) and, more precisely, reduced type 1 helper T cell (Th1) function (Weber et al., 2001) increase the risk of PML. During the disease, the number of JCV-specific $CD4^+$ T cells correlates with JCV clearance from the CSF of PML survivors (Gasnault et al., 2003), and PML survivors show a trend of earlier JCV-specific $CD4^+$ T cell responses than patients with disease progression (Gheuens

et al., 2011). Besides the work described in the attached publication, we have isolated and analyzed cells from a PML-IRIS lesion. One finding here was that $CD4^+$ T cells constitute also the majority of T cells in these lesions (Figure 3) (Aly et al., 2011).

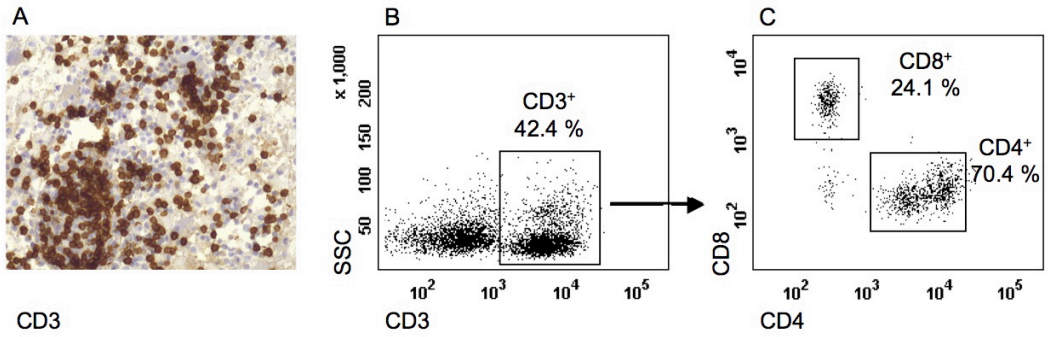


Figure 3: PML-IRIS lesion with massive infiltration of T cells. Immunohistochemistry reveals the pronounced perivascular and parenchymal infiltration of $CD3^+$ T cells (A). Flow cytometric analysis shows that T cells constitute 42.4% of the isolated cells (B). Out of these cells, 70.4% express CD4 (C) (Aly et al., 2011).

HLA

Unlike B cells, T cells in general only recognize antigen when it is presented to them on autologous major histocompatibility complex (MHC) molecules, termed HLA in humans. For $CD4^+$ T cells, MHC class II molecules, which are expressed by professional antigen presenting cells like dendritic cells or macrophages, present extracellular antigen. These proteins have been endocytosed and ingested before they appear nine to 25 amino acids (aa) long in the antigen binding groove, the α_1 and β_1 subunits of the MHC class II molecule. An important difference between antigen recognition by $CD4^+$ and $CD8^+$ T cells is the length of the presented peptide: MHC class I molecules have closed binding grooves, and usually present shorter peptides with only eight to ten aa, while MHC class II molecules accommodate longer peptides in open binding grooves. When activated, $CD4^+$ T cells communicate with B cells and induce their transformation into antibody-producing plasma cells, while $CD8^+$ T cell activation leads to a more pronounced release of perforin and granzymes inducing apoptosis in the presenting cell.

In humans, chromosome 6 contains the HLA class II genes encoding for, among others, DRB1, DRB3, DRB4, DRB5, DQB1, DQA1, DPA1 and DPB1 genes. Besides HLA-induced graft rejection after transplantation, the variations of HLA types among individuals are also associated with different susceptibilities to infections, autoimmune disease, or cancer. For instance, HLA DRB1*04:01^{+} HIV-1 infected individuals have shown slower disease progression (Kroner et al., 1995), whereas in autoimmunity the HLA DR15 haplotype accounts for the strongest genetic risk of developing MS (Hillert & Olerup, 1993; Sospedra & Martin, 2005), and is associated with an earlier disease onset (Masterman et al., 2000).

Markers for JCV exposure

JCV exposure can be determined by different diagnostic measures: JCV-specific IgG is found with high variability in the sera of 39% to 85% of Caucasian individuals (Egli et al., 2009; Weber et al., 1997; Kean et al., 2009; Knowles et al., 2003), while JCV DNA is not found in the peripheral blood of immunocompetent individuals (Koralnik et al., 1999). Although at much lower rates, urinary viral shedding can be another parameter verifying latent infection and persistence of JCV with a prevalence of about 20% (Egli et al., 2009). So far, there is no test to clearly identify JCV negative individuals (Tan & Koralnik, 2010), since even a negative antibody titer, being the most sensitive parameter for JCV exposure, does not assure JCV negativity. There have been MS patients without detectable antibody titers, who showed urinary viral shedding, and therefore evidence for exposure to the virus (Gorelik et al., 2010). A possible new method for the assessment of immune reactions to JCV is T cell proliferation after stimulation with virus-like particles (VLP) or viral peptides.

Aims of our study

In order to characterize immunodominant viral epitopes and improve the understanding of JCV immune control in healthy individuals, we examined the frequency and magnitude of CD4^{+} T cell proliferation upon stimulation with overlapping viral peptides spanning all open reading frames. These data might serve for the identification of risk factors for PML or provide information for the development of therapeutic approaches. Besides evaluation of proliferation as a new marker for

JCV positivity, we also performed a comparison to JCV-specific antibody levels and urinary viral shedding. Since HLA class II contributes significantly to peptide recognition of $CD4^+$ T cells, we also grouped all donors according to their HLA class II type, and included an analysis of HLA-dependent recognition of viral peptides.

Brief summary of methods

Peripheral blood mononuclear cells (PBMC) used for all proliferation assays were attained by Ficoll density gradient centrifugation and either used fresh or after storage in liquid nitrogen. We analyzed the proliferation of T cells from of 49 healthy donors (HD) after stimulation with JCV peptides. Phytohemagglutinin and tetanus toxoid (TTxd) served as positive controls, and T cell medium without any supplement was used as negative control. The JCV peptides were created to represent all potential JCV epitopes, therefore 204 13-16mer peptides with a five aa overlap were synthesized on the basis of the aa sequence of all JCV proteins. These peptides were arranged in 42 peptide pools, and each pool was seeded with eight replicates to detect peptide-specific T cells even if they were present only at very low frequencies. The proliferation was measured in a [^{3}H]-thymidine incorporation assay after one week of incubation. The stimulation index (SI) was calculated as counts per minute (cpm) of a well with antigen per mean cpm of all negative control wells. Wells with an SI>3 for fresh cells and SI>2 for frozen cells were considered positive. The phenotype of proliferating cells was determined by carboxyfluorescein succinimidyl ester (CFSE) assays and intracellular cytokine staining for interferon-γ production in a flow cytometric analysis.

We determined the presence of JCV VP1-specific IgG antibodies by enzyme-linked immunosorbend-assay (ELISA) with JCV VLP-coated plates exposed to serum or Ficoll supernatant of HD. Urinary shedding of JCV DNA was measured by quantitative real-time PCR (RT-qPCR) at the Department of Microbiology at the University of Barcelona. Additionally, the Department of Transfusion Medicine at the University Medical Center Hamburg-Eppendorf typed all donors for HLA class II molecules. For more detailed descriptions of the methods the reader is referred to the publication attached.

Summary of results with discussion

Proliferation upon stimulation with JCV showed high inter-individual variability among the donors with respect to the number of positive wells and the magnitude of positivity in individual wells. Also the peptides recognized by single donors varied substantially. Figure 2A in the attached publication depicts the proliferation after stimulation with JCV peptides, as well as negative and positive controls of three representative HD. By the use of 13-16mer peptides, our assay was designed to favor $CD4^+$ T cell proliferation. We chose radioactive [^{3}H]-thymidine incorporation as the most sensitive tool for the measurement of proliferation and were therefore not able to distinguish between $CD4^+$ and $CD8^+$ T cell proliferation. To prove $CD4^+$ rather than $CD8^+$ T cell proliferation, CFSE labeling and intracellular cytokine staining for the detection of interferon-γ production was performed. These assays confirmed that $CD4^+$ T cells respond preferentially to stimulation with JCV peptide pools (Figure 2B in the attached publication). Besides proliferation as a potential indicator of JCV exposure, seropositivity and urinary viral shedding were used to detect JCV infection. Similar to the publication by Gorelik et al. (2010), we identified only a weak correlation between JCV-specific IgG in serum and JCV DNA in urine in our samples.

To assess positivity with respect to JCV infection, we applied two arbitrarily defined thresholds for JCV positivity in each of the three assays. They were defined by the number of positive wells demonstrating a cellular immune response, the degree of extinction in an ELISA for JCV IgG indicating seropositivity and the amount of JCV DNA in urine proving urinary viral shedding. Depending on the threshold, positivity was between 28.6% and 77.6% in proliferation assays. IgG detection ranged from 42.6% to 89.4%, and urinary excretion varied between 36.4% and 45.5% depending on the defined threshold. Obviously, an individual showing urinary JCV excretion must be JCV exposed, whether or not she/he also tested positive in proliferation assays and serology. In contrast, it is very difficult to prove JCV negativity. Definite negativity could be postulated, when all three markers give negative results, but again, there is not even a universal threshold for a negative result. The samples used in our study were derived from different cohorts, since we were not able to obtain urine and serum from the anonymized donors of the PBMCs used in our proliferation assays. Therefore, further investigation with analysis of all

three parameters in the same individuals will be required to address the question of definite JCV negativity.

In order to account for different aspects of JCV-specific T cell responses, we considered three parameters into our calculation of immunodominant peptides: The first parameter was the magnitude of the proliferative response of each peptide pool. It is represented by the SI and describes the strength of the actual proliferation in each pool. In order to include only positive wells, this measure was calculated as the sum of all positive SI (∑(SI>3)) (Figure 3A in the attached publication, upper graph). The second parameter for immunodominance was the percentage of positive wells out of all seeded wells per peptide pool (Figure 3A in the attached publication, middle graph). Since the same amount of cells was seeded into each well, this measure served as an approximation for the precursor frequency within a certain range. The third parameter was the frequency of donors recognizing a distinct peptide pool, represented by the percentage of reactive individuals (Figure 3A in the attached publication, lower graph). Multiplying these three parameters displayed the reactivity score (RS), reflecting the immunogenic capacity of a peptide pool within the tested cohort (Figure 3B in the attached publication). Undoubtedly, these arbitrarily defined parameters can only serve as an approach towards immunodominance *in vivo*, where multiple other factors including the localization of the epitope on the surface or within the virus, the processing of the JCV proteins and the formation of neutralizing antibodies contribute to effective epitope recognition. However, we included three very important aspects into our analysis: the proliferative capacity of a $CD4^+$ T cell, precursor frequency in an individual, and the prevalence of epitope recognition in a cohort. Only peptides fulfilling all three criteria were considered immunodominant. It appears very likely that these epitopes also play an important role in viral control *in vivo*.

The highest RS were achieved by pool 14, 17, 32, and 33 (Figure 3B in the attached publication). They are therefore considered most immunogenic and most likely contain the immunodominant peptides. In order to determine which of the 5 peptides contained in each pool was responsible for the proliferation of the respective pool, the proliferation towards the single peptides from pools 14, 17, 32, and 33 was assessed. Among the 20 peptides contained in these four pools, peptides 41 ($VP1_{(123-137)}$), 81 ($VP2_{(30-43)}$), 82 ($VP2_{(39-51)}$), and 162 ($LTAg_{(415-429)}$) caused higher proliferative responses than the other peptides within these pools. We were not able

to detect a single peptide responsible for the proliferation in pool 32. Both recognition of peptide 81 and 82 as well as 154 and 155 may be similar because of their overlap by five aa and thus potential T cell recognition of the same aa sequence. The ideal epitope might have been located between peptide 81 and 82, or 154 and 155.

We were able to show that T cells from healthy individuals can recognize various JCV-derived epitopes. With seroprevalences up to 85% (Weber et al., 1997) it is not surprising that the human immune system is well adapted to JCV, and therefore broadly recognizes this ubiquitous virus. Another finding is the identification of promising epitopes with regard to immunodominance. This might suggest future research on JCV-specific PML immunotherapy, either by the construction of vaccines preventing PML, or the *ex vivo* activation and expansion of $CD4^+$ T cells against important JCV epitopes.

Since the recognition of each peptide pool by different donors also varies according to the HLA type of the individual, all individuals enrolled in this study were HLA-typed and HLA-restricted proliferation upon stimulation with peptide pools and single peptides was analyzed. 48 of the 49 HD were heterozygous for HLA DRB1*alleles, and therefore most of them appeared in two of the following groups (Figure 4 in the attached publication): DR1 (n=8), DR4 (n=15), DR7 (n=13), DR11 (n=9), DR13 (n=12), DR15 (n=13), DR17 (n=10), and other DRB1 haplotypes (n=15). In order to facilitate comparison, all groups were normalized to a virtual group of 10 HD (Figure 4B and 5B in the attached publication).

Notably, distinct HLA groups recognized different peptide pools: for instance the general proliferation in pool 14 was mainly due to $DR7^+$ and $DR17^+$ HD, while pool 33 was recognized mostly by $DR7^+$, $DR11^+$, and $DR13^+$ HD (Figure 4B in the attached publication). While proliferation after stimulation with TTxd revealed no significant differences among HLA groups, JCV-specific proliferation of $DR1^+$ and $DR4^+$ HD was lower than the one of $DR7^+$ or $DR17^+$ individuals (Figure 4C and 4D in the attached publication). Taking into account that binding motifs within the peptide binding grooves vary substantially according to the HLA DR haplotype of the HD, recognition of different peptides is a natural consequence. It would, however, be expected that at least some epitopes of a common pathogen like JCV are recognized in the context of each of the common HLA-class II haplotypes. Hence, the finding of

lower reactivity in individuals positive for HLA-DRB1*04:01 and to some extent also DRB1*01:01 is interesting.

Next, we examined HLA dependent specificity for individual peptides. HLA class II molecules trigger epitope recognition by $CD4^+$ T cells, and according to the recognition of peptide pools, we found differences among HLA class II groups in reactivity against single peptides (Figure 5B in the attached publication). It appears likely that the immunodominant peptide 41 was mainly recognized by $DR11^+$ and $DR13^+$ HD whithin the whole cohort, while the recognition of peptide 81 can be attributed to HD in the groups DR7, DR15, and DR17. Immunodominant peptides 82 and 162 were selected by the reactivity of distinct HLA groups as well, but additionally peptides 153 or 161, which did not appear in the whole cohort of HD, induced proliferation in certain HLA groups. Generally, $CD4^+$ T cells recognized many JCV epitopes, but the clarity of HLA-restricted peptide recognition might be compromised by the fact that most HD were DRB1* heterozygous and it was impossible to create groups of only one DRB1 allele without the second HLA DR allele contributing to peptide recognition. Clarifying which HLA-class II allele presents which of the immunodominant JCV peptides should be addressed by future studies employing e.g. JCV peptide-specific T cell clones.

In a more detailed analysis of the low RS conducted by $DR4^+$ HD, we subdivided the group of $DR4^+$ into ten HD with DRB1*04:01 and 5 HD with other $DR4^+$ alleles (referred to as DRB1*04:other). Interestingly, the low proliferation towards JCV peptide pools in the whole $DR4^+$ cohort was mainly due to extremely low proliferation of $DRB1*04:01^+$ HD, while the group DRB1*04:other showed robust RS (Figure 6A in the attached publication). Differences between the two groups were significant with regard to peptide pools, while the general proliferative capacity measured with TTxd was almost similar (Figure 6B and 6C in the attached publication). The exceptional role of DRB1*04:01 also became clear in the urinary viral shedding: none of the seven tested HD excreted viral DNA, while in both other groups, the DRB1*04:other, and the non-$DR4^+$ HD, four out of five and six out of ten, respectively, showed JCV DNA in urine (Figure 6D in the attached publication). Still, seropositivity was given in DRB1*04:01 HD (Figure 6E in the attached publication). In order to refute cross-reactivity between the polyomaviruses JCV and BKV, which could lead to a false positive result in $DRB1*04:01^+$ HD, JCV VP1 antibodies were also tested in competition with BKV VP1 protein. No significant cross-reactivity was

detected in any of the tested cohorts, confirming that IgG measured in Figure 6E in the attached publication was indeed specific for JCV. Since nine of the ten DRB1*04:01$^+$ donors were heterozygotes and each DRB1*04:01$^+$ donor showed a similar low RS, the negative effect of DRB1*04:01 alleles appears dominant. For instance, an individual with both alleles DRB1*04:01 and DRB1*15:01, would not strongly proliferate according to DRB1*15:01, but as low as other HD in the DRB1*04:01 cohort. The exact mechanism behind this observation still needs to be investigated. However, HLA positive dominant association with disease progression, control of infections, or responsiveness to drugs have been reported frequently, in particular with regard to DRB1*04:01 (Hoffmann et al., 2008; Newton et al., 2004; Wu et al., 2010).

The fact of negative urinary shedding, together with the marginal RS in proliferation of DRB1*04:01$^+$ HD might indicate that these individuals had not been exposed to the virus. However, we detected strong JCV-specific IgG in the same individuals who had also been tested in the assays mentioned before. Apparently, the immune response of DRB1*04:01$^+$ individuals against JCV included more antibody-producing plasma B cells than proliferating CD4$^+$ T cells. It can only be speculated whether cross-linking on the B cell receptor, binding to toll-like receptors, or even another mechanism activated B cells without involvement of CD4$^+$ T cells. On the other hand, involvement of CD4$^+$ T cells without consequent proliferation, but instead a shift towards humoral immune responses can be triggered by CD4$^+$ type 2 helper T cells (Th2) releasing interleukin (IL)-4 and IL-5. HLA dependency of such a shift towards Th1 over Th2 T cells, favoring CD8$^+$ and macrophage activation has been reported for an antigen of M. tuberculosis (Agrewala & Wilkinson, 1999). Wen et al. (2001) postulated that cells from HLA-DR4 transgenic mice developed Th2-like phenotypes upon stimulation with β-pancreatic antigens.

The major aim of our study was the mapping of immunodominant JCV epitopes. We were the first to identify four JCV peptides that cause strong CD4$^+$ T cell responses in healthy individuals. This result represents important information for the potential development of vaccines against JCV, and thus the prevention of PML. A second application of the aforementioned data is the development of a more efficient screening for JCV infection. Such a test may involve the detection of virus-specific CD4$^+$ T cells by HLA class II tetramers loaded with immunodominant peptides. Thirdly, knowledge about immunodominant epitopes might serve as the

basis for therapeutic strategies, e.g. an *ex vivo* expansion of JCV-specific T cells and subsequent autologous reinfusion. We also identified the cohort of DRB1*04:01^{+} HD, presenting an unusual pattern of immune responses against JCV, which might come along with an altered risk for PML. With our findings, we uncovered relevant novel questions about JCV immune control, and provide a comprehensive basis for future studies.

References

Agrewala, J.N. & Wilkinson, R.J. (1999) Influence of HLA-DR on the phenotype of CD4+ T lymphocytes specific for an epitope of the 16-kDa alpha-crystallin antigen of Mycobacterium tuberculosis. *European journal of immunology*, 29 (6), pp.1753–1761.

Altschuler, E.L. & Kast, R.E. (2005) The atypical antipsychotic agents ziprasidone [correction of zisprasidone], risperdone and olanzapine as treatment for and prophylaxis against progressive multifocal leukoencephalopathy. *Medical hypotheses*, 65 (3), pp.585–586.

Aly, L., Yousef, S., Schippling, S., Jelcic, I., Breiden, P., Matschke, J., Schulz, R., Bofill-Mas, S., Jones, L., Demina, V., Linnebank, M., Ogg, G., Girones, R., Weber, T., Sospedra, M. & Martin, R. (2011) Central role of JC virus-specific CD4+ lymphocytes in progressive multi-focal leucoencephalopathy-immune reconstitution inflammatory syndrome. *Brain : a journal of neurology*, 134 (9), pp.2687–2702.

Anon (2013) Tysabri Safety Update [Online] URL: http://www.biogenidec.ch/medizinische_fachkreise.aspx?ID=10389 [Accessed on September 09, 2013, 09:30 AM].

Antinori, A., Cingolani, A., Lorenzini, P., Giancola, M.L., Uccella, I., Bossolasco, S., Grisetti, S., Moretti, F., Vigo, B., Bongiovanni, M., Del Grosso, B., Arcidiacono, M.I., Fibbia, G.C., Mena, M., Finazzi, M.G., Guaraldi, G., Ammassari, A., Monforte, A.D., Cinque, P. & De Luca, A. (2003) Clinical Epidemiology and Survival of Progressive Multifocal Leukoencephalopathy in the Era of Highly Active Antiretroviral Therapy: Data from the Italian Registry Investigative Neuro AIDS (IRINA). *Journal of neurovirology*, 9 (s1), pp.47–53.

Astrom, K.E., Mancall, E.L. & Richardson, E.P. (1958) Progressive multifocal leuko-encephalopathy; a hitherto unrecognized complication of chronic lymphatic leukaemia and Hodgkin's disease. *Brain : a journal of neurology*, 81 (1), pp.93–111.

Berger, J.R. (2011) The clinical features of PML. *Cleveland Clinic Journal of Medicine*, 78 (Suppl_2), pp.S8–S12.

Berger, J.R., Pall, L., Lanska, D. & Whiteman, M. (1998) Progressive multifocal leukoencephalopathy in patients with HIV infection. *Journal of neurovirology*, 4 (1), pp.59–68.

Blake, K., Pillay, D., Knowles, W., Brown, D.W., Griffiths, P.D. & Taylor, B. (1992) JC virus associated meningoencephalitis in an immunocompetent girl. *Archives of disease in childhood*, 67 (7), pp.956–957.

Bloomgren, G., Richman, S., Hotermans, C., Subramanyam, M., Goelz, S., Natarajan, A., Lee, S., Plavina, T., Scanlon, J.V., Sandrock, A. & Bozic, C. (2012) Risk of natalizumab-associated progressive multifocal leukoencephalopathy. *The New England journal of medicine*, 366 (20), pp.1870–1880.

Bofill-Mas, S., Clemente-Casares, P., Major, E.O., Curfman, B. & Girones, R. (2003) Analysis of the Excreted JC Virus Strains and Their Potential Oral Transmission. *Journal of neurovirology*, 9 (4), pp.498–507.

Boster, A.L., Nicholas, J.A., Topalli, I., Kisanuki, Y.Y., Pei, W., Morgan-Followell, B., Kirsch, C.F., Racke, M.K. & Pitt, D. (2013) Lessons Learned From Fatal Progressive Multifocal Leukoencephalopathy in a Patient With Multiple Sclerosis Treated With Natalizumab. *JAMA neurology*, pp.1–5.

Cavanagh, J.B., Greenbaum, D., Marshall, A.H. & Rubinstein, L.J. (1959) Cerebral demyelination associated with disorders of the reticuloendothelial system. *Lancet*, 2 (7102), pp.524–529.

Chesters, P.M., Heritage, J. & McCance, D.J. (1983) Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *The Journal of infectious diseases*, 147 (4), pp.676–684.

Cinque, P., Koralnik, I.J., Gerevini, S., Miro, J.M. & Price, R.W. (2009) Progressive multifocal leukoencephalopathy in HIV-1 infection. *The Lancet Infectious Diseases*, 9 (10), pp.625–636.

Clifford, D.B., De Luca, A., DeLuca, A., Simpson, D.M., Arendt, G., Giovannoni, G. & Nath, A. (2010) Natalizumab-associated progressive multifocal leukoencephalopathy in patients with multiple sclerosis: lessons from 28 cases. *Lancet neurology*, 9 (4), pp.438–446.

De Luca, A., Ammassari, A., Pezzotti, P., Cinque, P., Gasnault, J., Berenguer, J., Di Giambenedetto, S., Cingolani, A., Taoufik, Y., Miralles, P., Marra, C.M. & Antinori, A. (2008) Cidofovir in addition to antiretroviral treatment is not effective for AIDS-associated progressive multifocal leukoencephalopathy: a multicohort analysis. *AIDS (London, England)*, 22 (14), pp.1759–1767.

Du Pasquier, R., Kuroda, M., Schmitz, J., Zheng, Y., Martin, K., Peyerl, F., Lifton, M., Gorgone, D., Autissier, P. & Letvin, N. (2003) Low frequency of cytotoxic T lymphocytes against the novel HLA-A* 0201-restricted JC virus epitope VP1p36 in patients with proven or possible progressive multifocal leukoencephalopathy. *Journal of virology*, 77 (22), p.11918.

Egli, A., Infanti, L., Dumoulin, A., Buser, A., Samaridis, J., Stebler, C., Gosert, R. & Hirsch, H.H. (2009) Prevalence of Polyomavirus BK and JC Infection and Replication in 400 Healthy Blood Donors. *The Journal of infectious diseases*, 199 (6), pp.837–846.

Freim Wahl, S.G., Folvik, M.R. & Torp, S.H. (2007) Progressive multifocal leukoencephalopathy in a lymphoma patient with complete remission after treatment with cytostatics and rituximab: case report and review of the literature. *Clinical neuropathology*, 26 (2), pp.68–73.

Friedman, R. (2011) *Malaria Drug Fails to Fulfill Promise in PML*. Neurology Today.

Gallia, G.L., Houff, S.A., Major, E.O. & Khalili, K. (1997) Review: JC virus infection of lymphocytes--revisited. *The Journal of infectious diseases*, 176 (6), pp.1603–1609.

Gasnault, J., Kahraman, M., de Goër de Herve, M.G., Durali, D., Delfraissy, J.-F. & Taoufik, Y. (2003) Critical role of JC virus-specific CD4 T-cell responses in preventing progressive multifocal leukoencephalopathy. *AIDS (London, England)*, 17 (10), pp.1443–1449.

Gheuens, S., Pierone, G., Peeters, P. & Koralnik, I.J. (2010) Progressive multifocal leukoencephalopathy in individuals with minimal or occult immunosuppression. *Journal of neurology, neurosurgery, and psychiatry*, 81 (3), pp.247–254.

Gheuens, S., Bord, E., Kesari, S., Simpson, D.M., Gandhi, R.T., Clifford, D.B., Berger, J.R., Ngo, L. & Koralnik, I.J. (2011) Role of CD4+ and CD8+ T-Cell Responses against JC Virus in the Outcome of Patients with Progressive Multifocal Leukoencephalopathy (PML) and PML with Immune Reconstitution Inflammatory Syndrome. *Journal of virology*, 85 (14), pp.7256–7263.

Gorelik, L., Lerner, M., Bixler, S., Crossman, M., Schlain, B., Simon, K., Pace, A., Cheung, A., Chen, L.L., Berman, M., Zein, F., Wilson, E., Yednock, T., Sandrock, A., Goelz, S.E. & Subramanyam, M. (2010) Anti-JC virus antibodies: implications for PML risk stratification. *Annals of neurology*, 68 (3), pp.295–303.

Gosert, R., Kardas, P., Major, E.O. & Hirsch, H.H. (2010) Rearranged JC virus noncoding control regions found in progressive multifocal leukoencephalopathy patient samples increase virus early gene expression and replication rate. *Journal of virology*, 84 (20), pp.10448–10456.

Hillert, J. & Olerup, O. (1993) HLA and MS. *Neurology*, 43 (11), pp.2426–2427.

Hoffmann, S., Cepok, S., Grummel, V., Lehmann-Horn, K., Hackermüller, J., Hackermueller, J., Stadler, P.F., Hartung, H.-P., Berthele, A., Deisenhammer, F., Wassmuth, R., Wasmuth, R. & Hemmer, B. (2008) HLA-DRB1*0401 and HLA-DRB1*0408 are strongly associated with the development of antibodies against interferon-beta therapy in multiple sclerosis. *American journal of human genetics*, 83 (2), pp.219–227.

Jensen, P.N. & Major, E.O. (2001) A classification scheme for human polyomavirus JCV variants based on the nucleotide sequence of the noncoding regulatory region. *Journal of neurovirology*, 7 (4), pp.280–287.

Johnson, R.T. (1998) *Viral infections of the nervous system*. Lippincott-Raven.

Kean, J.M., Rao, S., Wang, M. & Garcea, R.L. (2009) Seroepidemiology of Human Polyomaviruses W. J. Atwood ed. *PLoS Pathogens*, 5 (3), p.e1000363.

Khanna, N., Wolbers, M., Mueller, N.J., Garzoni, C., Pasquier, Du, R.A., Fux, C.A., Vernazza, P., Bernasconi, E., Viscidi, R., Battegay, M., Hirsch, H.H. & Swiss HIV Cohort Study (2009) JC Virus-Specific Immune Responses in Human Immunodeficiency Virus Type 1 Patients with Progressive Multifocal Leukoencephalopathy. *Journal of virology*, 83 (9), pp.4404–4411.

Knowles, W.A., Pipkin, P., Andrews, N., Vyse, A., Minor, P., Brown, D.W.G. & Miller, E. (2003) Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. *Journal of medical virology*, 71 (1), pp.115–123.

Koralnik, I.J., Boden, D., Mai, V.X., Lord, C.I. & Letvin, N.L. (1999) JC virus DNA load in patients with and without progressive multifocal leukoencephalopathy. *Neurology*, 52 (2), pp.253–260.

Koralnik, I.J., Pasquier, Du, R.A., Kuroda, M.J., Schmitz, J.E., Dang, X., Zheng, Y., Lifton, M. & Letvin, N.L. (2002) Association of prolonged survival in HLA-A2+ progressive multifocal leukoencephalopathy patients with a CTL response specific for a commonly recognized JC virus epitope. *Journal of immunology (Baltimore, Md : 1950)*, 168 (1), pp.499–504.

Koralnik, I.J., Wüthrich, C., Dang, X., Rottnek, M., Gurtman, A., Simpson, D. & Morgello, S. (2005) JC virus granule cell neuronopathy: A novel clinical syndrome distinct from progressive multifocal leukoencephalopathy. *Annals of neurology*, 57 (4), pp.576–580.

Koralnik, I.J. (2006) Progressive multifocal leukoencephalopathy revisited: Has the disease outgrown its name? *Annals of neurology*, 60 (2), pp.162–173. Available from: <http://doi.wiley.com/10.1002/ana.20933>.

Kroner, B.L., Goedert, J.J., Blattner, W.A., Wilson, S.E., Carrington, M.N. & Mann, D.L. (1995) Concordance of human leukocyte antigen haplotype-sharing, CD4 decline and AIDS in hemophilic siblings. Multicenter Hemophilia Cohort and Hemophilia Growth and Development Studies. *AIDS (London, England)*, 9 (3), pp.275–280.

Liu, C.K., Wei, G. & Atwood, W.J. (1998) Infection of glial cells by the human polyomavirus JC is mediated by an N-linked glycoprotein containing terminal alpha(2-6)-linked sialic acids. *Journal of virology*, 72 (6), pp.4643–4649.

Major, E.O. (2010) Progressive Multifocal Leukoencephalopathy in Patients on Immunomodulatory Therapies*. *Annual review of medicine*, 61 (1), pp.35–47.

Marzocchetti, A., Tompkins, T., Clifford, D.B., Gandhi, R.T., Kesari, S., Berger, J.R., Simpson, D.M., Prosperi, M., De Luca, A. & Koralnik, I.J. (2009) Determinants of survival in progressive multifocal leukoencephalopathy. *Neurology*, 73 (19), pp.1551–1558.

Masterman, T., Ligers, A., Olsson, T., Andersson, M., Olerup, O. & Hillert, J. (2000) HLA-DR15 is associated with lower age at onset in multiple sclerosis. *Annals of neurology*, 48 (2), pp.211–219.

Molloy, E.S. & Calabrese, L.H. (2009) Therapy: Targeted but not trouble-free: efalizumab and PML. *Nature reviews. Rheumatology*, 5 (8), pp.418–419.

Molloy, E.S. & Calabrese, L.H. (2012) Progressive multifocal leukoencephalopathy associated with immunosuppressive therapy in rheumatic diseases: evolving role of biologic therapies. *Arthritis and rheumatism*, 64 (9), pp.3043–3051.

Monaco, M.C., Atwood, W.J., Gravell, M., Tornatore, C.S. & Major, E.O. (1996) JC virus infection of hematopoietic progenitor cells, primary B lymphocytes, and tonsillar stromal cells: implications for viral latency. *Journal of virology*, 70 (10), pp.7004–7012.

Newton, J.L., Harney, S.M.J., Wordsworth, B.P. & Brown, M.A. (2004) A review of the MHC genetics of rheumatoid arthritis. *Genes and immunity*, 5 (3), pp.151–157.

Ou, W.C., Wang, M., Fung, C.Y., Tsai, R.T., Chao, P.C., Hseu, T.H. & Chang, D. (1999) The major capsid protein, VP1, of human JC virus expressed in Escherichia coli is able to self-assemble into a capsid-like particle and deliver exogenous DNA into human kidney cells. *The Journal of general virology*, 80 (Pt 1), pp.39–46.

Padgett, B.L., Walker, D.L., ZuRhein, G.M., Eckroade, R.J. & Dessel, B.H. (1971) Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. *Lancet*, 1 (7712), pp.1257–1260.

Pfister, L.A., Letvin, N.L. & Koralnik, I.J. (2001) JC virus regulatory region tandem repeats in plasma and central nervous system isolates correlate with poor clinical outcome in patients with progressive multifocal leukoencephalopathy. *Journal of virology*, 75 (12), pp.5672–5676.

Polman, C.H., O'Connor, P.W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D.H., Phillips, J.T., Lublin, F.D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M.A., Sandrock, A.W.AFFIRM Investigators (2006) A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *The New England journal of medicine*, 354 (9), pp.899–910.

Randhawa, P., Uhrmacher, J., Pasculle, W., Vats, A., Shapiro, R., Eghtsead, B. & Weck, K. (2005) A comparative study of BK and JC virus infections in organ transplant recipients. *Journal of medical virology*, 77 (2), pp.238–243.

Richardson, E.P. (1961) Progressive multifocal leukoencephalopathy. *The New England journal of medicine*, 265, pp.815–823.

Richardson, E.P. (1974) Our evolving understanding of progressive multifocal leukoencephalopathy. *Annals of the New York Academy of Sciences*, 230, pp.358–364.

Selik, R.M., Karon, J.M. & Ward, J.W. (1997) Effect of the human immunodeficiency virus epidemic on mortality from opportunistic infections in the United States in 1993. *The Journal of infectious diseases*, 176 (3), pp.632–636.

Sorensen, P.S., Bertolotto, A., Edan, G., Giovannoni, G., Gold, R., Havrdova, E., Kappos, L., Kieseier, B.C., Montalban, X. & Olsson, T. (2012) Risk stratification for progressive multifocal leukoencephalopathy in patients treated with natalizumab. *Multiple Sclerosis*, 18 (2), pp.143–152.

Sospedra, M. & Martin, R. (2005) Immunology of multiple sclerosis. *Annual review of immunology*, 23, pp.683–747.

Stoner, G.L., Ryschkewitsch, C.F., Walker, D.L. & Webster, H.D. (1986) JC papovavirus large tumor (T)-antigen expression in brain tissue of acquired immune deficiency syndrome (AIDS) and non-AIDS patients with progressive multifocal leukoencephalopathy. *Proceedings of the National Academy of Sciences of the United States of America*, 83 (7), pp.2271–2275.

Tan, C.S., Dezube, B.J., Bhargava, P., Autissier, P., Wüthrich, C., Miller, J. & Koralnik, I.J. (2009) Detection of JC virus DNA and proteins in the bone marrow of HIV-positive and HIV-negative patients: implications for viral latency and neurotropic transformation. *The Journal of infectious diseases*, 199 (6), pp.881–888.

Tan, C.S. & Koralnik, I.J. (2010) Progressive multifocal leukoencephalopathy and other disorders caused by JC virus: clinical features and pathogenesis. *Lancet neurology*, 9 (4), pp.425–437.

Weber, F., Goldmann, C., Krämer, M., Kaup, F.J., Pickhardt, M., Young, P., Petry, H., Weber, T. & Lüke, W. (2001) Cellular and humoral immune response in progressive multifocal leukoencephalopathy. *Annals of neurology*, 49 (5), pp.636–642.

Weber, T., Trebst, C., Frye, S., Cinque, P., Vago, L., Sindic, C.J., Schulz-Schaeffer, W.J., Kretzschmar, H.A., Enzensberger, W., Hunsmann, G. & Lüke, W. (1997) Analysis of the systemic and intrathecal humoral immune response in progressive multifocal leukoencephalopathy. *The Journal of infectious diseases*, 176 (1), pp.250–254.

Wen, L., Chen, N.Y., Tang, J., Sherwin, R. & Wong, F.S. (2001) The regulatory role of DR4 in a spontaneous diabetes DQ8 transgenic model. *The Journal of clinical investigation*, 107 (7), pp.871–880.

Whiteman, M.L., Post, M.J., Berger, J.R., Tate, L.G., Bell, M.D. & Limonte, L.P. (1993) Progressive multifocal leukoencephalopathy in 47 HIV-seropositive patients: neuroimaging with clinical and pathologic correlation. *Radiology*, 187 (1), pp.233–240.

Wu, J.S., Qiu, W., Castley, A., James, I., Mastaglia, F.L., Christiansen, F.T., Carroll, W.M., Joseph, J. & Kermode, A.G. (2010) Modifying effects of HLA-DRB1 allele interactions on age at onset of multiple sclerosis in Western Australia. *Multiple sclerosis (Houndmills, Basingstoke, England)*, 16 (1), pp.15–20.

Zonios, D.I., Falloon, J., Bennett, J.E., Shaw, P.A., Chaitt, D., Baseler, M.W., Adelsberger, J.W., Metcalf, J.A., Polis, M.A., Kovacs, S.J., Kovacs, J.A., Davey, R.T., Lane, H.C., Masur, H. & Sereti, I. (2008) Idiopathic CD4+ lymphocytopenia: natural history and prognostic factors. *Blood*, 112 (2), pp.287–294.

ZuRhein, G. & Chou, S.M. (1965) Particles resembling papova viruses in human cerebral demyelinating disease. *Science (New York, NY)*, 148 (3676), pp.1477–1479.

Danksagung

An erster Stelle danke ich Herrn Professor Roland Martin für die Bereitstellung des spannenden Themas, die Möglichkeit zur Durchführung der Arbeit in seinem Labor und die engagierte und strukturierte Betreuung. Insbesondere danke ich ihm für die große Unterstützung und Förderung seit Beginn meiner Zeit in seinem Labor bis heute. Klares wissenschaftliches Denken, Planen und Hinterfragen, erfolgreiches Arbeiten und überzeugendes Schreiben habe ich durch ihn kennen gelernt.

Ebenfalls möchte ich Frau Dr. rer. nat. Mireia Sospedra-Ramos meinen Dank aussprechen. Auch sie hat meine Arbeit maßgeblich mitbetreut. Ich konnte in fachlichen Diskussionen mit ihr viel lernen und freue mich, dass ich mich mit all meinen Fragen immer an Sie wenden konnte. Ich danke ihr sehr für die Unterstützung und die wertvolle Mitbetreuung meiner Arbeit.

Mein Dank richtet sich auch an Petra Breiden, mit der die Laborarbeit immer viel Freude gemacht hat und lange Arbeitstage oft zum Vergnügen wurden. Von ihr habe ich unzählige praktische Laborfertigkeiten gelernt.

Nicht zuletzt danke ich Dr. med. Ilijas Jelcic. Er hat das Projekt mit aufgebaut und mich über all die Jahre mit Fachkompetenz sowie großem Interesse begleitet. Ich habe die Arbeit mit ihm immer sehr geschätzt.

Frau Professor Gisa Tiegs danke ich für all die Unterstützung, die ich im Rahmen des Graduiertenkollegs erfahren habe. Sie hat uns Stipendiaten auf dem Weg in die Forschung begleitet und gefördert und sehr zu meiner Begeisterung für die Wissenschaft beigetragen.