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Cloning of the first human anti-JCPyV/VP1 neutralizing monoclonal antibody: Epitope definition and implications in risk stratification of patients under natalizumab therapy

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ABSTRACT

JC virus (JCPyV) has gained novel clinical importance as cause of progressive multifocal leukoencephalopathy (PML), a rare demyelinating disease recently associated to immunomodulatory drugs, such as natalizumab used in multiple sclerosis (MS) cases. Little is known about the mechanisms leading to PML, and this makes the need of PML risk stratification among natalizumab-treated patients very compelling. Clinical and laboratory-based risk-stratification markers have been proposed, one of these is represented by the JCPyV-seropositive status, which includes about 54% of MS patients. We recently proposed to investigate the possible protective role of neutralizing humoral immune response in preventing JCPyV reactivation. In this proof-of-concept study, by cloning the first human monoclonal antibody (GRE1) directed against a neutralizing epitope on JCPyV/VP1, we optimized a robust anti-JCPyV neutralization assay. This allowed us to evaluate the neutralizing activity in JCPyV-positive sera from MS patients, demonstrating the lack of correlation between the level of anti-JCPyV antibody and anti-JCPyV neutralizing activity. Relevant consequences may derive from future clinical studies induced by these findings; indeed the study of the serum anti-JCPyV neutralizing activity could allow not only a better risk stratification of the patients during natalizumab treatment, but also a better understanding of the pathophysiological mechanisms leading to PML, highlighting the contribution of peripheral versus central nervous system JCPyV reactivation. Noteworthy, the availability of GRE1 could allow the design of novel immunoprophylactic strategies during the immunomodulatory treatment.

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1. Introduction

JC virus (JCPyV) is a small, nonenveloped, double-stranded DNA virus belonging to Polyomaviridae family, *Orthopolyomavirus* genus, that infects only humans. Primary infection by JCPyV typically occurs in childhood or adolescence, with approximately 60% of adults being JCPyV-seropositive (Egli et al., 2009; Knowles et al., 2003; Major, 2010; Tan et al., 2010), and it is clinically silent with the kidney and lymphoid organs being considered as the possible sites of lifelong persistence (Eash et al., 2006). Occasionally, JCPyV infection may complicate in at-risk patients with defects in immune function, causing progressive multifocal leukoencephalopathy (PML), a rare fatal demyelinating disease of the central nervous system (CNS) due to lytic involvement of myelin-producing oligodendrocytes. Although the pathogenesis of PML has been widely studied, the mechanisms leading to CNS involvement with consequent destruction of oligodendrocytes is not completely understood. Many hypotheses have been suggested, including both JCPyV reactivation within CNS in oligodendrocytes latently infected since the first contact with the virus, and peripheral

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reactivation with subsequent CNS involvement (Bellizzi et al., 2012; Steiner and Berger, 2012; Tavazzi et al., 2012). Under a clinical point of view, PML was originally just an anecdotic report associated to B cell lymphoproliferative disorders (Astrom et al., 1958; Brooks and Walker, 1984), but it gained more importance during the HIV pandemic, with up to 5% of AIDS patients showing the disease (Cinque et al., 2009). More recently, an increase of PML incidence has been associated with novel immunomodulatory therapies including the use of "biological" drugs, such as monoclonal antibodies (mAbs) influencing the biological functions of receptors on the surface of immune cells (Aksamit, 2012; Major, 2010). The highest PML incidence (3.85/1000 patients) has been observed with natalizumab, a humanized mAb used in the treatment of relapsing-remitting multiple sclerosis (MS) cases (Ferenczy et al., 2012; Vermersch et al., 2011). In particular, natalizumab prevents the migration of lymphocytes into the CNS by blocking the interaction between the integrin α4 chain expressed on the surface of lymphocytes and the vascular cell adhesion molecule-1 expressed by endothelial cells (Yaldizli and Putzki, 2009; Yednock et al., 1992). Natalizumab has proven to be highly effective, with a marked reduction in the rate of clinical relapses and a slower MS progression, but, due to its correlation with PML, it was approved with a restricted distribution format in 2006 (Yaldizli and Putzki, 2009). For all these reasons, the PML risk stratification among natalizumab-treated patients is extremely important, therefore widely studied. The anti-JCPyV cellular immune response has been investigated evidencing the importance of both $CD4^+$ and $CD8^+$ T cells in JCPyV control (Koralnik, 2002; Perkins et al., 2012), as well as of a pro-inflammatory cytokine pattern (Weber et al., 2001). However, these observations are not easily transferred into a clinically useful routine assay. As a consequence, the only immune parameter actually reported is the JCPyV seropositivity status, evaluated by detecting antibodies directed against JCPyV/VP1 (the main surface JCPyV protein) using a single validated assay (STRATIFY JC virus™, Biogen Idec, Boston, MA (Bozic et al., 2011; Gorelik et al., 2010)). As an example, Bloomgren et al. have recently proposed a quantification of the risk directly related to three factors: (i) positive status to anti-JCPyV antibodies (e.g., positive STRATIFY JC virus™ assay), (ii) prior use of other immunosuppressive therapy, and (iii) duration of natalizumab treatment (Bloomgren et al., 2012). This risk-factor algorithm may help to identify patients for whom natalizumab therapy is most appropriate and may reduce the incidence of PML, but it has some limitations. First of all, about 54% of MS patients are JCPyV-seropositive (a figure very close to the 60% observed in the general population) limiting the usefulness of this marker in the stratification of at-risk patients (Bozic et al., 2011). Moreover, it has been described that JCPyV-viruria can occur in apparently JCPyV-seronegative patients, maybe due to the fact that some MS patients received earlier immunomodulatory therapy (the false-negative rate reported was 2.7% (95% confidence interval, 0.9–6.2) and for this reason it has been suggested that at-risk negative patients be retested every 6 months) (Bozic et al., 2011).

In our opinion, other not yet investigated factors should also be taken into account when considering the humoral response, and its possible use in risk stratification. As described for other persistent viral infections (for example HCV and HIV) in which the role of the humoral response was considered minor for long time, the humoral response should not be considered as a whole. Each response is made up of single antibody subpopulations endowed with different, often contrasting, biological activities which may influence the evolution of a given infection (Chuang et al., 2013; Hiatt et al., 2013; Mancini et al., 2012b; Sautto et al., 2012). We have recently proposed to investigate this aspect of the anti-JCPyV humoral response both for a better comprehension of the role of JCPyV reactivation in the genesis of PML and, possibly, for a better PML risk-stratification of natalizumab-treated patients (Mancini et al., 2012a).

In this study, we report the cloning of the first anti-JCPyV/VP1 neutralizing human monoclonal antibody (named GRE1). The availability of GRE1 allowed us to set up a robust neutralization assay, which we used to evaluate the neutralizing activity of STRATIFY JC virus™-positive sera from MS patients. Although to be confirmed on larger cohorts, with this proof-of-concept study we demonstrate that there is no correlation between the presence of anti-JCPyV antibody and anti-JCPyV neutralizing activity.

2. Materials and methods

2.1. Cloning of the neutralizing anti-JCPyV/VP1 human monoclonal antibody

To isolate human monoclonal antibodies directed against the JCPyV/VP1 protein, a human combinatorial phage display antibody library (IgG1/k isotype) was constructed as previously described (Clementi et al., 2012; De Marco et al., 2012; Solforosi et al., 2012). The library was generated starting from 8×10^6 bone marrow lymphoid cells of a JCPyV/VP1-seropositive donor. For the selection procedures (*panning*), we used a commercially available monomeric recombinant VP1 (Abcam, UK) and BSA was used as negative control antigen. The panning was performed as previously described (Burioni et al., 2010; Clementi et al., 2012). A total of five rounds of panning were performed, and phages from the final three rounds was converted into a soluble Fab-expressing phagemid system (Burioni et al., 1998, 2009b, 2010; Mancini et al., 2006; Solforosi et al., 2012). Briefly, Fab preparations were obtained with freeze–thawing procedure from an induced overnight culture of transformed *Escherichia coli* XL1-blue strain (Agilent Technologies, USA). Five milliliter of super broth (SB) containing ampicillin (50 μg/mL; Sigma Aldrich, Germany) and tetracycline (10 μg/mL; Sigma Aldrich, Germany) was inoculated with transformed bacteria, and grown for 7 h at 37 °C in a rotary shaker. Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mmol/L; Sigma Aldrich, Germany) was added to the growing bacteria, that were further incubated overnight at 30 °C. Cells were then harvested by centrifugation, resuspended in 1 mL of PBS/1% BSA, and subjected to freeze–thawing procedure (3 rounds). Cell debris were pelleted by centrifugation at 13,000 g at room temperature, and the supernatant was used without further processing in ELISA. The ELISA was performed using an adaptation of the already described protocol in literature (Lin et al., 2013). All clones showing an $OD_{450} > 0.8$ in ELISA against the monomeric VP1 were considered positive and further characterized. BSA and a commercially available recombinant BKPyV/VP1 (Abcam, UK) were used as control antigens.

The heavy and the light chains of the selected clones were sequenced and analyzed by IMGT (http://www.imgt.org/) and BLAST tools (http://www.ncbi.nlm.nih.gov/igblast/).

2.2. Production and purification of the anti-JCPyV/VP1 mAb as Fab fragment

The production and purification of the selected anti-JCPyV/VP1 Fab fragment, named GRE1, was performed as previously described (Burioni et al., 2002, 2009a). Briefly, Fab molecules were purified inoculating the selected bacterial clone in 1 L of SB containing ampicillin (50 μg/mL) and tetracycline (10 μg/mL), and growing it at 37 °C for 7 h in a rotary shaker. After induction with 1 mmol/L IPTG, the culture was then incubated overnight at 30 °C. Cells were harvested by centrifugation, resuspended in 25 mL of PBS and sonicated. Cell debris were eliminated by centrifugation (13,000*g* for 30 min), and the filtered supernatant was

loaded onto an immunoaffinity anti-human Fab column. The final yield and the purity of the Fab was analyzed by SDS–PAGE and Western blot by using standard protocols (Mahmood and Yang, 2012).

The purified Fab was then tested both in ELISA against the recombinant VP1 (Lin et al., 2013) and in an immunofluorescence (IF) assay on JCPyV Mad-4 strain-infected COS7 cells. A fluorescein isothiocyanate (FITC)-conjugated anti-human Fab preparation (Sigma Aldrich, Germany) was used as secondary antibody. Uninfected cells were used as negative control.

2.3. Production and purification of the anti-JCPyV/VP1 mAb as whole IgG1

The anti-JCPyV/VP1 IgG1 production was performed by GenScript (USA), through the transient expression of the antibody in suspension CHO cells using serum-free medium, followed by one-step purification. Briefly, the variable regions of the heavy and the light chain were cloned into a whole IgG1 expression proprietary vector. IgG1 production was performed in CHO cells, grown in serum-free Freestyle CHO expression medium (Invitrogen, USA) and maintained in Erlenmeyer Flasks at 37 °C with 5% CO_2 (Corning Inc., MA) on an orbital shaker. One day before transfection, the cells were seeded at an appropriate density in Corning Erlenmeyer Flasks. On the day of transfection, the vector was mixed with polyethylenimine (PEI) (Polysciences, Germany) and added to the cells. The supernatant collected on day 6 was used for the purification step. It was centrifuged, filtered and loaded onto a 5 mL HiTrap™ ProteinA column (GE Healthcare, Sweden) at 3 mL/min. After washing and elution with appropriate buffer, the fractions were collected and neutralized with 1 M Tris–HCl, pH 9.0. The final yield and the purity of the purified IgG1 was analyzed by SDS–PAGE and Western blot by using standard protocols, and tested both in ELISA and IF, as described above.

2.4. Anti-JCPyV/VP1 epitope mapping

2.4.1. Western blot and dot blot

To define the nature of the epitope (linear or conformational) bound by the anti-JCPyV/VP1 mAb, a Western blot and a dot blot were performed. For the Western blot, 200 ng of recombinant VP1 was run by SDS–PAGE gel and transferred to a nitrocellulose paper. The residual binding potential of the nitrocellulose was blocked by incubation overnight with 5% no fat milk in PBS, and the protein was probed with the selected GRE1 mAb or with a commercially available anti-JCPyV/VP1 mouse monoclonal antibody (34756 mAb) (Abcam, UK), as control. After incubation for 1 h at room temperature, the mAbs were removed, and the nitrocellulose paper was washed 5 times with PBS-Tween20, 10 min each time. The nitrocellulose was then incubated with HRP-conjugated anti-human IgG1 or anti-mouse IgG antibody. Unbound antibodies were removed by washing 5 times as previously described. Bound IgG was detected by radiography. Incubation of the nitrocellulose with only the secondary antibodies was performed as negative control.

For the dot blot, 200 ng of wild type recombinant VP1 and 200 ng of VP1 treated with SDS and β-mercaptoethanol was transferred to nitrocellulose paper and treated as described above for Western blot.

2.4.2. Competitive ELISA

A competitive ELISA was performed using 34756 mAb and GRE1. In details, recombinant VP1 protein was used to coat ELISA plates at 100 ng/well in PBS, overnight at 4 °C (Bugli et al., 2001). The plate was blocked with PBS/1% BSA for 1 h at 37 °C. Forty microliter of two fold serial dilution (from 10 μg/mL to 0.007 μg/mL) of the competing IgG (GRE1 or 34756 mAb) were added in duplicate to the wells and incubated for 2 h at 37 °C. The mAb used as probe (depending on the mAb used as competitor) was added at a final concentration giving approximately 60% of the maximum OD_{450} in ELISA, and the mixture was incubated for 30 min at 37 °C. The plate was washed 5 times with PBS + 0.1% Tween20 and the binding of the probe to the antigen was revealed with the appropriate HRP-conjugated antibody. The plate was incubated at 37 °C for 45 min and then washed 5 times with PBS + 0.1% Tween20. Forty microliter of TMB (tetramethylbenzidine) diluted in an equal volume of hydrogen peroxide were added to each well. The reaction was stopped after 10 min by adding 40 μL of 1 N sulfuric acid. OD_{450} was determined using a microplate reader. Control absorbance was defined as the absorbance (450 nm) obtained in the assay when each probe was used alone. Final results were determined as percent inhibition with the following formula: percent inhibition = $100 \times ((OD_{450}$ of probe alone $- OD_{450}$ of probe with competitor IgG$)/OD_{450}$ of probe alone).

2.4.3. Alanine scanning

To identify the residues involved in the interaction VP1-GRE1, its binding was tested on a panel of alanine point-mutated Mad-1 strain-derived VP1 molecules (except for original alanine residues that were mutated in glycine). To perform site-directed mutagenesis, a JCPyV/VP1 nucleotide sequence (ATCC® 45027) was cloned into pcDNA™ 3.1/V5-His TOPO® TA vector (Life Technologies, Germany), and the GeneArt® site-directed mutagenesis system was used (Life Technologies, Germany).

A total of 24 VP1 mutants were generated. The VP1 mutants were cloned into pCAG-JCV vector (a kind gift of Dr. Akira Nakanishi (Nakanishi et al., 2008)) by *Bste*II and *Pac*I sites, to allow their expression with the minor JCPyV capsid proteins (JCPyV/VP2 and JCPyV/VP3), in order to obtain the complete conformation of the viral capsid (pCAG-JCV was described for the construction of the JCPyV like-particles). The binding activity of GRE1 was then tested on the wild type VP1 and on VP1 mutants, and analyzed by fluorescence-activated cell sorting (FACS). In brief, human epithelial kidney (HEK) 293T cells were cultured in DMEM and supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). The cells were transfected with 2 μg of pCAG-JCV vector containing the VP1 mutant nucleotide sequence. After centrifugation and fixation with reagent A (FIX & PERM® Cell Fixation and Permeabilization Kits, Life Technologies), the transfected cells were incubated for 30 min at room temperature with GRE1 IgG (1 μg/mL) or GRE1 Fab (5 μg/mL) in reagent B (FIX & PERM® Cell Fixation and Permeabilization Kits, Life Technologies, Germany). The cells were then washed and incubated for 30 min at room temperature with a FITC-conjugated anti-human antibody (Sigma–Aldrich, Germany). The cells were washed and analyzed by FACS. Untransfected cells were also included in each experiment as negative control. 34756 mAb was used to evaluate the transfection efficiency for each VP1. Since we lack a conformational control mAb, only the mutants already described in some viral particles shed in urine or in PML patients (Delbue et al., 2009; Gee et al., 2004; Gorelik et al., 2011; Neu et al., 2010; Sunyaev et al., 2009; Zheng et al., 2005), (indicating that the mutation in these residues does not influence the correct protein conformation) were further considered in the analysis. The binding of GRE1 to the different VP1 mutants was expressed as binding percentage compared to the binding to the unmutated wild-type protein.

2.4.4. Hydrogen deuterium exchange mass spectrometry

Lyophilized monomeric JCPyV Polyomavirus major capsid VP1 protein was dissolved in PBS to give approximately 41.7 μM concentration. All samples containing free VP1, as well as those containing VP1 bound with antibodies were prepared in order to

perform analysis. In particular, experiments with mAb/VP1 complex were performed by mixing 625 pmol of VP1 with the same amount of GRE1 IgG or of 34756 mAb in order to give an antigen/mAb equimolar ratio.

Hydrogen/deuterium exchange (HDX) experiments were fully automated using a PAL autosampler (CTC Analytics). It enabled exchange start and quench, control of proteolysis temperature (4 °C) and duration (2 min), injection of the deuterated peptides, management of the injection and washing valves and triggering acquisition of the mass spectrometer and HPLC pumps. A Peltier-cooled box (4 °C) contained two Rheodyne automated valves (6-port for injection and 10-port for washing), a desalting cartridge (peptide Micro Trap from Bruker-Michrom) and a HPLC column (Poroshell 120 EC-C18, 1 × 50 mm, 2.7 μM from Agilent Technologies). Deuteration was initiated by a 5-fold dilution of VP1 or mAb/VP1 complex with D_2O. Porcine pepsin (Sigma–Aldrich, Germany) at 0.1 mg/mL in glycine–HCl 100 mM pH 2.5 was used to quench back-exchange and digest the deuterated proteins. After peptides desalting using an Agilent Technologies HPLC pump with TFA 0.03% in water at 200 μL/min, peptides were separated using another Agilent Technologies HPLC pump with a 15–100% B gradient in 20 min (A: TFA 0.03% in water; B: acetonitrile 90%, TFA 0.03% in water). The peptides masses were measured using an electrospray-TOF mass spectrometer (Agilent 6210).

The peptides were previously identified by tandem mass spectrometry, using a Bruker APEX-Q FTMS (9.4 T). Mass Hunter (Agilent Technologies, USA) and Data Analysis (Bruker) software were used for data acquisitions. HD Examiner (Sierra Analytics, USA) software was used for HDX data processing.

2.5. Set-up of the neutralization assay with the anti-JCPyV/VP1 antibody

The availability of GRE1 allowed us to use it as positive neutralization control in the set up of a robust neutralization assay. In two distinct version of the assay, the infected cells were detected by immunofluorescence using monoclonal antibodies directed against two different viral targets, an early protein (T antigen) and a late protein (VP1). More in details, African green monkey kidney cells (COS7) were cultured in Dulbecco's minimal essential medium (DMEM) (Life Technologies, Germany) supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin), at 5% CO_2. JCPyV Mad-4 strain (ATCC VR-1583) was used for infection (Bofill-Mas et al., 2003; Frye et al., 1997; Major et al., 1987). The viral stock was titrated using a immunofluorescent assay, following an already described protocol (Marriott and Consigli, 1985). In brief, COS7 cells (5×10^4/well) were seeded onto a 24-well plate (Sigma Aldrich, Germany), and grown overnight. Two-hundred microliter of twofold serial dilutions of the viral stock were added to the cells. After adsorption for 2 h at 37 °C, the medium was replaced with the maintenance medium (DMEM with 2%FBS), and incubated at 37 °C for 72 h. The cells were then washed in PBS and fixed in ice-cold methanol–acetone 1:1 (v/v) for 15 min, and then stained with 34756 mAb or with Pab2003 (a specific murine anti-JCPyV/Tag antibody, a kind gift of Prof. R.J. Frisque (Bollag et al., 2000)). After 1 h at 37 °C, a secondary fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma Aldrich, Germany) was added. After 30 min at 37 °C, the plate was examined with a fluorescence microscope and the titer was calculated by counting the fluorescent cells, and expressed as fluorescence forming units per mL (FFU/mL) of the original stock.

The neutralizing activity of GRE1 was tested in a Mad-4 strain-based neutralization assay, as Fab fragment and whole IgG1, using an adaptation of several already described protocols (Atwood, 2001; Frisque et al., 1979; Gosert et al., 2011; Hara et al., 1998; Marriott and Consigli, 1985; Nelson et al., 2012; Randhawa et al., 2009). Briefly, COS7 cells (5×10^4/well) were seeded onto 24 well plate (Sigma Aldrich, Germany), and grown overnight. Two-hundred microliter of linear concentrations (twofold dilutions from 20 μg/mL to 0.0002 μg/mL) of purified Fab or IgG1 (or of 34756 mAb, as a control) were mixed with 200 μL of medium containing approximately 100 FFU of viral particles, and incubated for 1 h at 37 °C. The mixture was added to COS7 target cells plated as described above for the viral titration, and incubated for 2 h at 37 °C. Finally, the supernatant was removed, 500 μL of maintenance medium were added to each well and cells were incubated at 37 °C for 72 h. Each dilution of purified Fab or IgG was tested in triplicate. Cells infected without the addition of the antibody were used as 100% infection control. The number of infected cell was calculated as FFU (described above). The neutralization activity was expressed by percent reduction of the number of fluorescent infected cells compared to 100% infection control.

2.6. JCPyV neutralization assay on sera from MS samples

The neutralization assay described below was used for testing the neutralizing activity of 31 sera samples from 28 MS patients, already tested for anti-JCV positivity using the STRATIFY JC virus™ assay. Sera samples, collected from MS patients at different times during the natalizumab therapy for diagnostic purpose in clinical routine, were stored in small aliquots at −80 °C until use.

In brief, after complement inactivation at 56 °C for 1 h, 200 μL of a single dilution (final dilution: 1:200, that is the same dilution used in the STRATIFY JC virus™ assay) of each sera sample was mixed with 200 μL of medium containing approximately 100 FFU (defined as described above), and incubated for 1 h at 37 °C. This mixture was added to COS7 target cells and incubated for 2 h at 37 °C. Finally, the supernatant was removed and 500 μL of fresh medium were added to each well and the cells were incubated for 72 h at 37 °C. Each serum sample was tested in triplicate. Cells infected without the addition of the sera samples were used as 100% infection control. The number of infected cell was calculated as described above. The neutralization activity was expressed as percent reduction of the number of fluorescent infected cells relative to the value of the infection control. GRE1 and 34756 mAb were used as positive and negative control, respectively.

3. Results

3.1. Selection of an anti-JCPyV/VP1 human monoclonal antibody (GRE1)

A human combinatorial phage display antibody library (IgG1/k isotype) was constructed with an estimated size of 2×10^7 members. The library was generated from the lymphocytes of a patient whose serum was strongly (diluted sample 1:200 $OD_{450} \approx 2.8$) positive for anti-JCPyV/VP1 antibodies in ELISA (Lin et al., 2013). The phage library was then immunoselected against the same recombinant monomeric VP1. After the third round of panning, a selective enrichment was observed, leading to a maximum of 3.1×10^7 pfu/mL in the fifth round. Phage from the final three rounds was converted to a soluble Fab expressing phagemid system. One-hundred and five clones were analyzed, with 11 (10.5%) clones reacting against JCPyV/VP1 and not on BSA, used as negative control antigen. Interestingly, none of the clones showed any reactivity against BKPyV/VP1, a protein featuring up to 75% sequence homology with JCPyV/VP1 (Eash et al., 2006).

The sequencing of the selected clones evidenced that all shared the same heavy and light chains sequences, and the single clone was named GRE1. Sequence analysis of the variable regions evidenced that the heavy chain of GRE1 originates from the VH4-31

Table 1
GRE1 mAb IMGT junction analysis.

Sequence ID	V-GENE and allele	V-REGION identity % (nt)	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR-IMGT length	AA JUNCTION
GRE1	Homsap IGHV4-31*06 F	90.69% (263/290 nt)	Homsap IGHJ6*03 F	Homsap IGHD3-10*01 F	2	[10.7.19]	CARDRGDSSGSSYYKYYMDVW
GRE1	Homsap IGKV1-39*01 F	98.57% (275/279 nt)	Homsap IGKJ1*01 F	–	–	[6.3.9]	CQQSYSTPRTF

The table shows VDJ genes of the variable portion of GRE1.

germline gene, and it is paired with a VL1-39-derived light chain (Table 1). The GRE1 heavy chain showed a 90.69% homology with germline sequence, evidencing its origin from a somatic mutation process. As usually observed with phage-display-derived mAbs, the GRE1 light chain showed a higher homology (98.57%) with the germline sequence. The analysis of the junction did not evidence any peculiar feature, with an average CDR3 length for both heavy (17 amino acidic residues) and light chains (9 amino acidic residues) (Rock et al., 1994; Yamada et al., 1991).

3.2. GRE1 binding features as whole IgG1

To study GRE1 as whole IgG1 molecule, the variable region of the heavy and light chain was cloned into an IgG1 expression vector. As expected, GRE1 IgG1 showed a higher affinity than GRE1 Fab on JCPyV/VP1 ($\approx 10^{-11}$ M for IgG1 compared to $\approx 10^{-9}$ M for Fab fragment) (Fig. 1A) in ELISA, and both format did not show any reactivity against BKPyV/VP1 and BSA (Fig. 1B). The specificity of GRE1 for JCPyV/VP1 was also confirmed in an immunofluorescence assay on JCPyV Mad-4 strain-infected COS-7 cells (Fig. 1C and D), showing a diffuse nuclear granular pattern.

3.3. GRE1 epitope mapping

Several approaches were followed to define the region on VP1 bound by GRE1. Firstly, a competitive ELISA was performed using GRE1 IgG1, and 34756 mAb. Interestingly, the two antibodies did not show any reciprocal competition in the binding to VP1 (data not shown), demonstrating that the two mAbs recognize two different epitopes. A Western blot assay with VP1 in denaturing conditions, and a dot blot with denatured and non denatured VP1 evidenced that GRE1 is able to recognize only non denatured VP1, showing that its epitope is conformational; on the other hand, 34756 mAb was able to bind both VP1 (wild type and denatured one) showing the linear nature of its epitope (Fig. 2A).

To identify the aminoacid residues on VP1 which influence GRE1 binding, an alanine scanning approach was followed. The highly homologous JCPyV/VP1 (Swiss ID:P03089) and BKPyV/VP1 (Swiss ID:P03088) amino acid sequences were aligned in order to identify the most divergent residues (in terms of polarity and charge) to be mutated in alanine. A total of 33 residues were identified, and 24 of them were mutated in alanine (or in glycine, where the original residue was alanine). The binding of GRE1 Fab and GRE1 IgG1 to the different mutants was expressed as binding percentage compared to the unmutated protein (100% of binding). All mutants were bound by 34756 mAb, which was used to evaluate the transfection efficiency for each VP1 mutants. In particular, three different mutants (A151G, F159A, V257A) showed a high expression variability, as demonstrated by standard deviation (Fig. 2B). G243A and N251A mutants showed a low expression level, and were not considered further in GRE1 binding analysis. Since the lack of conformational control in this study, only mutants already described in literature as not abolishing JCV infectivity (Delbue et al., 2009; Gee et al., 2004; Gorelik et al., 2011; Neu et al., 2010; Sunyaev et al., 2009; Zheng et al., 2005) were considered in further structural analysis. In particular, a total of ten mutants (I62A, S65A, E69A, A127G, D130A, N131A, A133G, A151G, F159A, A175G) caused more than 50% reduction of GRE1 IgG1 binding. Analogously, GRE1 Fab was inhibited by eight (I62A, S65A, E69A, A127G, A133G, A151G, F159A, A175G) out of the ten mutants interfering with IgG1, but it was also influenced by an additional mutant (R266A), already described as involved in VP1 binding to sialic acid. As shown in Fig. 2B, residues A151G, F159A and A175G were not further considered for structural analysis, as their role have never been investigated before, whereas E69 has already been described as altering VP1 folding (Gee et al., 2004). Although not previously described in literature, mutant I62A was further considered since its importance was also confirmed in this study by HDX experiments. As a consequence, residues I62, S65, A127, D130, N131 and N133 were mapped on VP1 structure (Fig. 2C–E).

The data obtained with alanine scanning were confirmed by HDX experiments (Zhang et al., 2011). In particular, deuteration profiles of free VP1 as well as VP1 bound with GRE1 or 34756 mAb were analyzed through mass spectrometry. HDX-mass spectrometry analysis resulted in 69% protein coverage (48 peptides) with some of these peptides overlapping, and thus increasing the VP1 resolution. HDX-mass spectrometry analysis evidenced that all the covered regions showed similar deuteration profiles in the context of the free VP1 or when bound with GRE1, except two overlapping peptides encompassing amino acid residues 58–67 and 62–67. In particular, the first peptide showed a deuteration level of 46% and 40% in the free VP1 protein and in 34756 mAb, respectively, compared to the 33% of deuteration level for the VP1-GRE1 complex. Similarly, for the second overlapping peptide a deuteration level of 53% and 49% in the free VP1 protein and in 34756 mAb, respectively, compared to the 36% of deuteration level for the VP1-GRE1 complex have been obtained. These data thus suggest that the region encompassing amino acid residues 58–67 of VP1 is involved in GRE1 binding considering the different deuteration profiles obtained in the VP1-GRE1 complex compared to that of the free antigen or when bound with the 34756 mAb (data not shown).

Considering the data obtained in the HDX-mass spectrometry analysis, the 58–67 amino acid region of VP1 is directly involved in the binding of the GRE1 mAb to the monomeric form of the VP1 protein. Taking into account the data obtained in the alanine scanning approach, it is possible to conclude that further distant regions, such as those involving amino acid residues A127, D130, N131 and A133 of VP1, are also involved in the binding of GRE1 mAb to the pentameric form of VP1. This is consistent with the pentameric structure of VP1, as the 58–67 amino acid region located in one monomer lies near the regions encompassing amino acids A127, D130, N131 and A133 of the adjacent monomer.

3.4. GRE1 anti-JCPyV neutralizing activity as Fab fragment and as whole IgG1

GRE1 neutralizing activity was tested using both the Fab fragment and the whole IgG1 molecule. GRE1 Fab showed a strong neutralizing activity, with a 50% inhibitory concentration (IC_{50}) of 0.4 μg/mL (Fig. 1E). On the other hand, GRE1 IgG1 showed an even

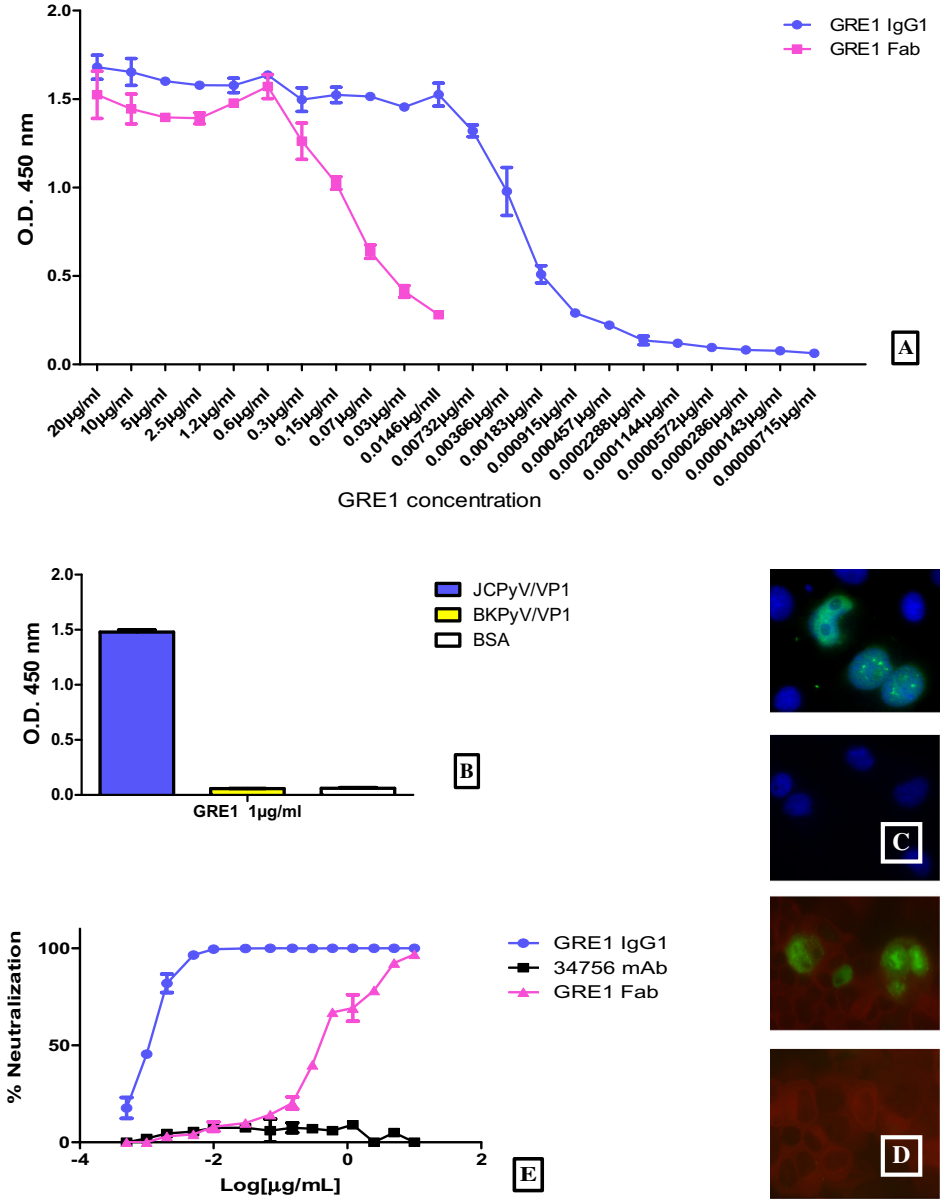

Fig. 1. GRE1 binding features and neutralizing activity. (a) Different binding features of GRE1 IgG1 (blue line) and GRE1 Fab (pink line) on recombinant JCPyV/VP1; (b) lack of reactivity on highly homologous BKPyV/VP1 (yellow column) and on BSA (white column); (c) immunofluorescence assay (IF) with DAPI stain on Mad-4 infected cell to highlight the nuclei (upper panel) and on uninfected cells (lower panel); (d) IF assay with Evan's blue stain on Mad-4 infected cell (upper panel) and on uninfected cells (lower panel); (e) neutralizing activity curve of GRE1 IgG1/Fab (blue and pink line, respectively) and 34756 mAb (black line) against Mad-4 strain.

higher neutralizing activity, with a 0.001 µg/mL IC_{50} (Fig. 1E). As expected, 34756 mAb did not show any neutralizing activity, evidencing that its epitope is distinct from the one recognized by GRE1 and it distant from the amino acidic residues involved in the sialic acid binding. The same results were obtained using both anti-JCPyV/VP1 (GRE1 or 34756 mAb) or anti-JCPyV/Tag antibody (Pab2003) as detecting mAbs in immunofluorescence assay for the count of FFU (Gosert et al., 2011).

3.5. Evaluation of the anti-JCPyV neutralizing activity in sera samples from STRATIFY JC virus™-tested MS patients

The fine characterization of GRE1 neutralizing activity, and the set up of a robust neutralization assay allowed us to perform a pilot study investigating the neutralizing activity of 31 samples from 28 MS patients previously tested in the STRATIFY JC virus™ assay. More in details, 21 of the 31 (67.7%) samples were positive while 10 samples (32.3%) were negative (Table 2).

In the neutralization assay, 11 (52.4%) STRATIFY JC virus™-positive samples showed a strong neutralizing activity (NA > 85%), four (19%) showed a weak NA (50% < NA < 85%), whereas, interestingly, 6 (28.6%) did not show any NA (NA < 50%). None of the ten STRATIFY JC virus™-negative samples showed any NA.

4. Discussion

In recent years, JC virus (JCPyV) gained new clinical relevance as etiologic agent of PML, due to its association with the administration of novel immunomodulatory "biological" drugs (Tavazzi et al., 2012). Particular attention was reserved to natalizumab, a humanized mAb highly effective in the treatment of relapsing-remitting MS but associated to a not negligible incidence (up to 3.85/1000)

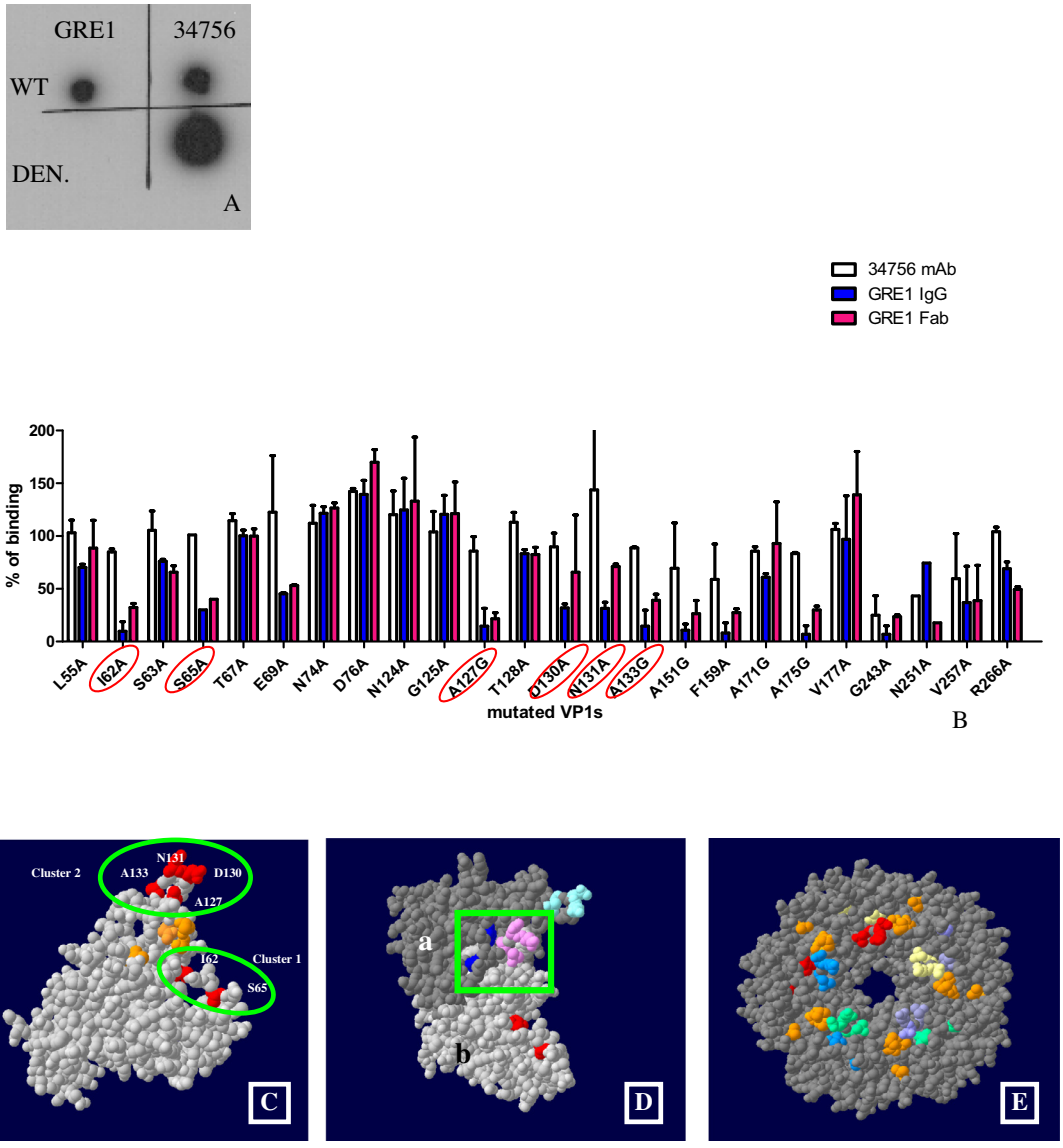

Fig. 2. GRE1 epitope characterization. (a) Dot blot analysis of GRE 1 and of control mAb 34756 on native (WT) and denatured (DEN) VP1 protein. (b) Binding analysis of GRE1 IgG1 (blue columns), GRE1 Fab and control mAb 34756 on VP1 mutants analyzed by FACS. Values are reported as percentage of the binding on unmutated VP1 protein. Mutants already reported in the literature as non abolishing JCV infectivity, and featuring more than 50% decrease of binding are circled in red. The importance of residue I62 was evidenced by HDX experiments performed in this study. (c) Structural analysis of the residues affecting (in red) GRE1 IgG1 binding on an available crystal model of VP1 monomer; the residues involved in the binding to sialic acid are reported in orange; (d) Two adjacent monomers of one pentamer are showed; to distinguish the two monomers, two different shades of gray were used. In particular, the two different clusters of residues identified in a monomeric form were highlighted in two different colors monomer a: cluster 1 is in red, cluster 2 is in pink; monomer b: cluster 1 is in blue and cluster 2 is in light blue). As evidenced by the green square, cluster 2 and cluster 1 (pink and blue respectively) of two adjacent monomers lay very close. (e) Structural analysis of the residues affecting GRE1 IgG1 binding on an available crystal model of a VP1 pentamer. The residues affecting the binding are reported in different colors (blue, green, violet, yellow and red) in order to distinguish the residues on distinct VP1 monomers. Also in this case, the residues involved in the binding to sialic acid are all reported in orange.

Table 2
Anti-JCV serological status and neutralizing activity of the samples from MS patients tested in this study.

STRATIFY JC virus™	N°. of samples (%)	Neutralizing activity (NA) (% within each STRATIFY JC virus™ group)		
		Strong (NA > 85%)	Weak (50% < NA < 85%)	No NA (NA < 50%)
Positive	21 (67.7%)	11 (52.4%)	4 (19%)	6 (28.6%)
Negative	10 (32.3%)	0	0	10 (100%)
Total	31 (100%)	11	4	16

of PML among treated patients (Ferenczy et al., 2012). Little is known about the molecular mechanisms leading to PML in some JCPyV-infected patients, and it would be of extreme importance to identify prognostic markers that could be used in natalizumab-treated patients. Recent studies have highlighted the role of cellular immune response in the control of JCPyV infection (Koralnik, 2002; Perkins et al., 2012), but these findings are not easily transferred into a routine assay for the identification of

high-risk patients. It is not surprising that most of the clinical studies performed to date have investigated and proposed the JCPyV positive serostatus as possible risk-stratification laboratory marker. A well-designed commercially available serological assay (STRATIFY JC virus™) has made the use of anti-JCPyV serology easier in the initial screening of MS patients to be treated with natalizumab, but it is important to be aware of the fact that this approach has some limitations. First of all, up to 60% of individuals in the general population (and about 54% of the MS patients (Bozic et al., 2011)) have been infected by JCPyV, limiting to the remaining 40% the potential benefits of this serology-based screening flow-chart (Egli et al., 2009; Knowles et al., 2003). Moreover, also in the case of a JCPyV-negative patient, it has to be borne in mind that an apparently JCPyV-seronegative status may be due to the previous use of immunomodulatory drugs, as recently demonstrated by the detection of JCPyV viruria in JCPyV-negative MS patients. Interestingly, anti-JCPyV antibodies were detected in cerebrospinal fluid in some JCPyV-seronegative patients, thus suggesting that other serological markers have to be considered (Lin et al., 2013).

Under this perspective, commenting a proposed clinical risk-stratification algorithm (Bloomgren et al., 2012), we recently suggested to investigate the level of neutralizing activity within the anti-JCPyV humoral response (Mancini et al., 2012a). The role of the humoral response in many persistent viral infections was considered minor for a long time, and only after its molecular dissection in the single components the real role of discrete antibody subpopulations was better evidenced. As a matter of fact, several anti-infectious mAbs endowed with broad neutralizing activity have entered or are entering clinical trials.

Given that, in this proof-of-concept study we evaluated the possible correlation between the overall anti-JCPyV antibody response and its neutralizing activity in sera samples of MS patients. Until now, it has been difficult to set up highly reproducible and robust neutralization assays for JCPyV, due to the time consuming and variability of virus replication when working *in vitro* with this virus. Our work was made markedly easier by the generation of the first human anti-JCPyV/VP1 neutralizing mAb (GRE1) which was taken as control of neutralization activity present in patient humoral response. Interestingly, GRE1 showed a high JCPyV/VP1-specificity (the most important viral surface protein involved in the binding to receptors), confirmed by its lack of reactivity on the highly homologous BKPyV/VP1 protein. As described in the literature for most of the neutralizing mAbs described against different viruses, GRE1 recognize a conformational epitope (Clementi et al., 2011; Mancini et al., 2009; Perotti et al., 2008). Moreover, an anecdotic report describes the anti-JCPyV humoral immune response as mainly directed against conformational epitopes, as shown by the lack of reactivity against denatured VP1 protein (Wang et al., 1999). The residues influencing GRE1 binding, tested both as Fab fragment and whole IgG1 molecule, allowed to define the bound region.

By alanine scanning assay, we identified ten and nine mutants affecting GRE1 IgG1 and GRE1 Fab binding, respectively. However, due to the lack of a conformational control, only mutations previously described in viral particles sequenced in the course of a real infection were considered, indicating that they do not significantly influence the correct protein folding (Delbue et al., 2009; Gee et al., 2004; Gorelik et al., 2011; Neu et al., 2010; Sunyaev et al., 2009; Zheng et al., 2005). For this reason, a total of three mutants (S65A, A127G, A133G) were considered to have an effect on both GRE1 Fab and GRE1 IgG1 binding, while two other mutants (D130A, N131A) only influence GRE1 IgG1, possibly due to IgG-related steric hindrance (Diotti et al., 2012). The mutation I62A was not described in literature, but the importance of this residue was confirmed by mass spectrometry experiment.

To better characterize the epitope recognized by GRE1 under the structural point of view, the mutated residues influencing GRE1 binding were indentified on an available crystal structure of VP1 (Neu et al., 2010) (http://www.rcsb.org/pdb/explore/explore.do?structureId=3NXG). Intriguingly, when the residues were mapped on the monomeric form of the JCPyV/VP1, two separated clusters were identified (cluster 1: I62, S65; cluster 2: A127, D130, N131, A133). Using VMD software (Humphrey et al., 1996), the distance between them was measured considering their centers of mass; clusters on the same monomers were at 26.4 Å, which is not compatible with an average epitope size (Fig. 2C). Conversely, in the pentameric form (the structural organization of VP1 on viral particles) the cluster 1 of a monomer and cluster 2 of adjacent monomer are much closer and therefore potentially bound by a single antibody (9.5 Å) (Fig. 2D and E). As already described for other conformational neutralizing antibodies directed against multimeric surface proteins (Julien et al., 2013), the bound epitope include residues distant on the single monomer, but very close between adjacent monomers.

More interestingly, the residues described above are in proximity to those important for the binding to sialic acid, the main JCPyV receptor, thus giving a structural basis to the observed neutralizing activity of GRE1 describe below.

The detected residues give also a structural clue to the noteworthy specificity and the very strong neutralizing activity featured by GRE1. In particular, the residues I62 and S65 are within a region that was already empirically used to generate monospecific anti-JCPyV sera in rabbits (Aoki et al., 1996).

Twelve JCPyV subtypes (Sugimoto et al., 2002) have been described to date, and they are all identical in the region bound by GRE1, thus suggesting its broad reactivity against different JCPyV strains. Analogously, other regions of the protein are conserved but in contrast to the region recognized by GRE1, these regions do not interfere with the viral cycle of JCPyV, as demonstrated by the fact that some VP1-binding sera do not show any neutralizing activity. It would be very interesting to investigate also the role of these non-neutralizing epitopes in JCPyV replicative cycle.

In this paper, we also report a pilot study on sera from MS patients, previously tested for their anti-JCPyV serostatus, evidencing the lack of correlation between seropositivity and neutralizing activity. In particular, even using same serum dilution as that used in STRATIFY JC virus™ assay, a relevant fraction (28.6%) of all STRATIFY JC virus™-positive patients did not show any anti-JCPyV neutralizing activity.

In conclusion, this is the first paper describing a fully human neutralizing anti-JCPyV/VP1 mAb and its epitope. Moreover, we evidenced that the anti-JCPyV neutralizing response is not correlated with the overall anti-JCPyV humoral response, and we suggest to investigate this finding in clinically more relevant cohorts of patients. In our opinion, several practical consequences may originate by transferring the observations of this proof-of concept study to PML patients. First of all, the serum anti-JCPyV neutralizing activity could be correlated to the actual risk of developing PML during treatment, possibly allowing a better risk stratification of natalizumab-treated patients. As recently proposed for the anti-JCPyV serological testing (Bozic et al., 2011), the anti-JCPyV neutralizing activity could also be evaluated on CSF samples, when available. As a possible consequence, these future studies may help to shed light on the JCPyV-related physiopathological mechanisms leading to PML, highlighting the relative contribution of peripheral JCPyV versus central nervous system reactivation. Finally, but not less important, the availability of a fully human broadly-neutralizing anti-JCPyV mAb, such as GRE1, may also allow the design of novel immunoprophylactic strategies (Burioni et al., 2008a,b) based on its administration to at-risk patients during immunomodulatory therapeutic regimens.

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