J. Neurovirol. (2016) 22:736–746
DOI 10.1007/s13365-016-0445-4

Host cell virus entry mechanisms enhance anti-JCV-antibody switch in natalizumab-treated multiple sclerosis patients

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Received: 12 December 2015 / Revised: 30 March 2016 / Accepted: 4 April 2016 / Published online: 11 May 2016

Abstract Estimating the individual risk for the development of progressive multifocal leukoencephalopathy (PML) in anti-John Cunningham virus (JCV) antibody-negative patients with multiple sclerosis (MS) treated with natalizumab is a major challenge. A serological conversion occurring under treatment from anti-JCV antibody-negative to positive status may significantly increase this risk. We investigated changes in peripheral blood cells' gene expression induced by natalizumab treatment in anti-JCV antibody-negative MS patients and tested blood transcriptional profile that characterizes patients predisposed to antibody switch under natalizumab treatment. After 3 years of natalizumab treatment, 24.6 % of anti-JCV antibody-negative MS patients switched to become anti-JCV antibody-positive (JCV switchers). Natalizumab induced 946 and 1186 significantly differentiating genes in JCV switchers and non-switchers, respectively. In JCV switchers, the signature was enriched by over-expression of genes associated with the first stages of viral entry to host cells including macropinocytosis ($p = 1.82E{-}06$), virus entry via endocytosis ($p = 1.60E{-}06$), clathrin-mediated endocytosis ($p = 1.13E{-}04$), and caveolar-mediated endocytosis ($p = 4.50E{-}04$) pathways. Further analysis to identify pre-existing transcriptional differences that characterize future JCV switchers prior to treatment initiation also demonstrated a transcriptional signature enriched by similar viral entry mechanisms. These findings, verified in an additional independent cohort of natalizumab-treated patients, could lead to future identification of patients that remain anti-JCV antibody-negative thus allowing safe continuation of treatment, as well as the development of future targeted therapeutic interventions to reduce the risk of PML.

Keywords JCV · Multiple sclerosis · Gene expression profiling · Natalizumab

Introduction

John Cunningham virus (JCV) is a common neurotropic polyomavirus that infects 80 % of the population worldwide and is a causative agent of progressive multifocal leukoencephalopathy (PML). This central nervous system (CNS) infection, for which there is no proven effective treatment, causes demyelization and leads to death or irreversible neurological disability. In immunocompetent individuals, viral replication within the CNS remains low, resulting in an asymptomatic infection; however in patients undergoing immunosuppression or immunomodulatory therapies, JCV may be reactivated and these patients have an increased risk of developing PML (Brooks and Walker 1984). This has been postulated to occur by the combination of increased B cell proliferation, a host cell for JCV, and decreased CNS immune surveillance (Bellizzi et al. 2013). The mechanisms that render some patients susceptible for developing PML, while other remain resistant, are thought to be associated with different immune system reactivity, as well as variability in virus-host interaction mechanisms. Several immunomodulatory drugs have a potential risk for inducing

Electronic supplementary material The online version of this article (doi:10.1007/s13365-016-0445-4) contains supplementary material, which is available to authorized users.

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PML including efalizumab, brentuximab vedotin, rituximab, and natalizumab (Ferenczy et al. 2012).

Natalizumab (Tysabri), a humanized monoclonal antibody directed against alpha4 integrin (very late activation antigen 4, VLA-4), is a leading drug for the treatment of relapsing-remitting multiple sclerosis (RRMS). Natalizumab reduces the relapse rate by 68 % during the first year of treatment, decreases disability progression by 42 % and has a marked beneficial effect on magnetic resonance imaging (MRI) outcomes (Polman et al. 2006). These therapeutic effects are mediated by inhibition of interaction between VLA4 and vascular cell adhesion molecule 1 (VCAM1), preventing T and B cell extravasation into the CNS followed by reduction of the ongoing inflammatory response. Additionally, natalizumab affects specific immune cell subsets, blocking CD4+ more than CD8+ T lymphocytes, and disproportionally increasing peripheral CD19+ mature and immature B lymphocytes (Putzki et al. 2010). However, the blockade of VLA-4 by natalizumab interferes not only with MS-related autoimmune processes but also with immune surveillance (Stüve et al. 2006). Despite the very effective treatment response to natalizumab, the primary drawback of natalizumab therapy is that it is associated with the development of PML (Berger and Koralnik 2005).

Estimations regarding the rate of sero-conversion from anti-JCV antibody-negative to positive in natalizumab-treated patients vary between studies, from 2 % sero-conversion per year (Gorelik et al. 2010), to significantly higher numbers reaching 26 % of sero-negative natalizumab-treated patients converting to sero-positivity after 1 year (Outteryck et al. 2012, 2013). Furthermore, serological testing has been shown to underestimate JCV infection rates, as more than one third of sero-negative patients have detectable JCV viremia or viruria. This has led to the suggestion that the current risk stratification strategy of serological testing prior to natalizumab treatment may not be sufficiently adequate and that additional serological testing and monitoring during treatment is required (Major et al. 2013; Berger et al. 2013).

Previous studies have shown that the risk of PML increases dramatically (44-fold) in natalizumab-treated patients that are anti-JCV antibody-positive, compared to anti-JCV antibody-negative (Bloomgren et al. 2012); therefore, identification of molecular markers that can detect, prior to treatment initiation, predisposition of patients to sero-convert from anti-JCV antibody-negative to positive during natalizumab treatment is of great significance. Moreover, identifying those who will not sero-convert is even of more importance for the continuation and safety of treatment. Hence, the aim of the current study was to identify early transcriptional changes occurring during natalizumab treatment, and detect pre-treatment transcriptional profile, that are associated with serological switch to the anti-JCV positive antibody status, increasing the susceptibility to PML.

Methods

Study design and setting

This was an observational prospective study to identify the transcriptional signature associated with anti-JCV antibody sero-conversion during treatment with natalizumab. RRMS patients from the Multiple Sclerosis Center of the Sheba Medical Center (Ramat Gan Israel) were assessed for their anti-JVC antibody status (using second generation enzyme-linked immunosorbent assay (ELISA)), and 142 patients identified as anti-JCV antibody-negative were treated with natalizumab (Tysabri, Biogen Idec) 300 mg administered intravenously once every 4 weeks, followed up for 3 years, and tested for anti-JCV antibody on a yearly basis. Patients who showed serological conversion of their anti-JCV antibody status from negative to positive were defined as JCV switchers. Of the 142 anti-JVC antibody-negative patients identified prior to initiation of natalizumab treatment, 60 patients were chosen randomly for analysis of transcriptional changes induced in peripheral blood mononuclear cells (PBMC) by natalizumab. The study design is shown in Fig. 1. PBMCs were obtained from this subset of patients at baseline—before initiation of natalizumab treatment—and after 1 year of treatment—and assessed for gene expression analysis as described below. The transcriptional changes induced by natalizumab after 1 year of treatment versus baseline were then compared between JCV switchers and non-switchers. Additionally, pre-treatment baseline samples were analyzed to identify pre-existing differences between JCV switchers and non-switchers. The results were verified on 28 samples of an additional independent group of anti-JCV antibody-negative RRMS patients obtained before initiation of natalizumab treatment.

Standard protocol approvals and patients consent

The study was approved by Sheba Medical Center Institutional Review and Ethical Board and all patients gave written informed consent. Study registration no: 8343-10-SMC.

Microarray gene expression

Total RNA was purified from PBMC using Trizol (Invitrogen, USA). Briefly, RNA integrity was determined using the RNA Experion automated electrophoresis system (Bio-Rad Laboratories Inc, Hercules, CA, USA). Probe synthesis using 3 μg total RNA was performed by the two-cycle

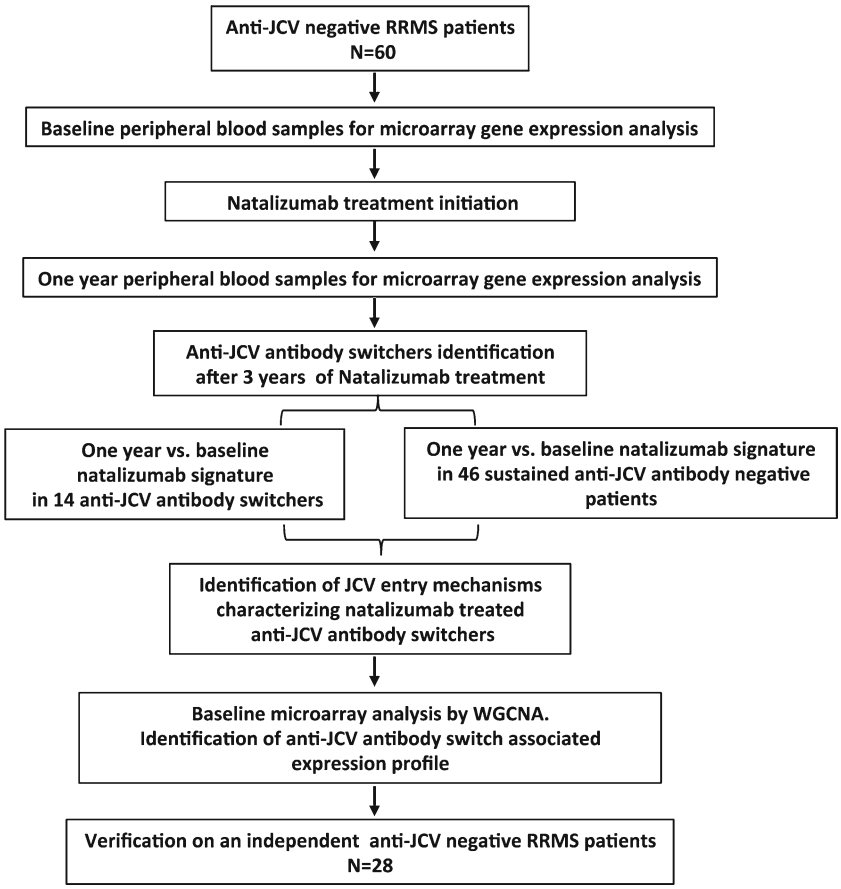


Fig. 1 Study design. The prospective study design is demonstrated. The study included 60 anti-JCV antibody-negative RRMS patients. After 3 years of natalizumab treatment, JCV switchers were identified. Blood samples obtained before and after 1 year of natalizumab treatment were subjected to comparative gene expression microarrays analysis between JCV switchers and JCV non-switchers. Baseline samples were analyzed to identify pre-existing transcriptional differences between future JCV switchers and non-switchers using weighted gene co-expression network analysis (WGCNA). Findings were verified in an additional group of 28 anti-JCV negative RRMS patients before initiation of natalizumab treatment

RNA amplification kit protocol and in vitro transcription was performed with the GeneChip IVT Labeling Kit (both Affymetrix Inc., Santa Clara, CA, USA). The biotin-labeled IVT–RNA was hybridized to a Genechip array (HU133A-2, including 22,000 probes corresponding to 14,500 human genes), washed in a GeneChip Fluidics Station 450 (Hewlett Packard, Palo Alto, CA, USA), and scanned using GeneArray™ scanner G2500A (Hewlett Packard) according to the manufacturer's protocol, as previously described (Gurevich et al. 2015).

Data pre-treatment, normalization, and statistical analysis

Following scanning of the microarrays, raw data was normalized by R Bioconductor Packages (R development Core Team 2013) as follows: (a) all arrays were normalized serially using a single sample microarray normalization approach designed for personal medicine workflows (SCAN Normalization) (Piccolo et al. 2012); (b) an empirical Bayes approach was used to address batch effect in the data as implemented in the Combat SVA package. The effect of treatment with natalizumab for 1 year in comparison to baseline was evaluated using paired *t* test analysis; differentially expressed genes (DEGs) were defined by a threshold of p value <0.01 following False Discovery Rate (FDR) correction for multiple testing. Analysis was done using Partek Genomic Software (www.partek.com).

Weighted Gene Co-expression Network Analysis (WGCNA)

To evaluate the baseline differences between patients who were JCV antibody-negative and later switched to become JCV antibody-positive and patients who remained JCV antibody-negative, weighted gene co-expression analysis (WGCNA) was applied. The general framework of WGCNA has been described previously (Zhang and Horvath 2005). Briefly, WGCNA is an unsupervised method allowing identification of co-expression patterns between genes of a network that organizes clusters of highly correlated genes in modules that can be related to sample traits. This method enables to reveal fine, coordinated gene expression changes that are hidden due to multiple testing limitations by methods that examine individual genes. In general, the WGCNA procedure consists of four steps: (1) generation of a similarity matrix of gene co-expression by determining the absolute

value of the Pearson correlation for all genes; (2) transformation of a similarity matrix to connectivity using a power adjacency function; (3) identifying network modules (highly correlated gene expression patterns across samples) using hierarchical clustering; (4) relating sample trait information to the identified modules. Gene co-expression networks were constructed using the WGCNA package in R statistical computing software (http://www.r-project.org); the precise procedure used for network construction is provided in Supplementary Material 1.

We correlated the identified gene co-expression modules to anti-JCV antibody switch status.

Classification analysis

Unsupervised classification of samples was performed using Principal Component Analysis (PCA) and hierarchical clustering algorithms implemented in the Partek software. The 60 baseline samples were used to construct a blood gene expression-based classifier (training set), while additional 28 independent baseline samples (verification set) were used for subsequent testing of the classifier performance. The most discriminative genes, used for classification algorithms, were chosen by applying a forward selection feature implemented in the Partek software. Multiple classification algorithms implemented in the Partek software were applied to the training set subgroups to construct classifiers that can correctly distinguish at baseline between future JCV switchers and non-switchers. The classifier with the best performance on the training set was chosen and subsequently validated on the verification set.

Functional analysis

Biological functional analysis of the DEGs and module genes was performed by Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com). Right-tailed Fisher's exact test was used to calculate a p value determining the probability that each biological function assigned to the gene data set is not due to random chance. The p values obtained by Fisher's analysis were applied for Benjamini-Hochberg FDR multiple testing corrections to keep the overall error rate at $p < 0.05$.

Two-step anti-JCV antibody testing

Anti-JCV antibody serological status and index were determined by the two-step second generation anti-JCV antibody assay STRATIFY JCV™ Test (Unilabs, Denmark) (Lee et al. 2013). In the first step, the anti-JCV antibody index (equivalent to titer) is classified: an antibody index that is lower than 0.20 is classified as negative, an index higher than 0.40 is classified as positive, and a value between 0.20 and 0.40 is classified as intermediate requiring further evaluation in second step confirmation test. In this second step, pre-incubation of samples with solution of JCV-like particles is performed and the percentage of inhibition is assessed. Inhibition ≤45 % is considered negative and inhibition >45 % is accounted as positive. The test was performed before initiation of natalizumab and on a yearly basis after treatment. All intermediate results were re-evaluated by repeating the test.

Flow cytometry

The proportion of CD4+, CD8+ T lymphocytes, and CD20+ B lymphocytes cell populations after 1 year of natalizumab treatment was determined using fluorescence-activated cell analysis (FACS) (FACscan Becton-Dickinson, San Jose, CA).

Results

Changes in anti-JVC antibody status following 3 years of treatment with natalizumab

One hundred and forty-two anti-JCV antibody-negative RRMS patients were treated with natalizumab and were tested for anti-JCV antibody status annually. At 1 year of follow-up, 6.3 % (9/142 patients) had switched to become anti-JCV antibody-positive. At 2 years, additional 10.6 % (15 patients) sero-converted, and at 3 years an additional 7.7 % (11 patients) switched to become anti-JCV antibody-positive. Overall, after 3 years, the anti-JCV antibody status of 24.6 % (35/142 patients) changed under natalizumab treatment, yielding an annual switch rate of 8.2 %.

Of the 142 anti-JCV antibody-negative RRMS patients who were treated with natalizumab, 60 patients (42 females, aged 38.1 ± 0.9 years, Expanded Disability Status Scale (EDSS) 3.4 ± 0.2, disease duration 11.2 ± 0.2 years) were randomly selected to undergo the evaluation comparing the change in PBMC transcriptional gene expression signature between baseline (prior to initiation of natalizumab treatment) and 1 year of treatment. Of these 60 patients, 23.3 % (14 patients, 7 females, mean age 34.7 ± 3.6 years, EDSS 2.9 ± 0.4, mean disease duration 10.6 ± 2.4 years) switched to become anti-JCV antibody-positive within 3 years. The average time to anti-JCV antibody switch was 22.8 ± 1.5 months, with two patients (3.3 %) converting within the first year, six patients (10 %) within the second year, and six patients (10 %) within the third year of natalizumab treatment, Supplementary Material 2. Anti-JCV antibody titer analysis demonstrated that of the 14 JCV switchers, 11 patients (78.5 %) had a positive anti-JCV index (>0.4) and 3 patients had an intermediate index (0.2 to 0.4) further defined as positive after obtaining a

high level of inhibition in the second step confirmation test. Forty-six patients (76.6 %) remained anti-JCV antibody-negative after 3 years of natalizumab treatment (35 females, age 39.5 ± 0.7 years, EDSS 3.5 ± 0.2, disease duration 11.4 ± 1.1 years). No significant differences were found between switchers and non-switchers in relation to demographic and clinical variables.

Natalizumab-induced gene expression signature: 1 year following treatment vs. baseline

The transcriptional gene expression signature of JCV switchers ($n = 14$) following 1 year of natalizumab treatment compared to pre-treatment baseline included 946 DEGs, 637 of which were up-regulated and 309 down-regulated genes; in non-switching patients ($n = 46$), the transcriptional signature comprised 1186 DEGs, 621 up-regulated and 565 down-regulated ($p < 0.05$ after FDR correction). A total of 445 gene-transcripts were common between both signatures, 264 DEGs were up-regulated and 181 DEGs were down-regulated. Functional analysis of the transcriptional changes induced by natalizumab is presented in Table 1. Similar operating mechanisms were demonstrated in anti-JCV antibody switchers and non-switchers in the following pathways:

B cell development-related pathways The most significantly enriched pathways were associated with B cell development-related pathways including phosphoinositide 3-kinase (PI3K) signaling in B lymphocytes, B cell development, B cell receptor signaling, and interleukin 4 (IL-4) signaling. These pathways were enriched with at least 49 well-recognized B cell-related DEGs including CD79A, CD79B, B cell receptor (BCR), CD19, CD24, CD40, tyrosine-protein kinase (BLK), B cell linker (BLNK), as well as B cell transcription factors such as Early B-Cell Factor 1 (EBF1), POU domain class 2-associating factor 1 (POU2AF1), paired box 5 (PAX5), and SPI-B.

Immune response Pathways related to immune response were also highly enriched in both patients groups. These include nuclear factor of activated T cells (NFAT) in regulation of immune response, N-formyl-methionyl-leucyl-phenylalanine (fMLP) signaling in neutrophils, primary

Table 1 Functional analysis of the transcriptional changes induced by natalizumab

Process	Canonical pathway	*p* value JCV switchers	*p* value JCV non-switchers
B cell development	PI3K signaling in B lymphocytes	7.66E−10	4.24E−06
	B cell development	4.17E−07	1.19E−07
	B cell receptor signaling	1.40E−06	4.52E−06
	Gap junction signaling	3.61E−04	5.85E−05
	IL-4 signaling	6.97E−04	4.66E−03
Immune response	Role of NFAT in regulation of the immune response	2.15E−07	2.25E−05
	fMLP signaling in neutrophils	1.76E−07	5.95E−04
	Primary immunodeficiency signaling	1.58E−04	1.33E−04
	NF-κB activation by viruses	1.12E−04	4.41E−02
	Fcγ receptor-mediated phagocytosis in macrophages and monocytes	9.54E−04	1.35E−01
	PKCθ signaling in T lymphocytes	6.78E−04	5.82E−05
	Leukocyte extravasation signaling	2.74E−04	1.48E−01
Viral entry	Macropinocytosis signaling	1.82E−06	3.49E−04
	Virus entry via endocytic pathways	1.60E−06	4.99E−03
	Clathrin-mediated endocytosis signaling	1.13E−04	2.26E−02
	Caveolar-mediated endocytosis signaling	4.50E−04	2.43E−01
Intracellular signaling	VEGF signaling	6.39E−02	7.39E−04
	p70S6K signaling	2.31E−03	1.85E−05
	14-3-3-mediated signaling	1.58E−02	1.35E−04
	Phospholipase C signaling	2.32E−06	2.20E−03
	Integrin signaling	1.18E−06	2.32E−02
	Paxillin signaling	1.61E−04	2.46E−02
Cell metabolism	PPARα/RXRα activation	4.34E−03	1.21E−04
	Cell cycle: G1/S checkpoint regulation	1.68E−04	1.00E+00
	Regulation of cellular mechanics by calpain	6.12E−05	3.59E−01

immunodeficiency signaling and protein kinase C-theta (PKCθ) signaling in T lymphocytes.

Two pathways related to immune activity showed significant enrichment levels only in JCV switchers and lacked significance in JCV non-switchers. These were Fcγ receptor-mediated phagocytosis in macrophages and monocytes, and leukocyte extravasation signaling.

Intracellular signaling Intracellular signaling pathways were enriched in both patient groups and included vascular endothelial growth factor (VEGF) signaling, p70S6k signaling, 14-3-3 signaling, and phospholipase signaling. Two signaling pathways—the integrin signaling pathway and the paxillin signaling pathway—were significantly more enriched in JCV switchers. In these patients, an abundance of differentially expressed alpha and beta integrin molecules was noted, including down-regulation of integrin alpha-IIb (ITGA2B, CD41), integrin beta-3 (ITGB3, CD61), integrin beta-5 (ITGB5), integrin beta-2 (ITGB2, CD18), integrin alpha M (ITGAM), and up-regulation of integrin alpha 4 (ITGA4, CD49D, alpha 4 subunit of VLA4 receptor).

Cell metabolism The cell metabolism pathway of peroxisome proliferator-activated receptor alpha/retinoid X receptor alpha (PPARα/RXRα) activation was enriched in both switchers and non-switchers, whereas JCV switchers were more enriched in G1/S checkpoint regulation pathways and in pathways regulating cellular mechanics by calpain protease.

Viral entry The most interesting finding in natalizumab-induced signature was enrichment of genes related to pathways associated with mechanism of viral entry into host cells. These were significantly more enriched in JCV switchers as compared to non-switchers. Natalizumab treatment affected mechanisms of viral entry related to the first stage of viral infection as demonstrated by enrichment in macropinocytosis signaling pathway, virus entry via endocytic pathway, clathrin-mediated endocytosis signaling, and caveolar-mediated endocytosis signaling; the last was observed only in JCV switchers. Specifically, in JCV switchers, these pathways were enriched by up-regulated genes related to viral entry including transferrin receptor (TFRC), huntingtin-interacting protein 1-related (HIP1R), epidermal growth factor receptor pathway substrate 15 (EPS15), ITGA4, adaptor-related protein complex 1, gamma 2 subunit (AP1G2), ras-related protein rab-5b (RAB5B), HERC, flotillin (FLOT), and filamin B (FLNB). Several genes were down-regulated including clathrin light chain A (CLTA), actin-related protein 2/3 complex, subunit 2 (ARPC2), ras-related protein rab-11A (RAB11a), and cortactin (CTTN).

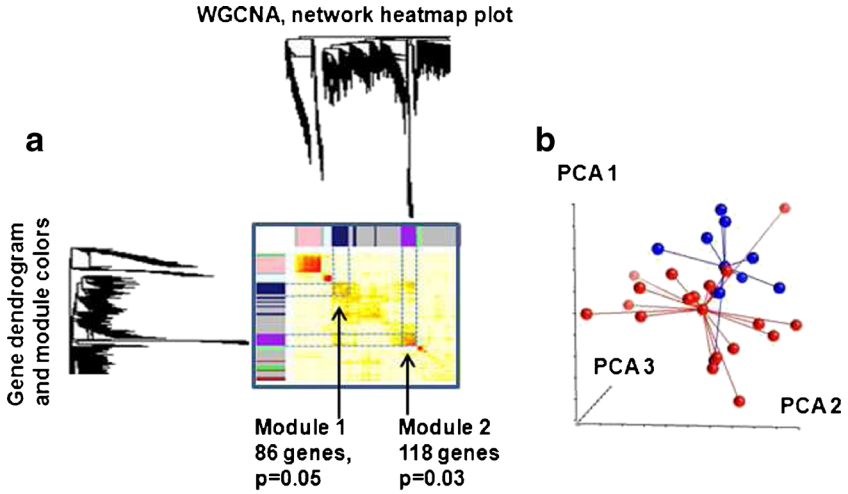
No significant correlations between the gene expression results and anti-JCV antibody index or time to sero-status switch were found in JCV switchers. To verify that the observed gene expression differences were not due to underlying differences in PBMC composition, we compared the percent of T and B cell sub-populations between anti-JCV switchers and non-switchers. PBMC sub-populations (mean ± SE) of CD4+ T cells (42.4 ± 3.1 vs 38.4 ± 3.2 %, $p = 0.20$), CD8+ T cells (20.2 ± 2.2 vs 22.8 ± 2.9 %, $p = 0.25$), and CD20+ B cells (24.2 ± 2.6 vs 22.8 ± 2.9 %, $p = 0.43$) were not significantly different between groups.

Pre-treatment gene expression analysis: comparison between future JCV switchers and non-switchers at baseline

To characterize the specific baseline gene expression profile of future JCV switchers and to differentiate it from that of non-switchers, differential gene expression analysis using WGCNA was done on PBMCs obtained at the baseline pre-treatment stage. This analysis identified co-ordinated changes organized in gene networks that included 13 modules, ranging in size from 29 to 744 genes. Two modules significantly correlated with a future JCV switch: module 1 included 86 genes, $r = -0.26$, $p = 0.05$ and module 2 included 118 genes, $r = -0.3$, $p = 0.03$, Fig. 2a. No significant correlations were found between these modules and demographic variables of gender and age. Functional enrichment analysis performed on these modules demonstrated that the most significantly enriched pathways in both modules were associated with mechanisms of viral entry into host cells.

Module 1 was significantly enriched by genes encoding for virus entry via the endocytic pathways ($p = 1.6E{-}03$), including clathrin, light chain B (CLTB), filamin A alpha (FLNA), adaptor-related protein complex 2 s (AP2S1), adaptor-related protein complex 2 m (AP2M1), as well as clathrin-mediated endocytosis signaling pathway ($p = 3.5E{-}03$) including CLTB, AP2M1, AP2S1, arrestin beta 2 (ARRB2), Serpin Peptidase Inhibitor, Clade A (SERPINA1), and caveolar-mediated endocytosis signaling ($p = 7.2E{-}03$) based on FLOT1, FLNA, and COPE.

Module 2 was most significantly enriched by genes encoding for virus entry via the endocytic pathway ($p = 3.6E{-}02$) including adaptor-related protein complex 1 (AP1G2), dynamin 2 (DNM2), and FYN genes; via the caveolar-mediated endocytosis signaling pathway ($p = 2.5E{-}04$) based on co-regulated expression of under-expressed RAB5B, FLOT2, DNM2, coatomer protein complex, subunit gamma 1 (COPG1), and FYN genes; and via clathrin-mediated endocytosis signaling pathway ($p = 1.6E{-}02$), including RAB5B, DNM2, AP1G2, cyclin G associated kinase (GAK), and hepatocyte growth factor-regulated tyrosine kinase substrate (HGS). All genes associated with viral entry mechanisms from both modules are shown in Table 2.



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Classification results	
Verification data set	N=28
Classification model	Nearest Centroid
Classifier genes	n=10
Sensitivity	0.78±0.13
Specificity	0.74±0.10
Average	0.76±0.08

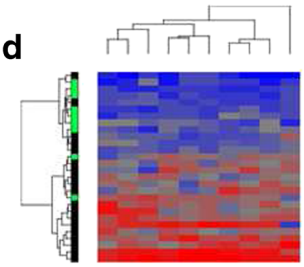

Fig. 2 Natalizumab pre-treatment gene expression analysis: comparison between future JCV switchers and non-switchers at baseline. **a** Weighted Gene Co-expression Network Analysis (WGCNA) was used to correlate gene expression profiles in RRMS patients before initiation of natalizumab and future anti-JCV antibody switch. Genes were clustered based on co-expression patterns represented by the dendrogram and gene expression heat map. Clusters of genes are referred to as modules, color coded on left and upper panels of the dendrogram. In the heat map, the intensity of *red coloring* indicates the strength of correlation between pairs on a linear scale. Genes that could not be assigned to a module are labeled *gray*. Two modules, module 1 and module 2 (marked by *dotted lines*), were found to significantly correlate with future conversion to anti-JCV antibody-positive. **b** Unsupervised PCA-based classification of baseline samples from the verification set ($n = 28$). PCA classification based on all 204 genes within the two identified WGCNA modules clearly distinguished between future JCV switchers (*blue dots*) and non-switchers (*red dots*). The distance between any pair of points is related to the similarity between the two observations in high-dimensional 3D space. **c** The results of classifier testing on independent pre-treatment samples from the verification set ($n = 28$). The nearest centroid algorithm classifier, based on 10 genes, was able to correctly predict future JCV status with an overall classification rate of 76.0 ± 8.1 %. **d** Unsupervised hierarchical clustering of the baseline samples from the verification set according to the expression of the 10 genes included in the classifier demonstrated two clusters, one of which was enriched by JCV switchers (*green color in left dendrogram*) and the other including mainly non-switching patients (*black color in left dendrogram*)

Verification of JCV entry mechanisms at baseline

Pre-treatment baseline gene expression findings were verified in PBMCs obtained from an independent cohort of 28 anti-JCV-negative RRMS patients prior to initiation of natalizumab treatment (verification set, 19 females, mean age 41.3 ± 2.7 years, disease duration 12.0 ± 1.4 years, EDSS 4.8 ± 0.4). In this population subset, 9 patients switched to become anti-JCV antibody-positive (Table 1) during natalizumab treatment, while 19 patients remained anti-JCV antibody-negative after 3 years of follow-up. The average time to anti-JCV antibody switch was 16.0 ± 2.2 months, with four (14.3 %) patients converting within 1 year, four within the second year, and one (3.5 %) patient converting within the third year of treatment.

First, we examined if the 204 genes identified within the two WGCNA modules related to anti-JCV antibody switch in our original pre-treatment (training) patients subset could differentiate JCV switchers from non-switchers in this independent verification set. Using an unsupervised classification PCA algorithm, we demonstrated that these patient groups are uniquely and differentially clustered by the modules genes (Fig. 2b). Next, to examine if the module-related genes could be used to predict future anti-JCV antibody switch, we searched for the most discriminative genes within the two modules, and based on these genes, examined multiple classification algorithms on our original baseline samples of 60 RRMS patients (training set). Subsequently, a 10-gene based nearest centroid classification algorithm was determined and correctly classified 71 ± 8.0 % of patients within our original training set. On the independent 28 patients verification set, the classifier demonstrated a similar overall classification rate of 75.0 ± 8.1 % in predicting future JCV sero-status (Fig. 2c). Unsupervised hierarchical clustering of the verification set samples according to

Table 2 Genes associated with viral entry mechanisms identified in the pre-treatment gene expression signature

Gene symbol	Gene name	Entrez ID	Pathway
AP1G2	Adaptor-related protein complex 1	8906	Clathrin-mediated endocytosis
AP2M1	Adaptor-related protein complex 2, m	1173	Clathrin-mediated endocytosis
AP2S1	Adaptor-related protein complex 2, s	1175	Clathrin-mediated endocytosis
ARRB2	Arrestin, beta 2	409	Clathrin-mediated endocytosis
CLTB	Clathrin, light chain B	1212	Clathrin-mediated endocytosis
DNM2	dynamin 2	1785	Clathrin/caveolar endocytosis
GAK	Cyclin G associated kinase	2580	Clathrin-mediated endocytosis
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate	9146	Clathrin-mediated endocytosis
RAB5B	RAB5B, member RAS oncogene family	5869	Clathrin/caveolar endocytosis
SERPINA1	Serpin peptidase inhibitor, member 1	5265	Clathrin-mediated endocytosis
FYN	FYN proto-oncogene	2534	Clathrin-mediated endocytosis
COPE	Coatomer protein complex, e	11316	Caveolar-mediated endocytosis
COPG1	Coatomer protein complex, subunit gamma 1	22820	Caveolar-mediated endocytosis
FLNA	Filamin A, alpha	2316	Caveolar-mediated endocytosis
FLOT1	flotillin 1	10211	Caveolar-mediated endocytosis
FLOT2	Flotillin 2	2319	Caveolar-mediated endocytosis

the expression of the 10 genes included in the classifier is presented in Fig. 2d, and shows two distinct clusters, one of which is enriched by JCV switchers and the other includes mainly non-switchers.

Discussion

The current study evaluated biological mechanisms associated with sero-conversion from anti-JCV antibody-negative to positive status in RRMS patients treated with natalizumab. The importance of understanding these biological mechanisms is highlighted by high sero-conversion rates observed in this study, as well as in recent previous reports. We found that 24.6 % of natalizumab-treated patients sero-converted within 3 years of treatment, similar to a previous report by Trampe et al. that found a rate of 9.8 % conversion after 7.7 months, and Outteryck et. al reporting an even higher sero-conversion rate of 28.2 % after 1 year of treatment (Outteryck et al. 2012, 2013; Trampe et al. 2012). Although these high rates may be partially due to de novo JCV infection, it is probably the result of re-activation of latent JCV by natalizumab. Indeed it is known that positive anti-JCV antibody serological status underestimates true JCV infection prevalence, as more than one third of sero-negative patients have detectable JC viremia or viruria (Major et al. 2013; Berger et al. 2013). The gut, lymphoreticular system, bone marrow, and the kidney are all likely sites of JCV latency (Chesters et al. 1983; Monaco et al. 1996). However, clinical risk of PML has been correlated to antibody status rather than viremia or viruria, emphasizing the clinical importance of antibody status. Although the anti-JCV antibody non-switchers group may be comprised by false negative patients, the best available two-step second generation anti-JCV antibody assay STRATIFY JCV^{TM} is characterized by high sensitivity and only 3 % rate of false negative results (Lee et al. 2013).

We found that even before initiation of natalizumab treatment, there were already differences between patients who remained anti-JCV antibody-negative and those who had sero-converted following natalizumab treatment. PBMC gene expression differences between these two patient groups related to virus entry mechanisms, suggesting a specific gene expression phenotype, which is probably genetically determined, that renders the patients who carry it susceptible to JCV sero-conversion.

The control of JCV replication in precursor B cells depends upon nuclear transcription factors of the host cells capable of recognizing DNA sequences in the viral regulatory region; this may lead to mobilization of B cells from the bone marrow and lymphoid tissues to the peripheral blood and subsequently to the brain (Jing et al. 2010; Lindberg et al. 2008). We suggest that natalizumab-induced switch of anti-JCV antibody status from negative to positive occurs due to activation of host B cell transcription factors. These nuclear transcription factors are usually inactive and are stimulated by natalizumab treatment as was demonstrated in previous publications (Ferenczy et al. 2012) as well as in our study. We found that natalizumab treatment activated genes related to B cell proliferation in both switchers and non-switchers. Specifically, our findings show up-regulation of B cell transcription factors including SPI-B, PAX5, EBF1, and POU2AF1, in addition to 49 downstream B cell-related

DEGs showing high enrichment for B cell activity and proliferation mechanisms (Fig. 3). SPI-B, a transcription factor normally promoting B cell maturation (Su et al. 1996), is especially interesting as it has been previously shown to have specific binding sites in the JCV viral promoter region and is important for production of JCV early and late viral genes (Marshall et al. 2010). As it is known that natalizumab treatment leads to changes in PBMC cell population proportions, and these changes may confound gene expression findings, we specifically tested and demonstrated that no differences in B and T cell populations were observed between anti-JCV antibody switchers and non-switchers.

The present data also demonstrate that for JCV seroconversion to occur under natalizumab treatment, specific changes in genes related to JCV viral entry mechanisms must take place. The first step of JCV infection happens as the virus contacts the host cells by binding to specific receptors including integrin molecules, a serotonin receptor, and a transferrin receptor (Elphick et al. 2004; Komagome et al. 2002). Accordingly to our findings, alpha integrin VLA-4, the target of natalizumab, and transferrin receptor (TFRC) are up-regulated in JCV switchers compared to non-switchers. VLA-4 also interacts with JCV through sialic acid residues to facilitate attachment to cell membrane. Together with transferrin receptor, these two highly expressed molecules contribute to increased viral insertion into host cells of future JCV switchers (Caruso et al. 2003; Komagome et al. 2002). We would like to note that in a previous report by Harrer et al. that examined gene expression effects of natalizumab treatment, in contrast to our findings, VLA-4 was found to be down-regulated (Harrer et al. 2011). However, as this was found in a mixed population of JCV switchers and non-switchers, it is possible that the over activation of VLA 4 in JCV switchers was not registered in that study because seroconverters represent only a small proportion of natalizumab-treated MS patients.

As it is known that natalizumab treatment leads to changes in PBMC cell population proportions, and these changes may confound the gene expression findings, we specifically tested and demonstrated that no differences in B and T cell sub-populations were observed between anti-JCV antibody switchers and non-switchers. These data support that the observed transcriptional differences are a true finding not related to PBMC composition. The next

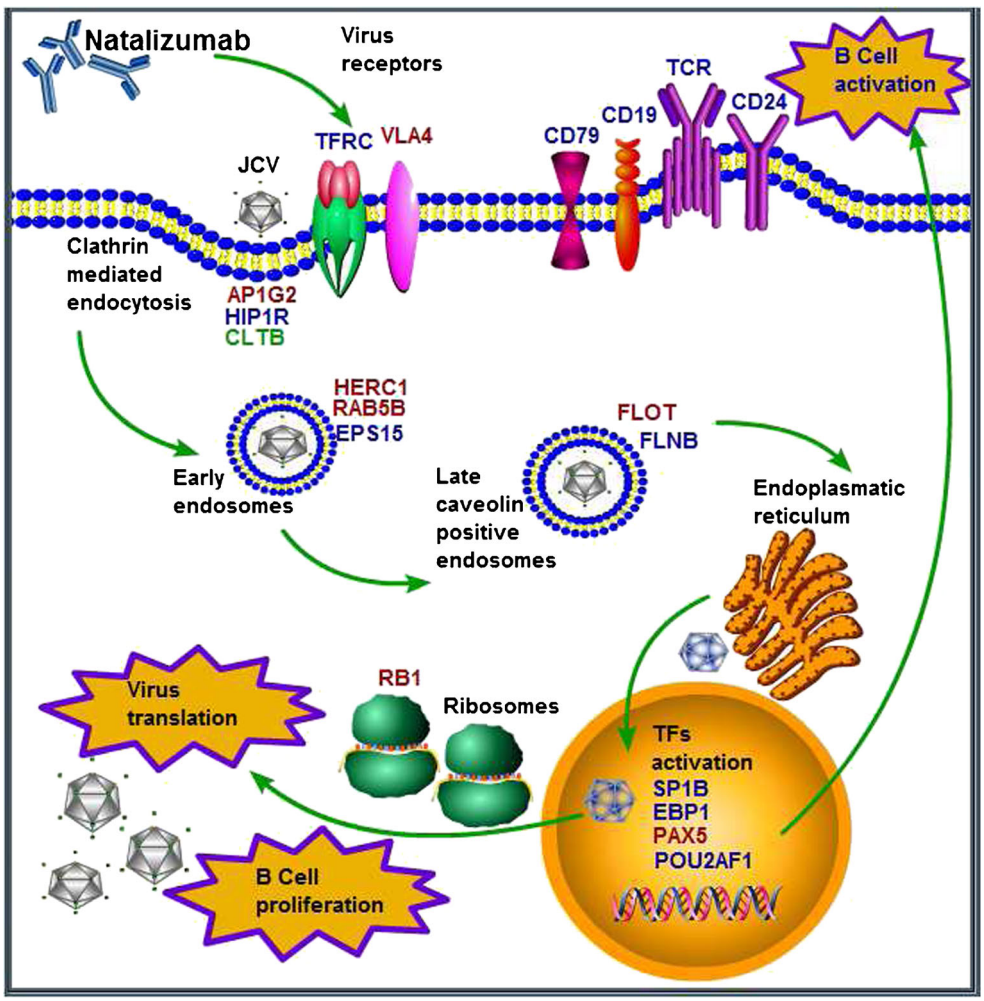

Fig. 3 Mechanisms associated with increased risk for anti-JCV antibody switch under natalizumab treatment. *Blue* corresponds to over-expressed genes in both JCV antibody switchers and non-switchers. *Red* represents over-expressed genes, and *green* represents down-expressed genes only in JCV antibody switcher group. Our findings demonstrate complex interactions of genes involved in all consecutive steps of JCV entry into host cells including JC virus receptor activation, implementation of early and late endosomes, and enhancement of B cells transcription factors resulting in activation of JCV and thus promoting its exposure to the host immune system and consequently leading to anti-JCV antibody production

step in JCV infection is receptor-mediated cellular entry. JCV uses a specific mechanism of invasion to host cells that depends on both clathrin and caveolin endocytosis pathways (Ferenczy et al. 2012). In JCV switchers, we observed enrichment of genes related to pathways associated with the mechanism of viral entry into host cells, namely macropinocytosis, endocytosis, clathrin-mediated endocytosis, and caveolar-mediated endocytosis. During receptor-mediated cellular entry via clathrin-dependent endocytosis, the JCV-receptor complex invaginates into a clathrin-coated pit that later forms an early endosome vesicle. In anti-JCV antibody switchers, we found up-regulation of clathrin adaptor proteins AP1G2 and HIP1R, both known to promote the formation of clathrin-coated vesicles and link vesicles to the cell cytoskeleton (Waelter et al. 2001). After JCV enters into the host cells, it is trafficked to early RAB5-positive endosomes and subsequently sorted to caveolin-1-positive late endosomes. Trafficking of JCV to early RAB5-positive endosomes is facilitated by EPS15, and dominant negative mutants of both RAB5B and EPS15 have been shown to block this process, highlighting their importance for effective JCV cell infection (Querbes et al. 2004). Flotilin and filamin are both members of the caveolin-positive late endosome and are required for the formation of caveolin vesicles (Stahlhut and van Deurs 2000). In the current study, these important early and late endosome genes including RAB5B, EPS15, FLOT, and FLNB were all over-expressed in JCV switchers, showing further evidence for stimulation of JCV infection in sero-converters. From the late endosome, JCV is transported to the endoplasmic reticulum, whereby it is partially disassembled, resulting in a virion that exits the endoplasmic reticulum and enters the cell nucleus through nuclear pores. In the host cell nucleus, JCV undergoes viral replication (Ferenczy et al. 2012). Our findings demonstrate complex interactions of genes involved in all consecutive steps of JCV entry into host cells with an overall activation of JCV infection following natalizumab treatment thus promoting JCV exposure to the host immune system and possibly leading to consequent antibody production. The studied genes are involved in the mechanism of viral entry that is shared by many naked viruses and thus the described mechanism may pertain to other viruses as well. We have also shown that similar mechanisms are associated with JCV antibody switch even before natalizumab treatment. WGCNA allowed us to determine a low but significant correlation between co-expressed genes organized in two separate modules that correlated with a future JCV sero-status switch. Both these modules were highly enriched in genes related to clathrin and caveolin endocytosis similar to our findings following natalizumab treatment. Notably, 16 genes associated with virus entry mechanisms, including FLOT, Filamin, APG12, and RAB5b, were also over-expressed in JCV antibody switchers following treatment. Taken together, these findings suggest that in future switchers, pre-defined expression of these genes is augmented by natalizumab treatment, subsequently facilitating exposure of latent JCV to the host immune system to induce antibody production.

To summarize, in the current study we provide the first proof of concept for transcriptional differences between RRMS patients who will undergo sero-conversion and those who will remain anti-JCV antibody-negative during natalizumab treatment. We identified viral-related host biomarkers that could be useful for future screening of patients who would be susceptible for sero-conversion, as well as for the development of targeted therapeutic interventions to prevent anti-JCV antibody switch, thereby reducing the risk of PML in these patients. Finally, our findings demonstrate that 77.6 % of natalizumab-treated RRMS patients remain anti-JCV antibody-negative after long-term (3 years) treatment. The non-switcher signature was characterized by down-regulation of clathrin/caveolin viral entry pathways allowing safe continuation of natalizumab treatment in the majority of patients.

Acknowledgments This research was supported by a research grant no: 8343-10-SMC from Medison Pharma SRL (Petach Tikva, 4917002, Israel).

This work was partially presented in the European Committee and Treatment for Multiple Sclerosis Meeting, Barcelona, Spain 7–10 October, 2015.

Compliance with ethical standards

Conflict of interests Prof. Achiron A, has provided consultation to Biogen Idec., Teva Pharmaceutical, MerckSerono, Novartis, and Genzyme. All other authors declare that they have no competing interests.

References

Bellizzi A, Anzivino E, Rodio DM, Palamara AT, Nencioni L, Pietropaolo V (2013) New insights on human polyomavirus JC and pathogenesis of progressive multifocal leukoencephalopathy. Clin Dev Immunol 2013:839719

Berger JR, Koralnik IJ (2005) Progressive multifocal leukoencephalopathy and natalizumab—unforeseen consequences. N Engl J Med 353:414–416

Berger JR, Houff SA, Gurwell J, Vega N, Miller CS, Danaher RJ (2013) JC virus antibody status underestimates infection rates. Ann Neurol 74:84–90

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

Brooks BR, Walker DL (1984) Progressive multifocal leukoencephalopathy. Neurol Clin 2:299–313

Caruso M, Cavaldesi M, Gentile M, Sthandier O, Amati P, Garcia MI (2003) Role of sialic acid-containing molecules and the alpha4beta1 integrin receptor in the early steps of polyomavirus infection. J Gen Virol 84:2927–2936

Chesters PM, Heritage J, McCance DJ (1983) Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. J Infect Dis 147:676–684

Elphick GF, Querbes W, Jordan JA, Gee GV, Eash S, Manley K, Dugan A, Stanifer M, Bhatnagar A, Kroeze WK, Roth BL, Atwood WJ (2004) The human polyomavirus, JCV, uses serotonin receptors to infect cells. Science 306:1380–1383

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

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

Gurevich M, Miron G, Achiron A (2015) Optimizing multiple sclerosis diagnosis: gene expression and genomic association. Ann Clin Transl Neurol 2:271–277

Harrer A, Wipfler P, Einhaeupl M, Pilz G, Oppermann K, Hitzl W, Afazel S, Haschke-Becher E, Strasser P, Trinka E, Kraus J (2011) Natalizumab therapy decreases surface expression of both VLA-heterodimer subunits on peripheral blood mononuclear cells. J Neuroimmunol 234:148–154

Jing D, Oelschlaegel U, Ordemann R, Hölig K, Ehninger G, Reichmann H, Ziemssen T, Bornhäuser M (2010) CD49d blockade by natalizumab in patients with multiple sclerosis affects steady-state hematopoiesis and mobilizes progenitors with a distinct phenotype and function. Bone Marrow Transplant 45:1489–1496

Komagome R, Sawa H, Suzuki T, Suzuki Y, Tanaka S, Atwood WJ, Nagashima K (2002) Oligosaccharides as receptors for JC virus. J Virol 76:12992–13000

Lee P, Plavina T, Castro A, Berman M, Jaiswal D, Rivas S, Schlain B, Subramanyam M (2013) A second-generation ELISA (STRATIFY JCV™ DxSelect™) for detection of JC virus antibodies in human serum and plasma to support progressive multifocal leukoencephalopathy risk stratification. J Clin Virol 57:141–146

Lindberg RL, Achtnichts L, Hoffmann F, Kuhle J, Kappos L (2008) Natalizumab alters transcriptional expression profiles of blood cell subpopulations of multiple sclerosis patients. J Neuroimmunol 194: 153–164

Major EO, Frohman E, Douek D (2013) JC viremia in natalizumab-treated patients with multiple sclerosis. N Engl J Med 368:2240–2241

Marshall LJ, Dunham L, Major EO (2010) Transcription factor Spi-B binds unique sequences present in the tandem repeat promoter/enhancer of JC virus and supports viral activity. J Gen Virol 91: 3042–3052

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

Outteryck O, Ongagna JC, Duhamel A, Zéphir H, Collongues N, Lacour A, Fleury MC, Berteloot AS, Blanc F, Giroux M, Vermersch P, de Sèze J (2012) Anti-JCV antibody prevalence in a French cohort of MS patients under natalizumab therapy. J Neurol 259:2293–2298

Outteryck O, Zéphir H, Salleron J, Ongagna JC, Etxeberria A, Collongues N, Lacour A, Fleury MC, Blanc F, Giroux M, de Seze J, Vermersch P (2013) JC-virus seroconversion in multiple sclerosis patients receiving natalizumab. Mult Scler 20(7):822–829

Piccolo SR, Sun Y, Campbell JD, Lenburg ME, Bild AH, Johnson WE (2012) A single-sample microarray normalization method to facilitate personalized-medicine workflows. Genomics 100:337–344

Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, Phillips JT, Lublin FD, Giovannoni G, Wajgt A, Toal M, Lynn F, Panzara MA, Sandrock AW, Investigators A (2006) A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med 354:899–910

Putzki N, Baranwal MK, Tettenborn B, Limmroth V, Kreuzfelder E (2010) Effects of natalizumab on circulating B cells, T regulatory cells and natural killer cells. Eur Neurol 63:311–317

Querbes W, Benmerah A, Tosoni D, Di Fiore PP, Atwood WJ (2004) A JC virus-induced signal is required for infection of glial cells by a clathrin- and eps15-dependent pathway. J Virol 78:250–256

R development Core Team (2013) R: a language and environment for statistical computing. R foundation for Statistical Computing, Vienna

Stahlhut M, van Deurs B (2000) Identification of filamin as a novel ligand for caveolin-1: evidence for the organization of caveolin-1-associated membrane domains by the actin cytoskeleton. Mol Biol Cell 11:325–337

Stüve O, Marra CM, Jerome KR, Cook L, Cravens PD, Cepok S, Frohman EM, Phillips JT, Arendt G, Hemmer B, Monson NL, Racke MK (2006) Immune surveillance in multiple sclerosis patients treated with natalizumab. Ann Neurol 59:743–747

Su GH, Ip HS, Cobb BS, Lu MM, Chen HM, Simon MC (1996) The Ets protein Spi-B is expressed exclusively in B cells and T cells during development. J Exp Med 184:203–214

Trampe AK, Hemmelmann C, Stroet A, Haghikia A, Hellwig K, Wiendl H, Goelz S, Ziegler A, Gold R, Chan A (2012) Anti-JC virus antibodies in a large German natalizumab-treated multiple sclerosis cohort. Neurology 78:1736–1742

Waelter S, Scherzinger E, Hasenbank R, Nordhoff E, Lurz R, Goehler H, Gauss C, Sathasivam K, Bates GP, Lehrach H, Wanker EE (2001) The huntingtin interacting protein HIP1 is a clathrin and alpha-adaptin-binding protein involved in receptor-mediated endocytosis. Hum Mol Genet 10:1807–1817

Zhang B, Horvath S (2005) A general framework for weighted gene co-expression network analysis. Stat Appl Genet Mol Biol 4:Article 17