Enhanced viral- and myelin-specific cellular immune responses without evidence of viral reactivation in multiple sclerosis patients treated with natalizumab: a longitudinal study

Samantha Jilek PhD, Emilie Jaquiéry MSc, Hans H. Hirsch MD, Andreas Lysandropoulos MD, Mathieu Canales, Laurence Guignard, Myriam Schlupe MD, Giuseppe Pantaleo MD, Renaud A. Du Pasquier MD

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Service of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland
Service of Neurology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland
Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Switzerland
Institute for Medical Microbiology, Department of Biomedicine, University of Basel, Switzerland

Running title: natalizumab and JCV-specific T cell responses

Corresponding author:
Renaud A. Du Pasquier
Service d’immunologie et allergie, BT-06
Centre Hospitalier Universitaire Vaudois
1011 Lausanne, Switzerland
Tel : + 41 21 314 1228; Fax: + 41 21 314 1161; Renaud.Du-Pasquier@chuv.ch
Jilek et al.

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Abstract

Background: Natalizumab, which is efficient in preventing relapses and progression of disability in multiple sclerosis, has been associated with cases of progressive multifocal leukoencephalopathy (PML). Therefore, it is of importance to better understand the mechanisms of this drug.

Methods: Twenty-four multiple sclerosis patients who started on natalizumab were followed prospectively. Blood and urine samples were tested for the presence of JC virus DNA using quantitative real-time PCR, before, and then, at regular intervals on natalizumab, for 18 months. At the same time points, using proliferation and enzyme-linked immunospot assays, the cellular immune responses against JC virus, Epstein-Barr virus, cytomegalovirus, myelin oligodendrocyte glycoprotein and myelin oligodendrocyte basic protein (MOBP) was determined. JC virus-specific humoral immune response was assessed with an enzyme immunoassay. The same experiments were performed in the blood of 16 patients with multiple sclerosis, before and 10 months after they began interferon beta therapy.

Findings: In natalizumab-treated patients, JC virus DNA was not detected in the peripheral blood mononuclear cell or plasma samples at any time point, however it was present in the urine of 6/24 patients. As compared to pre-treatment values, the cellular immune response was increased at six months to cytomegalovirus, at one, nine and 12 months to JC virus, and at 12 months only to Epstein-Barr virus and MOBP. In contrast, the humoral responses remained stable. In interferon beta-treated patients, there was no increase of the viral- or myelin-specific cellular immune responses.
Interpretation: Natalizumab enhances the viral- and myelin-specific cellular immune responses in the peripheral blood after one year, without evidence of viral reactivation.

**KEY WORDS:** natalizumab, JC virus, cellular immune response, multiple sclerosis
INTRODUCTION

Multiple sclerosis, the most common cause of chronic neurological disability in young adults, causes demyelination and axonal damage in the central nervous system (CNS). Migration of inflammatory cells from the peripheral blood into the CNS is considered as a critical step in the pathogenesis of the disease.\(^1\) Natalizumab (Tysabri\textsuperscript{®}, Biogen Idec and Elan) is a monoclonal antibody which binds to the $\alpha$ chain of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins that are expressed at the cell surface of hematopoietic cells.\(^2\) Binding of natalizumab to $\alpha 4\beta 1$ integrins prevents firm adhesion and diapedesis of activated lymphocytes through the blood-brain barrier.\(^3\) This drug has been shown to decrease the annual relapse rate at one year by 68% and the risk of sustained disability progression over two years by 42% as compared to placebo.\(^4\) However, as of December 11, 2009, 28 confirmed cases of PML have been reported (Biogen Idec Medical Information Service). PML is a severe demyelinating disease of the CNS caused by the polyomavirus JC, named after the first patient in the brain of whom the virus was isolated (John Cunningham), and is frequently fatal as there is currently no effective therapy. Thirty-five to 80% of the healthy adult population harbor JC virus antibodies.\(^5,6\) In the setting of severe immunosuppression, JC virus may escape the immune surveillance,\(^7\) and cause progressive CNS damage due to lytic replication in oligodendrocytes.\(^8\) The mechanism that may favour the onset of PML in rare natalizumab-treated patients remains obscure. However, in natalizumab patients, leukocyte counts are not decreased and since this drug has not been consistently associated with opportunistic infections other than PML, natalizumab cannot be considered as a classical immunosuppressant.\(^4,9\) One hypothesis poses that the blockade of ingress of JC virus-specific T cells through the blood brain barrier results in decreased immune surveillance of the CNS.\(^10\) Another hypothesis is that natalizumab induces an increased mobilization of bone marrow B cells infected with JC virus leading to viral replication and release of the virus initially into the periphery and eventually into the CNS.\(^11\)
In order to examine in details the virological, humoral and cellular immune response in natalizumab-treated patients, we performed a comprehensive, cross-sectional and longitudinal study. Using a quantitative real-time PCR (qRT-PCR), we determined the JC virus DNA load in natalizumab treated multiple sclerosis patients in the blood cells, plasma and urines at different time points. Indeed, JC virus is shed in the urine of 25% of the population, as it can reside in the kidneys.5, 12 In the blood, JC virus is associated with B lymphocytes, which reflects the fact that the bone marrow may be another reservoir of this virus.13 Thus, when JC virus is detected in the blood, it is more often in the peripheral blood mononuclear cells (PBMC) than than as cell-free virus in the plasma.12, 14, 15 To determine whether natalizumab acts only on JC virus or whether its effects would be less specific, we assessed the cellular immune responses against JC virus, but also against two other viruses (Epstein-Barr virus and cytomegalovirus) and two myelin proteins (myelin oligodendrocyte glycoprotein [MOG] and myelin oligodendrocyte basic protein [MOBP]). Epstein-Barr virus has been involved as a possible trigger of MS and the cellular immune response to this virus is dysregulated in these patients.16-18 Cytomegalovirus, which, such as Epstein-Barr virus, is a herpes virus, can establish latent infections, and is neurotropic. However, contrary to Epstein-Barr virus, it has neither been convincingly associated with MS, nor thought to play a deleterious role in MS.16, 17 Thus, we posed that, if the effects of natalizumab are not specific to JC virus, there would be a good chance to see a dysregulated response against one of these viruses. It has been hypothesized that the immune response to auto-antigens may decrease with time on natalizumab19 whereas other authors have suggested that T cell activation increased on this drug.20 Therefore, we took advantage of this longitudinal study to examine the cellular immune response against MOG and MOBP, two myelin proteins that have been reported as putative auto-antigen targets (S. Jilek and R.A. Du Pasquier, manuscript in preparation).21, 22 Finally, to determine whether the findings obtained in the natalizumab-treated cohort would
be specific to natalizumab, we assessed all the parameters described above in a control cohort of multiple sclerosis patients treated with interferon beta.
MATERIAL AND METHODS

Patients
From June 2007 to October 2008, we enrolled 24 consecutive relapsing-remitting multiple sclerosis patients starting natalizumab (300mg iv monthly) and followed them for up to 18 months (Table 1). The control group consisted of 16 multiple sclerosis patients treated with interferon beta. This study was accepted by our institution’s ethical commission and all subjects gave their written informed consent according to review board guidelines. At the time of enrolment, the diagnosis of multiple sclerosis was made using the revised McDonald’s criteria. Patients were considered as relapsing if a relapse had started less than four weeks prior to the blood sample draw. For each patient of the natalizumab group, blood and urine samples were drawn just before the first natalizumab injection (M0), then one month later (M1), then two months later (M3), then every three months up to 12 months (M12) and then six months later (M18). For the control group, blood was drawn twice respectively before [3 (2-6) months; median (interquartile range)] and after [10 (7-12) months; median (interquartile range)] onset of interferon beta therapy. However, in contrast to natalizumab patients, urine was not collected in these patients. Furthermore, interferon beta-treated patients do not need to come every month to our clinic, indeed they are controlled every six to nine months, which explains why their blood was not drawn as frequently as natalizumab patients one. Of note, one patient who was part of the interferon beta arm was subsequently switched to natalizumab, and thus enrolled in the latter arm (patient #24). Natalizumab treatment was started one year after the second blood draw that was performed for the interferon beta arm.

Sample collection
Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Hypaque (Amersham Biosciences, Otelfingen, Switzerland) and either resuspended
in RPMI medium (Invitrogen, Basel, Switzerland) for immediate use or frozen in freezing medium, as composed of 90% fetal calf serum (Invitrogen) and 10% dimethylsulfoxide (Sigma, Buchs, Switzerland), and stored in liquid nitrogen for further analysis. Plasma was harvested and kept at -80°C until use. Urine from natalizumab patients was collected simultaneously with blood draw and kept at -20°C until further use.

Quantification of JC virus DNA in PBMC, plasma and urines

Using quantitative real-time polymerase chain reaction (qRT-PCR), the determination of JC viral load was performed in PBMC, plasma and urines of natalizumab-treated patients as well as in PBMC and plasma of interferon beta-treated patients. DNA was extracted from peripheral blood mononuclear cells (PBMC), plasma, and urine with the QIAamp DNA blood mini kit (Qiagen, Switzerland). The set of primers and probe located in the large T gene sequence of the MadI JCV strain were specific for JC virus.24 The forward primer was JCT1: 5’-AGAGTGTTGGGATCCTGTGTTTT-3’ (nt 4298 to 4320 of MadI prototype sequence), the reverse primer was JCT2: 5’-GAGAAGTGGGATGAAGACCTGTTT-3’ (nt 4375 to 4352 of MadI sequence) and the probe was: JCT1.1: 6-FAM-5’-TCATCACTGGCAACATTTCCTTCATGGC-3’-TAMRA (nt 4323 to 4350 of MadI sequence). The qRT-PCR reaction was performed in a total of 25 µl volume, containing 12.5 µl 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 0.4 µM of each primer, 0.1 µM of probe and either 10 µl DNA solution containing 0.5 µg DNA (PBMC) or 10µl DNA solution resulting from DNA extraction of plasma or urines. qRT-PCR reaction was performed on a 7700 Sequence Detector System (Applied Biosystems). Cycling conditions included a 50°C, 2-min step to decontaminate any possible carryover contamination and a 95°C, 10-min step to denature the template DNA and activate the polymerase, followed by 40 cycles consisting of 15 sec at 95°C for denaturation and 1 min at 60°C for annealing and elongation.24 JC virus Mad I strain cloned in pUC18 vector between
two EcoRI sites was used for standard curve (kindly provided by I. Koralnik). Extreme caution was taken to perform all preparatory PCR steps, including DNA extraction, in a separate room completely isolated from any post-PCR samples to prevent contamination. Using this qRT-PCR, JCV DNA was detected in the CSF of a PML patient and in the PBMC of another one. Thus, these samples served as positive controls. The lower limit of JC virus DNA detection was 500 copies per millilitre of plasma and urine with a linear range from 500 to 10^8 copies/ml. The lower limit of JC virus DNA detection was 10 copies per microgram of PBMC DNA with a linear range from 10 to 2*10^6 copies per microgram of PBMC DNA. To confirm our results, plasma samples from natalizumab patients at the 18 months after treatment onset were sent for JC virus DNA qRT-PCR to the laboratory of diagnosis of infectious diseases at our institution. This laboratory serves as reference center for several pathogens, including JC virus, is active in the development of new tests, is recognized by Swissmedic (authorization 28411) and is accredited under ISO/CEI 17025 (STS328).

**JC virus-specific humoral immune response**

To assess for the presence of antibodies to JC virus in plasma of the patients, we performed an enzyme immunoassay (EIA), whose sensitivity has been shown to be high since it is sufficient to detect small changes in humoral immune responses in severely immunosuppressed AIDS patients with PML. The VP1 coding sequence JC virus was inserted into a pFastBac vector (Invitrogen, Basel, Switzerland) and recombinant baculovirus genomes were generated by using the Bac-to-Bac expression system (Invitrogen) and transfected in Sf 9 insect cells following the manufacturer’s instructions. JC virus-like particles (JCVLP) were purified from passage 3 infection cultures using optiprep gradients (H.H Hirsch and J. Samaridis, unpublished data). The 3-dimensional structure and quality of JCVLP was confirmed by electron microscopy and by hemagglutination titers. JCVLP were used as antigens in a standard EIA assay, as described elsewhere. The optical density at 492nm (OD492)
obtained with PBS alone was taken as background and subtracted, and positive and negative control sera were run in parallel with every assay. An OD492 above 0.110 was used to define a positive serologic response for IgG.

**Peptides**

For JC virus- and myelin-specific immune responses, pools of 15-mer peptides, overlapping by 10 amino acids, and encompassing the whole amino acid sequence of the VP1, the major capsid protein of JC virus, MOG and MOBP proteins were used (SynPep Corporation, Dublin, CA). Eight (JC virus), six (MOG) and four (MOBP) pools each containing nine 15-mers were used at a concentration 10 µg/ml and could elicit CD4+ as well as CD8+ T cells.27, 28 For Epstein-Barr virus- and cytomegalovirus-specific immune responses, purified viral lysates (ABI Inc, Columbia, MD) specifically eliciting CD4+ T cells, or pools of CD8+-restricted immunodominant peptides were used at 1µg/ml as previously described in details.17

**Detection of antigen-specific proliferating T cells**

To determine the presence of T cells proliferating specifically against JC virus, Epstein-Barr virus, cytomegalovirus or myelin proteins, we performed proliferation assays such as described in details previously.17 Briefly, upon isolation 200'000 PBMC were plated in quadruplicate in 96-well plates (Corning Life Science, Schiphol-Rijk, Netherland) in RPMI-5% human AB serum (Inotech, Dottikon, Switzerland) and stimulated with pools of JC virus peptides, myelin peptides, Epstein-Barr virus or cytomegalovirus lysates, or pools of Epstein-Barr virus or cytomegalovirus CD8+-restricted peptide epitopes. Peptide-free medium served as negative control and phyto-hemagglutinin lectin (PHA-L; Calbiochem, Dietikon, Switzerland) at 5mg/ml as positive control. Cells were incubated for 5 days at 37°C and then pulsed overnight with 1 µCi/well of methyl-[3H]thymidine (Hartmann Analytic, Braunschweig, Germany). After harvesting, nuclear incorporation of radioactivity was
measured in a scintillation beta counter (Topcount, Zurich, Switzerland). Proliferation responses were calculated as stimulation index (SI), as determined by the mean ratio of antigen-stimulated counts per minute (cpm) over background cpm. For JC virus, patients were considered as responding if they had at least one pool with a SI higher than 2.0. Data are presented as cumulative SI for both JC virus- and myelin-specific immune responses, where results of all peptide pools were added for a given patient.

Detection of antigen-specific interferon-γ-secreting T cells

To detect JC virus-, Epstein-Barr virus- or cytomegalovirus-specific activated T cells secreting interferon-γ, we used an enzyme linked immunospot (ELISPOT) assay, such as we have previously described. We did not look at myelin-specific interferon- γ secreting T cells since preliminary data have shown that these responses were very weak (S. Jilek and R.A. Du Pasquier, manuscript in preparation). Briefly, 200'000 PBMC were incubated in triplicates for 18 hours in the presence of JC virus peptide pools, Epstein-Barr virus or cytomegalovirus viral lysates or pools of Epstein-Barr virus or cytomegalovirus CD8+-restricted peptide epitopes. Peptide-free medium and PHA-L (5 mg/ml) served as negative and positive controls, respectively. Responses were expressed as net spot-forming cells (SFC) per 10⁶ PBMC. The assay was considered experimentally valid if the SFC, in the absence of peptide, was lower than 40 per 10⁶ cells. These background values were subtracted from the peptide-stimulated data before analysis. For JC virus-specific immune responses, data are presented as cumulative SFC/10⁶ celles where results of all peptides pools were added for a given patient.
Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA). The differences in the immune responses between the start of the study (onset of natalizumab or interferon beta treatment) and the various subsequent time points were analysed with the non-parametric paired Wilcoxon signed-rank test. Only patients having data for both M0 (baseline) and a subsequent given time point were used in the paired statistical calculation. Comparison of immune response frequency was assessed with 2-sided Fisher exact test. A p<0.05 was considered as significant.

Role of funding source

There were different sponsors for this study and none of them had a role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.
RESULTS

In the natalizumab arm of the study, six patients dropped out before the study end-point because of: secondary effects related to natalizumab treatment (2), accidental death (1), or loss of follow-up (3). Data for these patients that were gathered prior to drop-outs were included in the analysis of JC viral load as well as immune response data since all these six patients had baseline data, but dropped at different time points: one patient dropped after M0, one after M1, two after M3 and two after M9. In the interferon beta arm, two patients were lost to follow-up, meaning that no data could be gathered for the second time point, i.e. one year after treatment onset. In the natalizumab group, five patients developed seven relapses but there was no sustained progression of disability as measured using the Expanded Disability Status Scale (EDSS). Similarly, seven interferon beta-treated patients developed 15 relapses throughout the study, however there was no sustained progression of disability as shown by stable EDSS.

To examine whether there was a reactivation of JC virus in natalizumab-treated patients, we analysed the JC viral load in the PBMC, plasma and urine of natalizumab patients using qRT-PCR (Table 2). As a control, JC viral load was analysed in PBMC and plasma of interferon beta-treated patients. JC virus DNA was not detected in any PBMC or plasma sample of natalizumab- or interferon beta-treated patients throughout the entire study duration. Of note, plasma from the 16 patients who reached the M18 time point were analysed twice, once by us and once by an independent and accredited laboratory in our institution, who confirmed absence of JC virus in all, but one sample. In the latter sample, there was barely detectable JC virus DNA, however, since it was under the quantitative limit of detection of this laboratory (1000 copies/ml) and only slightly above the threshold of detection (approximately 100 copies/ml), this JCV DNA could not be reliably quantified. By contrast, JC virus DNA was
present in the urines of six natalizumab patients. In two patients JC virus was detectable at baseline and throughout the study (#05, 10), whereas for two patients (# 15, 18) urines were collected for the first time at M3 and M1, respectively and JC virus DNA was always detectable thereafter. In a fifth patient JC virus became detectable 12 months after natalizumab onset (#06), and in the last patient, there was a transient JC virus reactivation at three and six months after natalizumab onset (#20).

To assess for the presence of anti-JC virus antibodies in the plasma of the natalizumab- and interferon beta-treated patients, we performed JC virus-specific EIA at different time points (natalizumab: baseline (M0), month nine (M9) and month 12 (M12); interferon beta: M0 and M10). We found evidence of JC virus-specific IgG antibodies in 12/24 (50%) natalizumab patients and 6/14 (43%, p=0.75, Fisher’s exact test) interferon beta patients (Table 1). Presence or absence of antibodies was constant in all patients throughout the study. There was no difference among those who were seropositive [natalizumab patients: 0.21 (0.12-0.56), 0.26 (0.13-0.60) and 0.29 (0.12-0.52) at M0, M9 and M12, respectively; interferon beta patients: 0.24 (0.22-0.25) and 0.17 (0.14-0.19) at M0 and M10, respectively; median (interquartile range)]. These data rule out that JC virus primary infection occurred during the study. The rate of JC virus seropositivity in our study is consistent with recent serological studies of immunocompetent individuals.5, 6 One of six patients with JC viruria was found to have antibody titers below the cut-off defining seropositivity in healthy adults, suggesting that there may be an impaired humoral JC virus-specific immune response in this natalizumab treated patient.

Since JC virus-specific cellular immune response has been shown to play an important role in keeping JC virus on check,29 we assessed the JC virus-specific memory as well as effector T cell responses. Isolated PBMC from peripheral blood were used both in proliferation assays
for the assessment of antigen-specific memory T cells and in ELISPOT assay for the
evaluation of antigen-specific effector T cells. Twenty out of 24 (83%) natalizumab patients
and 10/16 (63%) interferon beta patients had detectable JC virus-specific cellular immune
responses as assessed by proliferation assays (p=0·16, Fisher’s exact test; Table 1). As
compared to M0, we found a significant increase in the JC virus-specific T cell proliferation
in natalizumab patients already one month after the first injection (M1, p=0·031, paired
Wilcoxon signed-rank test; Figure 1). Then, the proliferation of JC virus-specific T cells
decreased at M3 and M6, and increased again significantly at M9 and M12 (p=0·015 and
p=0·019, respectively; Figure 1). At M18, the JC virus-specific T cell responses were not
longer different from M0. Myelin-specific responses were either stable (MOG) or showed a
transient increase at M1 and M12 after natalizumab onset (MOBP) (p=0·023 and p=0·011,
respectively; Figure 1). MOG, and in particular its N-terminal portion, is known to elicit a
strong cellular immune response. Indeed, at baseline the MOG-specific cellular immune
responses were higher than the ones against MOBP. However, there was no further significant
increase in the MOG-specific T cell responses. Epstein-Barr virus or cytomegalovirus-specific
T cell proliferations were similar at all time points, except for Epstein-Barr virus-specific
CD8+ T cell proliferation which was enhanced at M1 (p=0·038; Figure 1) and
cytomegalovirus-specific CD4+ T cells which tend to be increased at M12 (p=0·052; Figure
1). Of note, we observed no increase in the T cell proliferation against any viral or myelin
antigens in the interferon beta group at M10 (Figure 2).

In order to determine to which extent the JC virus-specific T cells are activated in
natalizumab-treated patients, we analysed the secretion of IFN-γ by effector T cells with an
ELISPOT. We found that, as compared to M0, there was an increased frequency of JC virus-
specific T cells secreting IFN-γ at M12 (p=0·033; Figure 3). Interestingly such an increase
was also present against Epstein-Barr virus and was mediated by CD4+ and CD8+ T cells
(p=0.036 and p=0.021, respectively; Figure 3). Furthermore, an increased CMV-specific CD8+ effector T cell response was seen at M6 (p=0.0156; Figure 3). Again, no difference was seen in the group of patients with interferon beta treatment for JC virus-, Epstein-Barr virus- or cytomegalovirus-specific IFN-γ secretion, except for a significant decrease in the frequency of Epstein-Barr virus-specific CD4+ T cells secreting IFN-γ at M10 (p=0.004; Figure 4). The latter result stands in marked contrast with the significant increase of Epstein-Barr virus-specific CD4+ T cell response at the same time point in natalizumab-treated patients.
DISCUSSION:

In this prospective study of multiple sclerosis patients first time treated with natalizumab, we found no evidence of JC virus reactivation or primary infection as reflected by undetectable JC virus DNA in the blood (plasma or PBMC), stable levels of JC virus DNA in the urine of the 25% of patients who excreted it, and a constant rate of JC virus-specific antibodies in the blood. However, as compared to the pre-treatment values, we found significantly increased JC virus-, Epstein-Barr virus- and cytomegalovirus-specific memory and/or effector T cell responses up to 12 months after starting natalizumab. At the same time points, memory T cells specific for the myelin protein, MOBP were also increased. By contrast, there was no increase of any viral- or myelin proteins-specific T cell responses in the interferon beta-treated group, indicating that the responses observed in the natalizumab cohort were specific to this drug.

The lack of detection of JC virus DNA in the PBMC or plasma is in accordance with data gathered from large cross-sectional studies where authors did not find JC virus reactivation in natalizumab-treated patients up to 24 months of treatment.31-34 Similarly, our rate of 25% of JC virus DNA detection in the urine is consistent with values reported in healthy individuals.5,12 Contrasting with our data, authors of another recently published longitudinal study on natalizumab patients with a design relatively similar to ours frequently found JC virus DNA in different compartments. In urine, 12/19 (63%) patients had detectable JC virus at 12 months, a rate which declined to 6/12 (50%) at 18 months. In plasma, 1/19 (5%) and 3/15 (20%) were detected at 12 and 18 months, respectively, whereas in PBMC 1/19 (5%) and 9/15 (60%) were found at these time points, respectively.35 We cannot rule out that differences in the sensitivity of the qRT-PCR assays may partially explain these discrepancies: Chen et al. mention a sensitivity of 25 copies per millilitre for urine and
plasma. Such low copy numbers are technically challenging as they must be based on a reproducible, quantitative and at least 20-fold more efficient extraction as compared to standard tests such as ours with a cut off of 500 copies per millilitre. However, such considerations do not seem to apply to the more critical PBMC results as the limit of detection of JC virus DNA in the PBMC was 10 copies per microgram of cellular DNA in both studies. Additional studies are warranted to sort out these issues.

Contrasting with the absence of virological or humoral changes, we found enhanced JC virus-specific memory and/or effector T cell responses already at one month and then, at nine and 12 months after the introduction of natalizumab. Our results differ from the observations of Chen et al. who reported a relative decrease in the JC virus-specific T cell responses at month 12 as compared to month six after starting natalizumab therapy. This discrepancy could be due in part to the fact that we compared time points on natalizumab to pre-treatment values (M0), whereas Chen et al. had no data on the pre-treatment JC virus-specific T cell responses. In addition, these authors performed ELISPOT with in vitro expanded memory T cells, a procedure which may have introduced a not yet assessed bias and variability to the T cell repertoire. One has to acknowledge that the precursor frequency of JC virus-specific T cells being low, the ex vivo detection of these cells can be difficult, either by tetramer staining or by ELISPOT assays. Nevertheless, by pooling the T cell responses of the overlapping peptides of VP1, and by assessing these responses at different time points for each study patient, we were in a position to amplify potential differences in the JC virus-specific T cell reactivity.

The absence of detection of JC virus DNA by qRT-PCR does not exclude a transient JC virus reactivation between blood sampling that would have triggered the JC virus-specific T cell response. Indeed, the two peaks of JC virus-specific T cell responses at one month and
nine/twelve months may suggest a periodic reactivation of JC virus. However, this hypothesis seems unlikely as we checked frequently for a putative surge of JCV DNA by qRT-PCR in different compartments: every one to three months up to 12 months and, every six months from 12 to 18 months. Our data rather suggest that natalizumab treatment traps antigen-specific memory and effector T cells in peripheral blood. Indeed, the fact that not only JC virus- but also Epstein-Barr virus-, cytomegalovirus or MOBP-specific immune responses were increased in natalizumab-treated patients, usually at the same time points as was seen with JC virus, indicates that the enhanced cellular immune response is not restricted to JC virus. These results are in accordance with two recent studies demonstrating that natalizumab increases the percentage of activated T cells producing proinflammatory cytokines in the peripheral blood, even if theses authors did not look at antigen-specific cells.\textsuperscript{20, 36} Others have found increased Human Herpes virus-6-specific humoral immune response in the serum of natalizumab-treated patients.\textsuperscript{37} This immune hyperactivation may be explained by the fact that natalizumab may interfere with the immunological synapse\textsuperscript{38} and alter the profile of the immune response, such as shown by microarrays experiments.\textsuperscript{39} Finally, the observation of an increased antigen-specific T cell response may be directly related to the mode of action of natalizumab: indeed, by blocking $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, natalizumab prevents activated T lymphocytes from entering into the CNS or the gut, which may result in a sequestration of these activated lymphocytes in the peripheral blood.\textsuperscript{20, 36} Interestingly, a study has shown that there was a periodical increase in the expression of genes related to the immune response in natalizumab-treated patients, which was particularly evident at one and twelve months\textsuperscript{39}. Together with our findings, these data suggest that natalizumab may have a direct effect on the activity of T cells, independently of their antigen-specificity. By contrast, the viral- or myelin-specific T cell responses were unchanged on interferon beta. There was even a decline in Epstein-Barr virus-specific CD4+ T cells as compared to pre-treatment values. Indeed, among other mechanisms, interferon beta modulates the immune responses by suppressing the
generation of pro-inflammatory cytokines, such as IFN-$\gamma$, IL-12 and TNF-$\alpha$, by preventing MHC class II expression on antigen presenting cells, and by downmodulating the expression of co-stimulatory molecules at the surface of the cells.\textsuperscript{40}

The present study provides new insights on the biological activity of natalizumab. However, since none of our patients developed PML, we cannot claim that we have identified a biomarker for patients at risk of PML. In fact, it was previously shown that an increased JC virus-specific T cell response was a good prognostic factor in patients who were suffering from PML.\textsuperscript{29, 41} By analogy, we can venture the hypothesis that natalizumab-treated patients who harbour an enhanced JC virus-specific T cell response may be at lesser risk of developing PML. By contrast, it is the rare and unfortunate combination of JC virus reactivation and absence of JC virus-specific T cell immune responses that could favour the onset of PML in some natalizumab-treated patients.
Contributors:
SJ, RADM conceived and designed the experiments. SJ, EJ, HHH, AL, MC, LG performed the experiments. SJ, EJ, GP, RADM analysed the data. HHH, MS, GP, RADM contributed to reagents/materials/analysis tools. SJ, HHH, RADM wrote the paper.

Conflict of interest statement:
MS has served as a consultant for Merck-Serono, has received honoraria, payment for development of educational presentations and travel support from Merck-Serono, Biogen Dompé, Novartis, Sanofi-Aventis and Bayer Schering. RDP has received unrestricted grant from Biogen Dompé and Bayer Schering, served as expert for Biogen Dompé, Novartis and Merck-Serono, has received honoraria and payment for development of educational presentations from Biogen Dompé, Novartis and Merck-Serono and has received travel support from Merck-Serono, Biogen Dompé, Bayer Schering. SJ, EJ, AL, MC, LG, HHH and GP declared no conflicts of interest.

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REFERENCES


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Figure 1

Increase of viral and myelin-specific memory T cells in natalizumab-treated patients

T cell proliferative responses against JC virus VP1 protein, myelin proteins (MOG and MOBP), Epstein-Barr virus and cytomegalovirus in natalizumab-treated patients were examined. Data obtained at baseline (M0) and one (M1), three (M3), six (M6), nine (M9), 12 (M12) and 18 (M18) months after treatment initiation are displayed. For JC virus VP1, MOG and MOBP, data are presented as cumulative SI, which means that the results of the pools of overlapping peptides encompassing the amino acid sequence of a protein were cumulated at any given time point for each patient. Bottom graph: Data for JC virus VP1-specific T cell responses are shown for each individual patient throughout the whole study. Box and whiskers plots show median, 25th and 75th percentile, and range. For each time point, only patients with paired data were included in the figure. *, p<0·05; JCV, JC virus VP1; MOG, myelin oligodendrocyte glycoprotein; MOBP, myelin oligodendrocyte basic protein; EBV, Epstein-Barr virus; CMV, cytomegalovirus; SI, stimulation index.

Figure 2

Viral and myelin-specific memory T cells are stable in interferon beta-treated patients

T cell proliferative responses against JC virus VP1 protein, myelin proteins (MOG and MOBP), Epstein-Barr virus and cytomegalovirus in interferon beta-treated patients were examined. Data obtained at baseline (M0; -3 (-6 to -2) months) and 10 (7-12) months (M10) after treatment initiation are shown. For JC virus VP1, MOG and MOBP, data are presented as cumulative SI as explained in legend of Figure 1. Box and whiskers plots show median, 25th and 75th percentile, and range. For each time point, only patients with paired data were included in the figure. *, p<0·05; IFN beta, interferon beta; JCV, JC virus VP1; MOG, myelin oligodendrocyte glycoprotein; MOBP, myelin oligodendrocyte basic protein; EBV, Epstein-Barr virus; CMV, cytomegalovirus; SI, stimulation index.
Figure 3

**Increase of viral-specific effector T cells in natalizumab-treated patients**

T cell effector responses were determined using ELISPOT assays. Cellular immune responses, IFN-γ-mediated, against JC virus VP1 protein, Epstein-Barr virus and cytomegalovirus were examined in natalizumab-treated patients. Time points of analyses were the same as for Figure 1. JC virus VP1 results are presented as cumulative spot-forming cells (SFC)/10^6 cells, which means that the results of the pools of overlapping peptides encompassing the amino acid sequence of VP1 were cumulated at any given time point for each patient. Bottom graph: Data for JC virus VP1-specific T cell responses are shown for each individual patient throughout the whole study. Box and whiskers plots show median, 25th and 75th percentile, and range. For each time point, only patients with paired data were included in the figure. *, p<0.05; JCV, JC virus VP1; EBV, Epstein-Barr virus; CMV, cytomegalovirus; SFC, spot forming cells.

Figure 4

**Viral-specific effector T cells in interferon beta-treated patients**

T cell effector responses were determined using ELISPOT assays. Cellular immune responses, IFN-γ-mediated, against JC virus VP1 protein, Epstein-Barr virus and cytomegalovirus were examined in interferon beta-treated patients. Data obtained at baseline (M0; -3 (-6 to -2) months) and 10 (7-12) months (M10) after treatment initiation are shown (idem Figure 2). JC virus VP1 results are presented as cumulative spot-forming cells (SFC)/10^6 cells, such as explained in Figure 3. Box and whiskers plots show median, 25th and 75th percentile, and range. For each time point, only patients with paired data were included.
in the figure. **, p<0.01; IFN beta, interferon beta; JCV, JC virus VP1; EBV, Epstein-Barr virus; CMV, cytomegalovirus; SFC, spot forming cells.
Figure 1

Individual follow-up for JCV-specific T cell responses

months after natalizumab onset

months after natalizumab onset
Figure 2

- JCV
- MOG
- MOBP
- EBV CD4
- EBV CD8
- CMV CD4
- CMV CD8

months after IFN beta onset
Figure 3

Individual follow-up for JCV-specific T cell responses

months after natalizumab onset

months after natalizumab onset
Figure 4

- **JCV**
  - Cumulative SFC/10^6 cells
  - n=13

- **EBV CD4**
  - SFC/10^6 cells
  - n=10

- **EBV CD8**
  - SFC/10^6 cells
  - n=10

- **CMV CD4**
  - SFC/10^6 cells
  - n=5

- **CMV CD8**
  - SFC/10^6 cells
  - n=5

Months after IFN beta onset

n=13 n=10 **

n=10

n=5

n=5
Table 1
Clinical data of the 40 patients enrolled.

<table>
<thead>
<tr>
<th>Age at blood draw (years)</th>
<th>F:M ratio</th>
<th>Disease duration (years)</th>
<th>EDSS score</th>
<th>Total number of relapses before treatment onset</th>
<th>OCB</th>
<th>Treatment before NTZ onset</th>
<th>Patients in relapse at treatment onset</th>
<th>Drop-out</th>
<th>JCV humoral response</th>
<th>JCV cellular response</th>
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</thead>
<tbody>
<tr>
<td>natalizumab-treated patients (n=24)</td>
<td></td>
<td></td>
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<tr>
<td>39 ± 6</td>
<td>18:6</td>
<td>7.5 ± 7.7</td>
<td>3 ± 0.75</td>
<td>11 ± 4.5</td>
<td>16/19</td>
<td>n.d.</td>
<td>12 IFN beta</td>
<td>7</td>
<td>6</td>
<td>12/24</td>
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<td>interferon beta-treated patients (n=16)</td>
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<tr>
<td>37 ± 6</td>
<td>11:5</td>
<td>0.6 ± 5.3</td>
<td>2 ± 0.4</td>
<td>2 ± 1.5</td>
<td>15/16</td>
<td>naïve</td>
<td>5</td>
<td>2</td>
<td>6/14</td>
<td>10/16</td>
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</tbody>
</table>

1Numbers represent the median ± inter-quartile range
2n.d.: not determined
3natalizumab
4interferon beta
5glatiramer acetate
6The wash-out period between interferon beta / glatiramer acetate and natalizumab was 0.3 ± 0.3 months, whereas it was 7 ± 0.2 months between mitoxantrone and NTZ
7as determined by a stimulation index > 2.0 in proliferation assays
Table 2. Quantification of JCV DNA in natalizumab- and interferon beta-treated patients

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<th></th>
<th>M0</th>
<th>M1</th>
<th>M3</th>
<th>M6</th>
<th>M9</th>
<th>M12</th>
<th>M18</th>
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<td>0/13</td>
<td>0/19</td>
<td>0/20</td>
<td>0/17</td>
<td>0/20</td>
<td>0/18</td>
<td>0/16</td>
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<tr>
<td><strong>Plasma</strong></td>
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<td>0/20</td>
<td>0/21</td>
<td>0/18</td>
<td>0/20</td>
<td>0/18</td>
<td>0/16</td>
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<td>--</td>
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<table>
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<th>M0^3</th>
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</tr>
<tr>
<td><strong>Plasma</strong></td>
<td>0/14</td>
<td>0/14</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
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</tbody>
</table>

1 Number of JCV DNA copies per milliliter
2 3 ± 3 months before interferon beta treatment onset
3 10 ± 4 months after interferon beta treatment onset