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Research paper

Pharmacodynamic assessment of cell-bound natalizumab on PBMC samples stored in liquid nitrogen

Anja ten Brinke[a,c], Iris Claessen[a,c], Zoé L.E. van Kempen[b], Joep Killestein[b], Theo Rispens[a,c,*]

[a] Department of Immunopathology, Sanquin Research, Amsterdam, the Netherlands
[b] Department of Neurology, Amsterdam Neuroscience, VUmc MS Center Amsterdam, VU University Medical Center, Amsterdam, the Netherlands
[c] Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

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ABSTRACT

Natalizumab is a monoclonal IgG4 antibody used for treatment of relapsing remitting MS. Natalizumab interferes with lymphocyte migration by blocking alpha-4 integrin (CD49d). Saturation levels of alpha-4 integrin on circulating T cells by natalizumab have been associated with clinical effectiveness of therapy. However, in most cases, measurements have been carried out using freshly isolated PBMCs. The aim of this study was to set up and evaluate a method to measure relative levels of cell-bound natalizumab using frozen PBMC samples. A new method was set up to measure cell-bound natalizumab by flow cytometry on T cell subsets using fully saturated cells as a 100% reference. A comparison was made between spike samples and samples of natalizumab-treated MS patients freshly isolated and stored in liquid nitrogen. Cell-bound natalizumab could be measured (using an anti-IgG4 antibody) on cells stored in liquid nitrogen. Natalizumab was found to slowly dissociate from the cells during isolation and subsequent sample work-up. This dissociation was more pronounced for monovalent natalizumab resulting from Fab arm exchange (the predominant isoform in patients) than bivalent natalizumab straight from the vial. We established a correction factor to account for this phenomenon. The resulting method has good accuracy compared to assessing fresh cells. The inter-assay precision (%CV) is ca. 12% using frozen cells. In conclusion, we established a method to assess relative levels of cell-bound natalizumab on cells obtained from frozen PBMC samples.

1. Introduction

Natalizumab (Tysabri, Biogen Idec, Inc., and Elan Pharmaceuticals, Inc.) is an effective treatment in relapsing remitting multiple sclerosis (RRMS)(Polman et al., 2006). Natalizumab is a humanized recombinant antibody that binds alpha-4 integrin, also called CD49d antigen. Natalizumab selectively inhibits alpha-4 integrin-mediated adhesion of lymphocytes to endothelial receptors vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin-cell adhesion molecule 1 (MAdCAM-1). This decreases transmigration of lymphocytes across the blood-brain-barrier thereby suppressing inflammation in the central nervous system (CNS)(Yednock, 2006). While natalizumab is an effective treatment for RRMS, a serious adverse event is progressive multifocal leukoencephalopathy (PML), a potentially fatal John Cunningham virus (JCV)–related opportunistic infection of the CNS.(Bloomgren et al., 2012) In order to reduce the risk of PML, there have been studies in which the standard dosing regimen was adjusted to an extended interval (Bomprezzi and Pawate, 2014; Zhovtis Ryerson et al., 2016). These studies suggest that there might be a window of adjusted, lower dosing of natalizumab, where RRMS is sufficiently suppressed yet the risk of PML reduced.

A neglected aspect in these studies is the interpatient variation of circulating natalizumab concentrations, which can vary substantially (Rispens et al., 2011a; Vennegoor et al., 2013). Therefore, adjusting the dose based on individual pharmacokinetics might seem a more rational option. In order to support such an intervention strategy, it would be helpful to have detailed insight into the relationship between natalizumab pharmacokinetics and optimal blocking of the lymphocyte transmigration, for which levels of saturation of the CD49d antigen by natalizumab would be a reasonable proxy. Several studies have

Abbreviations: Relapsing remitting multiple sclerosis, (RRMS); Progressive multifocal leukoencephalopathy, (PML); John Cunningham virus, (JCV); Central nervous system, (CNS); Vascular cell adhesion molecule 1, (VCAM-1); Mucosal addressin-cell adhesion molecule 1, (MAdCAM-1); peripheral blood mononuclear cells, (PBMC)

* Corresponding author at: Department of Immunopathology, Sanquin Research, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands.
E-mail address: t.rispens@sanquin.nl (T. Rispens).

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demonstrated that saturation of the CD49d with natalizumab is correlated with the serum natalizumab concentration (Khatri et al., 2009; Harrer et al., 2017). These studies suggest that a concentration of natalizumab below ca. 1 μg/mL is associated with receptor saturation less than 50%. On the other hand, upon standard dosing regimen, after 12 months, the large majority of patients have levels of natalizumab exceeding 10 μg/mL at the time of the next infusion, which might result in receptor saturations reaching near 100% (Khatri et al., 2009; Vennegoor et al., 2013). Therefore, measurement of receptor saturation might provide valuable additional information to optimize natalizumab treatment.

So far, studies that measured the binding of natalizumab to circulating lymphocytes used freshly obtained PBMCs (Khatri et al., 2009; Sehr et al., 2016; Derfuss et al., 2017; Harrer et al., 2017; Punet-Ortiz et al., 2018; Foley et al., 2019). While this undoubtedly is a preferred option in terms of obtaining the most accurate results, is also forms a major hurdle in carrying out studies addressing the correlation of receptor saturation levels to clinical parameters and pharmacokinetics. In this study, we investigated the possibilities to determine levels of natalizumab binding to lymphocytes/receptor saturation using PBMCs that were stored frozen.

2. Materials and methods

2.1. Healthy volunteers and patients

Blood samples from anonymized healthy volunteers were drawn upon written informed consent. Furthermore, blood was taken from RRMS patients treated with natalizumab. Patients were recruited at the at the MS center of the Amsterdam UMC. All patients had a current diagnosis of RRMS according to the applicable panel criteria (Thompson et al., 2018), were over eighteen years of age and were currently treated with natalizumab with a minimum of six consecutive infusions. Patients participated in one or two of the following trials; one study extending the standard 4-week treatment interval based on individual natalizumab trough concentrations and one study researching the wearing-off effect of natalizumab in relation to natalizumab concentration and receptor saturation. Both studies were approved by the local institutional board of the Amsterdam UMC (reference 2016–161 and reference 2017.373 respectively). All patients gave written informed consent. Blood samples were taken through the i.v. drip before natalizumab infusion.

2.2. PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from citrated blood samples (9 ml Vacuette tubes with 3.8% coagulation sodium citrate, Greiner bio-one) within four hours after blood collection by density gradient centrifugation with Lymphoprep (Axis- Shield, Norway) and were used directly or were cryopreserved in liquid nitrogen in IMDM (Lonza Biowhittaker) with 20% normal calf serum (Gibco) and 10% DMSO (J. T. Baker) until use. After thawing 0.5*10^6 PBMCs were washed with PBS prior to staining for flowcytometry.

2.3. Preparing exchanged natalizumab

In vivo exchange of the IgG4 half molecules with natalizumab was mimicked in vitro by incubation of 30 μg/ml Tysabri (Biogen Idec BV) with a 20-fold excess of recombinant IgG4 adalimumab and 0.5 mM glutathione (Sigma Aldrich), overnight at 37 °C as described before (Rispens et al., 2011a). After overnight incubation the exchange reaction was stopped by adding in 1.5 mM 2-iodacetamide (Merck).

2.4. Sample preparation

To mimic patient samples, citrated whole blood from healthy volunteers was spiked with varying concentrations of natalizumab for 2 h or 24 h prior to PBMC isolation.

2.5. Natalizumab saturation

To obtain a completely saturated reference sample, PBMCs were post saturated with saturating concentrations natalizumab (2 μg/ml) for 15 min at room temperature, followed by extensive washing with PBS to remove excess natalizumab.

2.6. Natalizumab serum concentrations

Natalizumab serum concentrations were measured using a cross-linking assay, in which specific polyclonal rabbit anti-natalizumab F (ab)2 fragments are used as capture reagent and a mouse antihuman IgG4 (anti-hIgG4) monoclonal antibody is used for detection as described elsewhere (Rispens et al., 2011a).

2.7. Flow cytometry

The expression of alpha-4 integrin subunit and natalizumab binding to alpha-4 integrin on PBMCs was analyzed either together or in separate samples by flow cytometry using CD49d-PE (clone 9F10 (BD Biosciences) and in-house APC fluorescent labelled anti-hIgG4 clone MH164.4 in a concentration of 2 μg/ml (Sanquin, The Netherlands). These stainings were combined with antibodies against CD3-PerCP clone SK7 (BD Biosciences), CD4-PECy7 clone SK3 (BD Biosciences), CD8-BV510 clone SK1 (Biolegend) and CD45RO-BV421 UCHL-1 (BD Biosciences) and CCR7-FITC clone 150,503 (BD Biosciences) to allow analysis of the expression on different T cell subsets (naïve (N): CD45RO-CCR7+; central memory (CM): CD45RO+ CCR7+; effector memory (EM): CD45RO+ CCR7-; effector (EFF): CD45- CCR7-). First samples were stained with near-IR dead cell stain (Thermo Fischer Scientific Inc.) for 20 min at room temperature in the dark to exclude dead cells in the analysis. Subsequently the cells were stained with the antibodies in PBS containing 0.5% BSA and 0.01% NaN3 (PBA) for 30 min at room temperature, samples were washed after staining with PBA, and either resuspended in PBA and measured directly, or fixated with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature, washed with PBA and kept overnight at 4 °C before measuring on a Canto FACS flow cytometer (BD Bioscience). Analysis was performed with Kaluza software (Beckman Coulter).

2.8. Calculation of % receptor saturation by natalizumab

To obtain a completely saturated reference sample, PBMCs were post saturated with saturating concentrations natalizumab (2 μg/ml) for 15 min at room temperature, followed by extensive washing with PBS to remove natalizumab excess and also analyzed by flow cytometry for maximum natalizumab levels by anti-hIgG4. Natalizumab binding was corrected for background signals (i.e., MFI from reference tube without anti-hIgG4) and was expressed as percentage relative to saturated condition for each sample. As indicated in the main text, in the final protocol we applied a correction factor of 1.4 to arrive at a saturation level more closely reflecting that of the in vivo saturation levels.

3. Results

3.1. Detection of CD49d and natalizumab on CD4 and CD8 T cell subsets

In order to develop an assay for saturation of alpha-4 integrin subunit (CD49d) by natalizumab, we studied the feasibility to determine both the natalizumab levels and CD49d levels at once. Natalizumab, a humanized IgG4 monoclonal antibody was detected with a directly labelled anti-hIgG4 monoclonal antibody, while CD49d was detected with clone 9F10, a clone which has been described not to

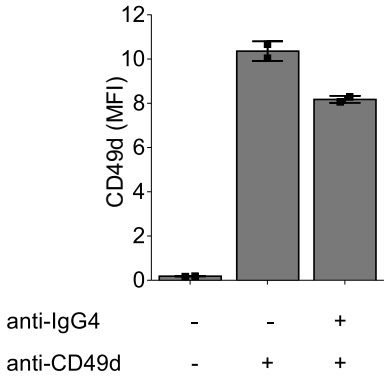

Fig. 1. Influence of anti-hIgG4 staining on CD49d detection. CD49d in the presence or absence of anti-hIgG4 on CD8 EM and EFF for PBMCs of healthy donors pre-incubated with natalizumab.

compete with natalizumab for CD49d binding (Defer et al., 2012). However when CD49d was detected simultaneously with the anti-hIgG4 staining for natalizumab levels the signal for CD49d was lower compared to staining for CD49d alone (Fig. 1), suggesting that the presence of anti-hIgG4 on natalizumab lead to steric hindrance for the CD49d antibody. Therefore we concluded it to be necessary to stain for natalizumab levels and CD49d levels in separate tubes.

Next, the CD49d expression was determined on the different CD4 and CD8 T cell subsets, e.g. naïve, effector, effector memory and central memory in healthy donors. We observed variable CD49d expression on these T cell subsets; expression was highest on CD8 effector and effector memory populations, which together represent around 40% of CD8 T cells (Fig. 2A,B). Furthermore, CD49d expression levels are lower in patients during treatment with natalizumab (Fig. 2C), in line with published results (Defer et al., 2012). Again, the expression of CD49d

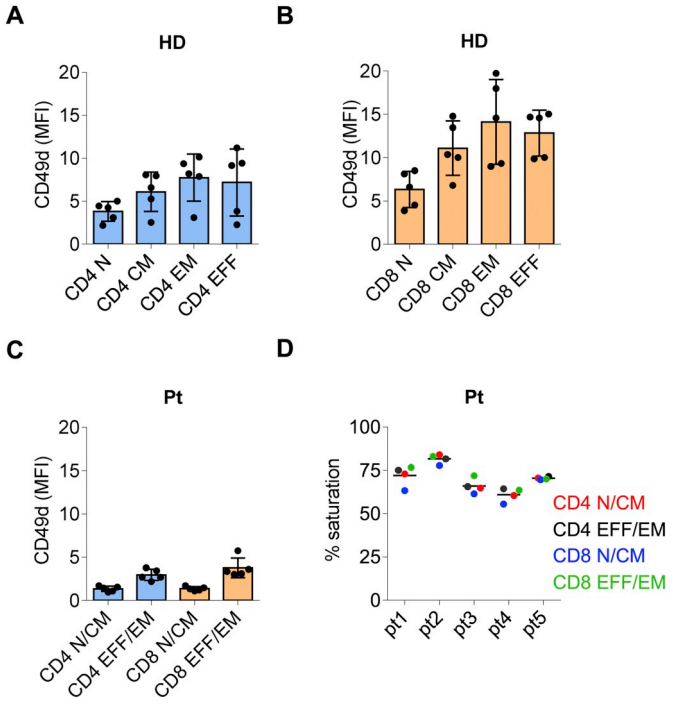

Fig. 2. CD49d highest on CD8 effector and effector memory cells. CD49d expression determined on T cell subsets from healthy donors (A,B) and natalizumab treated patients (C). $N = 5$, Mean +/− SD. D) % CD49d saturation by natalizumab calculated for different T cell subsets in natalizumab treated patients. Note: these data were generated using PBMC samples that have been frozen and thawed; using procedures described elsewhere in the text.

was highest on the combined CD8 effector/effector memory population.

3.2. Determination of degree of saturation of CD49d by natalizumab on T cell subsets

To calculate the percentage of CD49d molecules which are saturated/occupied with natalizumab, we have chosen an approach in which patient PBMCs are separated into a fraction that is directly analyzed for natalizumab/IgG4 binding, and another fraction that is incubated before analysis with saturating concentrations of natalizumab to obtain a 100% saturated reference. This approach has been used successfully by others (Khatri et al., 2009; Sehr et al., 2016; Harrer et al., 2017). As shown in Fig. 2D, the receptor saturation by natalizumab, expressed as percentage, is similar across the different T cell subsets and therefore does not depend on the differences in receptor densities. Therefore, because absolute signals are highest for CD8 effector/effector memory populations due to the higher CD49d expression (see above), we restricted subsequent analyses to these subsets in order to maximize precision.

3.3. Natalizumab binding after fab arm exchange

We anticipated a potential partial dissociation of natalizumab from cells during the isolation and subsequent sample work-up procedure. In particular, because natalizumab is an IgG4 antibody without a 'stabilized' hinge, it can engage in a process called Fab arm exchange (van der Neut Kolfschoten et al., 2007; Rispens et al., 2011b), which renders the antibody effectively monovalent (Fig. 3A), and results in weaker binding to cells. The dissociation was investigated by incubating isolated PBMCs with saturating amounts of natalizumab, and incubating for various times after washing the cells. Whereas for cells saturated with natalizumab straight from the vial hardly any dissociation was observed for up to six hours, a significant loss of signal was observed for natalizumab that underwent Fab arm exchange (the predominant isoform in patients) during this time period (Fig. 3B). The cumulative time for sample work-up is around 3 h. Therefore, we established a correction factor to account for this phenomenon for spiked samples from healthy donors, as well as patient samples (Fig. 3C). Both types of samples yield the same factor of 1.4, which was subsequently used to correct for this artefactual dissociation. Upon spiking various concentrations of natalizumab to PBMC samples of healthy donors, a dose-dependent signal was obtained (Fig. 3D). The decline in natalizumab signals was found to be fairly uniform across a wide range of natalizumab concentrations (Fig. 3D,E).

3.4. Fresh vs frozen

Next, we evaluated if it would be possible to obtain a reliable estimate of receptor saturation using frozen PBMC samples. For this purpose, we spiked natalizumab in blood samples of healthy donors and analyzed PBMCs isolated from these samples both directly after isolation as well as after freeze/thawing. We observed that values for receptor saturation were essentially the same for the freshly isolated vs freeze/thawed cells (Fig. 4A).

We also analyzed PBMC samples of patients treated with natalizumab directly after isolation and after freeze/thawing (Fig. 4B). Again, values obtained for receptor saturation were very similar for both conditions. We conclude that it is feasible to obtain a reliable estimate of natalizumab receptor saturation using frozen PBMC samples.

3.5. Assay performance

To obtain an idea about the reproducibility of the assay, we prepared a QC sample consisting of a sub-saturating amount of natalizumab spiked to PBMCs of a healthy donor, which was stored frozen as single-use aliquots. The average level of saturation for this sample was

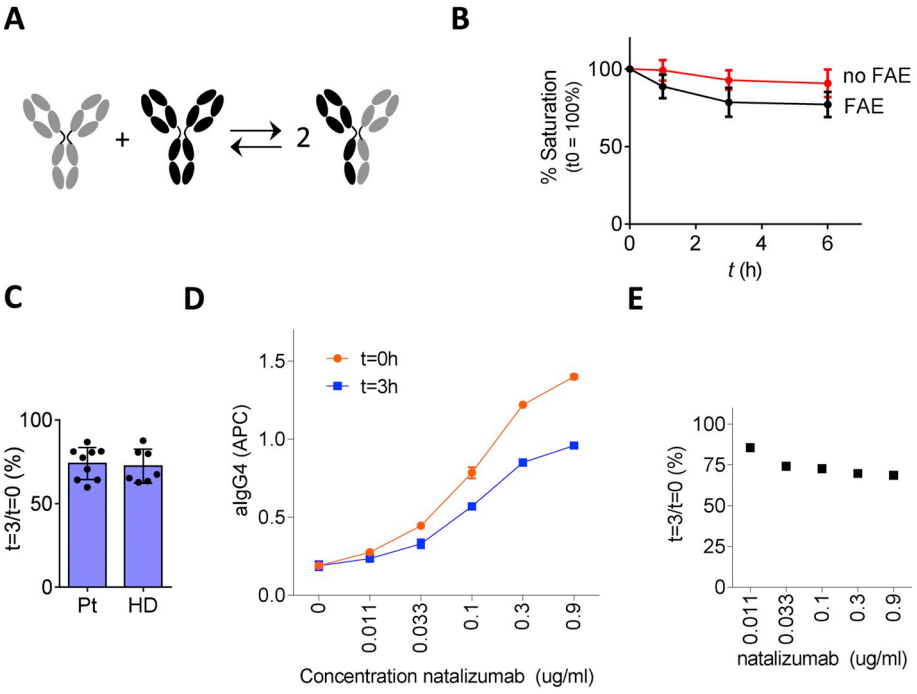

Fig. 3. Natalizumab dissociation. A) Fab arm exchange (FAE) of IgG4, resulting in effectively monovalent antibodies. B) Dissociation of natalizumab from PBMCs of healthy donors after saturating cells in time, $n = 5$. FAE: after Fab arm exchange with 15-fold excess of irrelevant IgG4; no FAE: straight from vial. C) Dissociation of natalizumab (FAE) for patient PBMCs incubated for 3 h after isolation ($n = 9$) or healthy donor PBMCs incubated for three hours as in B, $n = 7$. D) Comparison of dose-response curve of PBMCs incubated with subsaturating concentrations of natalizumab (FAE), representative example of $n = 3$. E) Data from D expressed as $t = 3/t = 0$.

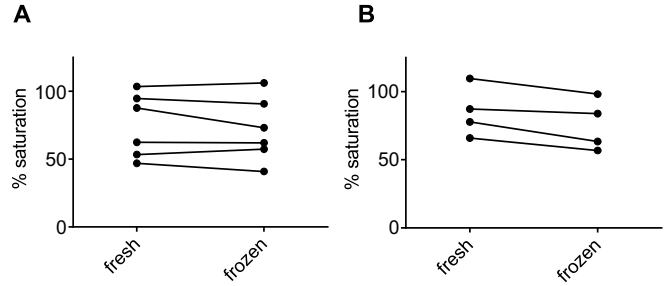

Fig. 4. Fresh vs Frozen. Natalizumab receptor saturation was compared for samples that were both analyzed directly after isolation and after freeze/thawing, for A) PBMC samples of healthy individuals spiked with natalizumab, and B) PBMC samples obtained from MS patients treated with natalizumab. Values calculated taking into account correction factor of 1.4 as explained in the text.

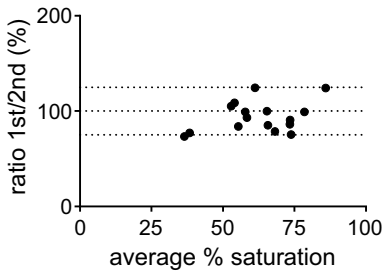

Fig. 5. Comparison of duplicate measurements of samples of patients treated with natalizumab that participated in an extended interval dosing study. The ratio of the first vs the second measurement is plotted (in %) vs the percentage saturation (average of both measurements). Data is compiled from multiple experiments. Dotted lines indicate 75 and 125%. Values calculated taking into account correction factor of 1.4 as explained in the text.

39.7%; the coefficient of variation (CV) for the inter-assay variation was 11.7% ($n = 19$).

For patient samples, an estimate of the overall CV was obtained from the average of the differences between duplicate runs for 16 different samples (levels of saturation ranging from 30.9–95.2%; Fig. 5): the average of the ratio highest vs lowest measurement per sample was 1.17, translating to ca. 12% CV.

We assessed receptor saturation in a patient with anti-natalizumab antibodies after infusion of natalizumab and observed a rapid decline in receptor saturation, which paralleled natalizumab serum concentrations (Fig. 6).

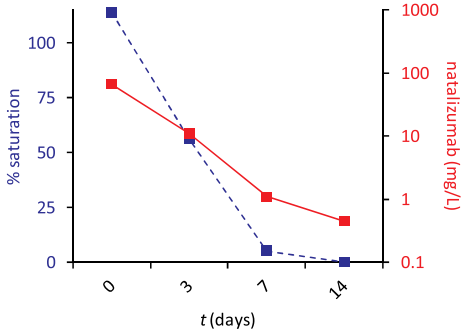

Fig. 6. Receptor saturation and natalizumab concentrations after an natalizumab infusion in a patient with anti-natalizumab antibodies. Receptor saturation values calculated taking into account correction factor of 1.4 as explained in the text. For comparison sake: in 14 patients without detectable anti-natalizumab antibodies, median natalizumab trough concentration was 24 mg/L (IQR 18.3–32.5); and median receptor saturation was 81% (IQR 77–85).

4. Discussion

In this paper we demonstrated the feasibility of obtaining good estimates of the degree of saturation of CD49d by natalizumab from frozen PBMC samples. It was found that the total work-up procedure of PBMC samples results in a partial loss of natalizumab by dissociation from cells. However, it proved possible to correct for this loss by means of a simple correction factor. Although evaluation of freshly isolated samples (especially whole blood samples, by-passing the PBMC isolation procedure altogether) will be more accurate, it is also much more difficult to achieve in practice. The possibility to store samples frozen for evaluation at a later time point makes cohort studies much more manageable and allows for retrospective analyses.

The dissociation of natalizumab was found to be related to the phenomenon of Fab arm exchange. It has been demonstrated that natalizumab participates in Fab arm exchange in vivo (Labrijn et al., 2009; Shapiro et al., 2011), making it relevant to incorporate Fab arm exchange into the assessment of any assay aimed to investigate ex vivo aspects of natalizumab in patients.

Whether or not other cell-bound molecules (e.g. vedolizumab) will also dissociate to similar degrees or not will depend on their individual characteristics such as affinity and avidity. Nevertheless, this paper demonstrates that even in case some dissociation takes place during elaborate cell handling, it may be possible to obtain a reasonable estimate of the original degree of binding.

We also established that levels of saturation were similar across different T cell subsets, which opened up the possibility to determine saturation levels using only T cell subsets with high CD49d expression. It is relevant to point out that although the % saturation was similar across the different T cell subsets, this translates into different *absolute* levels of remaining, unbound receptor molecules per cell. A similar percentage saturation may therefore in principle result in a different functional outcome for the different cell types. This could be of potential interest regarding the pathophysiology behind the decrease of PML risk found with extended interval natalizumab dosing (Ryerson and Chang, 2018). Former hypotheses state that a certain window of receptor saturation could result in a balance between immunosuppression to prevent MS inflammation and immunosurveillance to protect against PML. However, it might be more complicated than this as certain lymphocyte cell subsets could play a more vital role in JCV surveillance than others. Migration over the blood brain barrier could be dependent on absolute numbers of available receptors which means that certain subsets might be able to transport into the CNS as others will not despite a comparable percentage of receptor saturation. Further research regarding migration of different cell subsets in relation to unbound receptor molecules is needed to allow further understanding of PML protection in extended interval dosing of natalizumab.

In summary, we established a method to assess the relative levels of cell-bound natalizumab on cells obtained from frozen PBMC samples.

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Declaration of Competing Interest Statement

A. ten Brinke, I. Claessen, and Z.L.E. van Kempen report no disclosures. J. Killestein consulted for Novartis, Merck Serono, Bioven, Genzyme, Teva, and Roche; received speaker honoraria from Biogen, Novartis, Teva, Merck Serono, and Roche; and received research support from Schering AG, Biogen Idec, Merck Serono, Teva, Genzyme, Novartis, and Roche. T. Rispens received travel funding and/or speaker honoraria from Regeneron, Pfizer, and AbbVie, and received consulting fees and research support from Genmab.

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