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# Flow cytometry and drug-monitoring of natalizumab saturation of immune cells in multiple sclerosis[1)]

**Abstract**

**Background:** Natalizumab (Tysabri) is a blocking antibody specific to the α-4 integrin subunit and can be detected on the surface of immune cells by flow cytometry. We investigated if the determination of natalizumab saturation of immune cells has the potential to act as a biomarker for treatment effectiveness in multiple sclerosis (MS).

**Methods:** Natalizumab saturation of immune cells from 11 patients was measured before the start of treatment and after 4, 8, and 12 weeks of natalizumab therapy on T cells ($CD3^+$) and T cell subsets [naive ($CD45RA^+$/memory ($CD45R0^+$) $CD4^+$ and $CD8^+$] by flow cytometry.

**Results:** At weeks 4, 8, and 12 the average (n=9) natalizumab saturation of T cells approximated 80%. One patient with natalizumab neutralizing antibodies (NABs) and another patient with irregular infusion intervals were identified by abnormalities in the natalizumab saturation of cells. Different α-4 expression levels of T cell subpopulations were irrelevant to measuring cellular natalizumab saturation.

**Conclusions:** We showed that monitoring natalizumab saturation of T cells by flow cytometry is a useful and routine-qualified method to identify patients with a reduced treatment effect due to NABs or irregular infusion intervals. Further studies to determine a cut-off value for suboptimal natalizumab saturation of immune cells will also show the potential of this parameter concerning more individualized treatment schedules. Considering the risk of opportunistic infections it is very important to increase the safety of this highly effective MS therapy.

**Keywords:** biomarker; flow cytometry; multiple sclerosis; natalizumab; neutralizing antibodies.

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## Introduction

Natalizumab (Tysabri) is the first approved monoclonal antibody therapy for multiple sclerosis (MS). It directly binds to the α-4 (CD49d) subunit of very late activation antigen-4 (VLA-4; α-4/β-1; CD49dCD29) and thus blocks the transmigration of proinflammatory immune cells from the blood into the central nervous system [1, 2].

Natalizumab is generally very well tolerated and highly effective. It stabilizes disease activity by diminishing the relapse rate and the number and volume of new lesions on magnetic resonance imaging [3, 4]. However, there are some patients with persistent relapses, neutralizing antibodies to natalizumab (NABs), or both [5]. A rare but serious complication is progressive multifocal leukoencephalopathy (PML) caused by reactivation of the JC virus after 2 or more years of natalizumab therapy in most cases [6]. There is an urgent need for objective and routine-qualified biomarkers for treatment responsiveness and/or risk stratifications in JC virus positive patients after 2 years on natalizumab therapy.

As a blocking antibody, immune cell-bound natalizumab can be detected both indirectly and directly by flow cytometry, which is an interesting option for biomarker research. From various commercially available anti-human α-4 antibodies the epitope specificity of clone

[1)] Original German online version at: http://www.degruyter.com/view/j/labm.2012.36.issue-6/labmed-2012-0016/labmed-2012-0016.xml.

HP2/1, for example, largely overlaps with that of natalizumab. When using clone HP2/1 for the detection of α-4, cell-bound natalizumab can be detected indirectly by a pronounced reduction in the detectability of the α-4 integrin subunit on lymphocytes and monocytes [7, 8]. A direct detection of immune cell-bound natalizumab can be achieved by use of monoclonal fluorescence-labeled anti-human IgG4 antibodies because natalizumab is a humanized monoclonal antibody of the IgG4 subclass [9, 10].

Importantly, the often-quoted "blocking" of α-4 on immune cells is not the only effect of natalizumab. We and others have shown that α-4 partly disappears from the cell surface during natalizumab therapy [9, 11]. Accordingly, neither the direct nor the indirect detection of cell-bound natalizumab alone is suitable as a biomarker for conclusions about therapeutic effectiveness.

In this study, we optimized our flow cytometric method and investigated the natalizumab saturation of the α-4-integrin subunit of T cells and naïve and memory subpopulations in 11 patients during the first 3 months after initiation of natalizumab therapy. Analyzing the natalizumab saturation levels of lymphocytes from natalizumab-treated patients appears very promising as a biomarker candidate for treatment efficacy under the hypothesis that blocking all α-4-integrin subunits with natalizumab is associated with a maximum therapeutic effect.

# Materials and methods

## Patients

Eleven patients (8 women, 3 men; aged 29–58 years, median: 48), with clinically definitive relapsing-remitting MS according to the revised 2010 McDonald criteria [12], were included in this study. Patients were administered the standard dose of 300 mg natalizumab intravenously every 4 weeks. Routine checks for NABs were carried out by the specialist laboratory of the University Hospital of Neurology at the Medical School of Innsbruck on serum that was collected prior to the second and third natalizumab infusions [13]. For flow cytometric analyses, venous blood was collected at the start of therapy (before the first natalizumab infusion) and after 4, 8, and 12 weeks (each time directly before the next infusion). The study was approved by the local Ethics Committee (Ethics Committee for the Province of Salzburg 415-E774/6-2007) and all participants gave their written informed consent after full explanation of the purpose of the study.

## Sample processing

Venous blood was collected using Vacutainer® CPT™ tubes (Becton Dickinson AG, Basel, Switzerland). Peripheral blood mononuclear cells (PBMCs) were enriched by centrifugation at 1800×*g* for 20 min, washed, and diluted to a concentration of $1\times10^6$ cells/mL in a buffer solution (phosphate buffered saline, PBS, pH 7.2, 2.5% fetal calf serum, 0.1% sodium azide).

## Determination of natalizumab saturation

Saturation levels of lymphocytes with natalizumab were examined by flow cytometry as follows: $0.1\times10^6$ cells were incubated in vitro with saturating amounts of natalizumab (10 μg/mL, corresponding to 100% natalizumab saturation) or buffer only for 30 min on ice and unbound natalizumab was removed by repeated washing steps. PBMCs were stained with fluorescence-labeled monoclonal antibodies for 30 min on ice in the dark, washed, and fixed with 4% formaldehyde solution in PBS. Immune cell-bound natalizumab [anti-human IgG4 (α-huIgG4), clone HP6025, FITC] and the expression of the α-4-integrin subunit (CD49d, clone HP2/1, FITC) were examined on T cells (CD3, clone UCHT1, ECD) and naive (CD45RA, clone ALB11, PE) and memory (CD45R0, Clone UCHL1, ECD) $CD4^+$ (clone SFCI12T4D11, PC7) and $CD8^+$ (clone B9.11, PC5) T cell subpopulations using five-color flow cytometry (Cytomics FC500, Beckman Coulter, Vienna, Austria). The saturation level (in %) of PBMCs with natalizumab was calculated using the median fluorescence intensity (MFI) by the following equation: MFI anti-huIgG4 of buffer-treated cells/MFI anti-huIgG4 of natalizumab-saturated cells ×100.

## Statistics

Differences in the expression of α-4 between T cell subpopulations were analyzed using a two-sided unpaired Student t-test. A p-value $<0.05$ was considered statistically significant. Microsoft Excel was used for calculations (Microsoft Office 2007, Redmond, WA, USA).

# Results

## Optimization of the detection of cell-bound natalizumab

First, we determined the optimal concentration of anti-human IgG4-FITC (synonymous "anti-natalizumab") for

the detection of natalizumab on T cells ($CD3^+$) and T cell subpopulations (naive/memory $CD4^+$, naive/memory $CD8^+$) using natalizumab-naïve patient PBMCs collected before treatment was initiated. PBMCs were incubated in vitro with saturating amounts of natalizumab or buffer in order to achieve 100% or 0% natalizumab saturated α-4-integrin subunits on immune cells. Using 0.5 µg anti-natalizumab per $10^5$ PBMCs for staining of the in vitro saturated cells, the MFI for the detection of cell-bound natalizumab (Figure 1A, black circles) corresponded to that of direct detection of α-4 using anti-α-4 detection antibodies (Figure 1B, black diamonds). Moreover, Figure 1 also illustrates that indirect detection of α-4 via natalizumab and anti-natalizumab detection antibodies showed identical T cell subset-specific α-4 expression patterns (Figure 1A, black circles) – with higher α-4 levels on $CD8^+$ compared with $CD4^+$ T cells (p=0.001) and on memory compared with naive subsets ($CD4^+$, p=0.017; $CD8^+$, p<0.001) – than obtained for direct detection of α-4 using anti-α-4 detection antibodies (comparison of $CD4^+$/$CD8^+$, p<0.001; comparison of memory/naive: $CD4^+$, p=0.006; $CD8^+$, p<0.001) (Figure 1B, black diamonds). Untreated cells (white circles) stained with anti-natalizumab exhibited minor background values, with a mean MFI of 0.58 (±0.27) (Figure 1A, open circles).

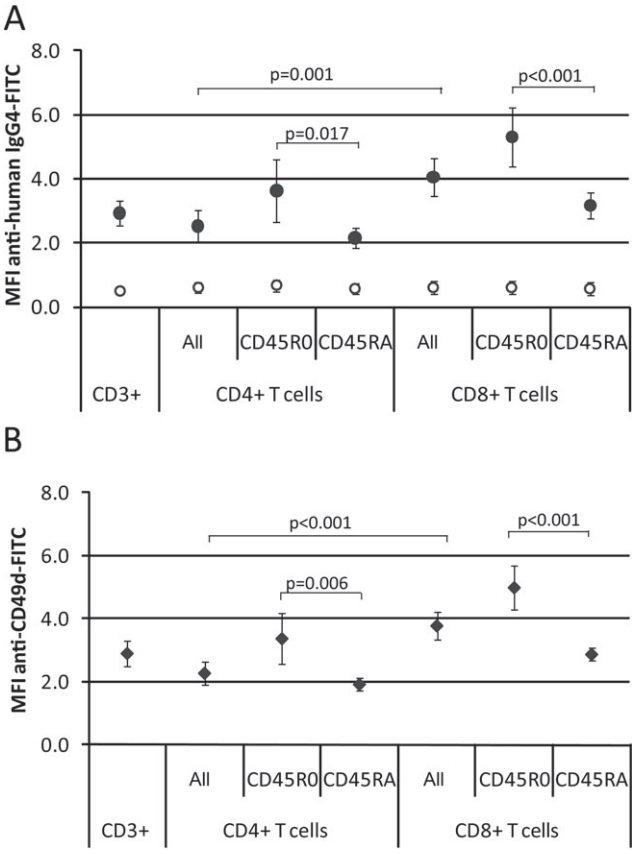


**Figure 1** Expression of α-4 and optimized detection of cell bound natalizumab. (A) Detection of cell bound natalizumab after in vitro incubation of PBMCs with saturating amounts of natalizumab (black circles) or buffer (white circles, negative control) using FITC-labeled monoclonal anti-human IgG4 antibodies on $CD3^+$ T cells and naive ($CD45RA^+$) and memory ($CD45R0^+$) $CD4^+$ and $CD8^+$ T cell subpopulations. (B) Detection of α-4 (C49d) expression (black diamond) on $CD3^+$ T cells and on naive ($CD45RA^+$), memory ($CD45R0^+$) $CD4^+$, and $CD8^+$ T cell subpopulations by FITC-labeled monoclonal anti-human α-4. Analyses were performed with PBMCs from natalizumab-naive patients before therapy was started. Bars indicate 95% confidence intervals.

## Natalizumab saturation of T cells during treatment

Subsequently, we investigated treatment-derived natalizumab saturation levels of the α-4 subunits on T cells from patients after 4, 8, and 12 weeks on therapy. Samples were collected directly before the next infusion after the specified 4-weekly intervals – with one single exception. In vitro saturation of natalizumab was performed with an aliquot of cells to assess the 100% natalizumab binding capacity of the cells and for calculating the percentage of treatment-derived T cell saturation levels.

Figure 2A shows a mean natalizumab saturation level of approximately 80% (n=9, black squares) on $CD3^+$ T cells 4 weeks after the first, second, and third infusion. In two patients, however, the percentage of the natalizumab saturation of their T cells deviated significantly. In one case (open ring) T cells were only 47% saturated after 4 weeks, and the saturation level even dropped to background levels in the subsequent two measurements. This patient had developed NABs within the first 4 weeks of treatment. A second patient (open diamond) showed strong deviations in the natalizumab saturation levels of his T cells, which could be interpreted as transient NABs. After an initial rise to normal natalizumab saturation levels after the first infusion, a sharp decline to background levels was observed after the second infusion, and recovery to 46.1% after the third infusion. In this patient, a too-long interval after infusions two and three of approximately 8 and 6 weeks caused the insufficient natalizumab saturation levels of T cells.

The different surface expression of α-4 between T cell subpopulations suggested a possible subset-specific sensitivity to natalizumab therapy. However, as depicted in Figure 2B, there were no significant differences in natalizumab saturation between $CD4^+$ (black line) and $CD8^+$ (gray line) T cell subpopulations and their corresponding

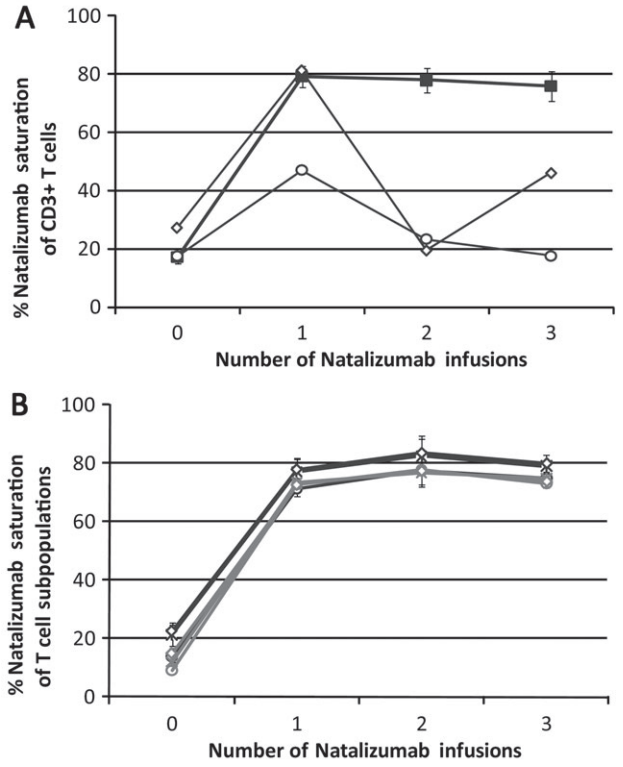


**Figure 2** Percent natalizumab saturation of T cells before and during natalizumab therapy. (A) Mean natalizumab saturation levels (n=9) of $CD3^+$ T cells before and after infusions 1, 2, and 3 (square marker icon). Differential course of natalizumab saturation levels in the case of natalizumab neutralizing antibodies (ring-shaped marker icon) and prolonged infusion intervals (diamond-shaped marker icon). (B) Mean natalizumab saturation levels (n=9) on $CD4^+$ (star-shaped marker icon, black) and $CD8^+$ (star-shaped marker icon, gray) T cells, and naive ($CD45RA^+$, diamond-shaped marker icon) and memory ($CD45R0^+$, ring-shaped marker icon) $CD4^+$ (black) and $CD8^+$ (gray) T cell subpopulations. Bars indicate 95% confidence intervals.

memory and naive subsets. Natalizumab saturation levels of 17.2% (standard deviation, SD±3.5) in $CD3^+$, 19.2% (SD±4.8) in $CD4^+$, and 12.8% (SD±2.9) in $CD8^+$ T cells at baseline (before the start of therapy) result from background MFI levels (mean 0.58, SD±0.3) of anti-human IgG4 antibody staining and are thus to be interpreted as background.

## Discussion

The possibility to detect natalizumab on the surface of immune cells makes investigating immune cell-bound natalizumab an interesting opportunity for biomarker research in MS. In previous studies, we have already established that monitoring of cell-bound natalizumab and/or unblocked α-4 is useful to observe changes in natalizumab surface levels of patient immune cells [8, 9, 14]. However, from these studies we also learnt that pre-treatment measurements were only of limited value as reference points for investigating changes in the course of treatment.

In the current study, we optimized our method and established the "percentage natalizumab saturation" of immune cells as a new parameter. We showed that the actual treatment effect could be estimated very well on the basis of natalizumab saturation of patient immune cells. T cells from 9 out of 11 patients were approximately 80% saturated in blood collected immediately before the subsequent infusion at the end of the 4-weekly interval. Natalizumab saturation levels of T cells from the remaining two patients strongly deviated during the 3-month observation period. One of these two patients had developed NABs and the other had irregular infusion intervals. With less than 50% of natalizumab saturation after the first infusion, we were able to demonstrate very early a potentially reduced therapeutic effectiveness due to NABs. Moreover, the further drop of natalizumab saturation levels to background levels after infusions two and three raised suspicion of high titer NABs, which was confirmed by serological testing. Meanwhile, natalizumab therapy was terminated in this patient because of high titer persistent NABs. The early and reliable detection of NABs by flow cytometry provides the treating neurologists with important information and allows for increased vigilance. This is of relevance because the disease can progress despite continued drug administration in the presence of NABs, and allergic reactions have also been reported in larger numbers [5]. According to the AFFIRM study, NABs occur in at least 9% of patients, two-thirds (6%) of whom suffer from high titer and persistent NABs.

Another potential advantage of our method is to identify patients with insufficient natalizumab saturation of lymphocytes caused by prolonged intervals between infusions, which also suggests the possibility of a patient-oriented adjustment of infusion intervals. Rispens et al. [15], for example, investigated natalizumab serum concentrations from patient blood collected after 4-weekly infusion intervals and reported individuals with exceptionally high natalizumab levels. Extremely high serum levels are probably associated with differences in the pharmacokinetic turnover of natalizumab between individual patients. This raises the question if the 4-weekly infusion interval probably over-satisfies the demand for natalizumab in such

patients. Monitoring natalizumab saturation in such individuals might provide an answer to this question.

α-4 is differently expressed on various immune cell populations [11, 16] and we hypothesized that investigating cellular natalizumab saturation might be more sensitive to higher subset-specific α-4 expression. Accordingly, we compared natalizumab saturation levels of high α-4 expressing memory $CD4^+$ and $CD8^+$ T cell subpopulations with low α-4 expressing naïve $CD4^+$ and $CD8^+$ T cells, but our assumption was not confirmed. We did not observe relevant differences in natalizumab saturation between T cell subsets and thus consider assaying $CD3^+$ T cells sufficient.

Analyzing natalizumab saturation levels of T cells by flow cytometry appears to be a useful and routine-qualified method to identify patients with a potentially inadequate therapeutic response due to NABs or irregular infusion intervals. Further studies about the therapeutic relevance of a full blockade of α-4 integrin subunits with regard to clinical effectiveness are needed. Moreover, establishment of a threshold and a lower limit for cellular natalizumab saturation levels might avoid unnecessary infusions and allow for a more patient-oriented treatment. Regarding the risk of opportunistic infections and in particular natalizumab-associated PML, it will be of major importance to further increase the safety of this highly effective therapeutic for the long-term treatment of MS.

### Conflict of interest statement

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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