Original Research Paper

The effect of disease modifying therapies on CD62L expression in multiple sclerosis

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Abstract

Background: The increasing armamentarium of disease-modifying therapies in multiple sclerosis is accompanied by potentially severe adverse effects. The cell-adhesion molecule CD62L, which facilitates leukocyte extravasation, has been proposed as a predictive marker for treatment tolerability. However, pre-analytical procedures might impact test results, thereby limiting its clinical usability. Whether the immediate analysis of CD62L expression of peripheral blood mononuclear cells can aid treatment decision making is yet unclear.

Objective: To investigate the effect of various disease-modifying therapies in multiple sclerosis on CD62L expression of $CD3^{+}CD4^{+}$ peripheral blood mononuclear cells in freshly collected blood samples.

Methods: We collected peripheral blood samples from patients with clinically isolated syndrome and multiple sclerosis (baseline/follow up $n = 234/n = 98$) and healthy controls ($n = 51$). $CD62L^{+}CD3^{+}CD4^{+}$ expression was analysed within 1 hour by fluorescence-activated cell sorting.

Results: $CD62L^{+}CD3^{+}CD4^{+}$ expression was significantly decreased in patients treated with natalizumab ($n = 26$) and fingolimod ($n = 20$) and increased with dimethyl-fumarate ($n = 15$) compared to patients receiving interferon/glatiramer acetate ($n = 90/30$) or no disease-modifying therapies ($n = 53$) and controls ($n = 51$) ($p < 0.001$). CD62L expression showed temporal stability during unchanged disease-modifying therapy usage, but increased after natalizumab withdrawal and decreased upon fingolimod introduction.

Conclusion: $CD62L^{+}CD3^{+}CD4^{+}$ expression is altered in patients treated with different disease-modifying therapies when measured in freshly collected samples. The clinical meaning of CD62L changes under disease-modifying therapies warrants further investigation.

Keywords: CD62L, lymphocytes, multiple sclerosis, clinically isolated syndrome, disease-modifying therapies, immunology

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Introduction

An increasing number of highly effective disease modifying therapies (DMTs) are available in multiple sclerosis (MS). Nevertheless, both treatment response and the occurrence of potentially severe side effects in individual patients remain highly unpredictable.[1] Therefore, a strong clinical need exists for body fluid markers that can aid in treatment decision making in terms of efficacy and tolerability.[1]

CD62L, or L-selectin, is a cell adhesion molecule expressed on all leukocytes, which facilitates lymphocyte extravasation and homing of naive T cells to peripheral lymphoid organs. The presence of CD62L ligands on oligodendrocytes and myelin in the central nervous system (CNS) might furthermore

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mediate leukocyte targeting to myelinated axons, e.g. in demyelinating diseases.[2] Recently, it was suggested that the expression of CD62L on $CD3^+CD4^+$ peripheral blood mononuclear cells (PBMCs) is decreased with the use of different DMTs in MS, especially natalizumab and fingolimod.[3–5] It was hypothesized that as a secondary result of this dysfunctional immune status, immune cells in these patients are not able to suppress viral activity as sufficiently, allowing viruses to induce disease. On this basis, CD62L expression could potentially serve as a risk marker for the development of viral infection under the use of DMTs.[3,6] Currently, the reduction of CD62L due to DMT usage has mainly been associated with the development of progressive multifocal leukoencephalopathy (PML), a severe John Cunningham (JC) virus-induced demyelinating disease.[3,4,6–9]

Notably, most studies towards CD62L in MS were performed on cryopreserved PBMCs, for which a pre-analytical procedure is required, which has been shown to impact the test results.[1,6,8,10] Two studies postulated that the CD62L expression is solely an indicator of immune cell stress and subsequent decreased cellular integrity caused by the freeze/thaw procedure.[6,10] The clinical applicability of CD62L as a treatment risk marker may be limited due to the influence of sample quality and the potential requirement of the pre-analytical steps, which may further differ among laboratories. Nevertheless, it is not yet clear if immediate measurement of CD62L may yield comparable results in prognosticating treatment tolerability, and how expression of this surface marker in freshly collected samples may be altered by various DMTs. Therefore, we here aimed to determine longitudinal CD62L expression of $CD3^+CD4^+$ PBMCs in freshly collected whole blood in MS patients using different types of DMTs, and compare it to healthy controls.

Patients, materials and methods

This study was approved by the ethics committee of the Medical University of Graz, Austria. All participants gave written informed consent.

Patients and controls

We included consecutive patients ($n = 234$) who were seen at the MS outpatient clinic of the Department of Neurology, Medical University of Graz, during regular clinical visits between March and December 2015, and met the following criteria: 1) diagnosis with clinically isolated syndrome (CIS) suggestive of MS, or definite MS, according to available criteria at time of inclusion;[11,12] 2) availability of one or multiple fresh full blood samples; and 3) availability of detailed clinical data.

Healthy controls ($n = 51$) consisted of volunteers who were seen at the 'Area health insurance of Graz' between April and October 2015, and met the following criteria: 1) legal age and maturity; 2) no diagnosis with neoplastic disease, acute or chronic infections, autoimmune diseases, acute or chronic diseases with organ damage, pregnancy, or severe anaemia (Hb < 9 mg/dl); and 3) availability of a freshly taken full blood sample.

Clinical assessment and follow up

Demographic and clinical data of patients were recorded at every visit by experienced neurologists. These included age, gender, age at disease onset, occurrence of relapses, degree of disability as determined by the Expanded Disability Status Scale (EDSS) score,[13] and medication.

Blood sampling and CD62L analyses

From each subject full blood samples, i.e. PBMCs, were collected in EDTA tubes. Leukocyte cell surface antigen analysis was performed by flow cytometry within 1 hour of sampling. Samples were transferred into fluorescence-activated cell sorting (FACS) tubes and incubated with antibodies (dilution per antibody 1:20 in sample) against CD3 (BD Pharmingen APC-H7 mouse anti-human CD3, cat. no. 341110, BD Biosciences), CD4 (BD Horizon V500 mouse anti-human CD4, cat. no. 560768, BD Biosciences) and CD62L (PE mouse anti-human CD62L, cat. no. 304806, BioLegend) for 30 minutes. Red blood cells were lysed with BD FACS Lysing Solution (cat. no. 349202, BD Biosciences). Upon incubation, samples were washed and pellets were resuspended in cell wash. All steps were performed at room temperature and protected from light. Readout of CD3, CD4 and CD62L was performed with the BD FACSCanto II system (BD Biosciences).

Statistics

Statistical analyses were performed using SPSS Statistics (version 23.0, IBM Corp. Armonk, New York, USA) and GraphPad Prism (version 5.00, GraphPad Software, San Diego, USA). Data were tested for normal distribution using the Shapiro-Wilk test. Group differences were determined by either chi-square test for categorical variables, or Mann-Whitney *U* test for continuous variables. Multiple comparisons were performed using Kruskal-Wallis test and post-hoc Dunn's multiple comparison test. Longitudinal

paired data were analysed using the Wilcoxon signed rank test. Correlations were determined by Spearman's rank-order correlation. Significance was set at 5% ($p < 0.05$).

Results

Cohort description

Demographic and clinical data of all subjects included are listed in Table 1. At time of the first analysis, 181/234 patients received long-term DMT: natalizumab ($n = 26$), fingolimod ($n = 20$), dimethyl fumarate ($n = 15$), interferon beta ($n = 90$) and glatiramer acetate ($n = 30$). Longitudinal blood samples were obtained in 98 patients (up to a total of seven samples per patient; total time interval between baseline and last sample collection median 113, interquartile range (IQR) 78–163 days), see Table 2 and Table 3 for data per subgroup.

For the entire patient group ($n = 234$), the clinical follow-up time was median 9.5, IQR 6.2–13.4 months upon baseline sampling.

Table 1. Demographic and clinical data of study subjects.

	CIS	MS	Controls	*p*-value
n Patients (% female)	48 (72.9)	186 (59.7)	51 (39.2)	0.002[a]
Age (years)	33.3 (26.7–44.1)	36.1 (30.5–45.3)	49.1 (34.1–60.9)	<0.001[b]
Age disease onset (years)	30.9 (25.1–40.2)	26.1 (21.0–31.4)	N/A	0.001[c]
Disease duration (years)	2.3 (1.2–4.8)	9.2 (5.0–15.3)	N/A	<0.001[c]
EDSS	1.0 (0.0–2.0)	1.8 (0.0–3.0)	N/A	0.005[c]
n DMT	40 (83.3)	141 (75.8)	N/A	n.s.[c]
DMT duration (years)	1.8 (0.4–4.1)	2.9 (1.0–6.7)	N/A	0.008[c]
Time since last relapse (years)	2.5 (1.3–4.9)	1.9 (0.5–5.8)	N/A	n.s.[c]
Time since last cortisone (years)	2.4 (1.2–5.0)	2.0 (0.5–4.9)	N/A	n.s.[c]
Time clinical follow-up (months)	8.0 (5.2–10.8)	10.2 (6.3–14.4)	N/A	0.022[c]
Time until last sampling ($n = 12/85$) (days)	117 (56–150)	113 (83–165)	N/A	n.s.[c]

CIS: clinically isolated syndrome; DMT: disease modifying therapy; EDSS: Expanded Disability Status Scale; MS: multiple sclerosis; *n*: number of subjects; N/A: not applicable; n.s.: not significant.
Unless otherwise described, data are given for time at the first sampling. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed by chi-square test[a], Kruskal-Wallis test[b], or Mann-Whitney *U* test[c].

Table 2. CD62L expression of study subjects per patient subgroup at baseline and follow up.

	CIS $n_{BL} = 48$ / $n_{FU} = 12$	MS $n_{BL} = 186$ / $n_{FU} = 79$	Controls $n_{BL} = 51$	*p*-value
$CD62L^+$ (% $CD4^+$) BL	84.6 (79.5–87.2)	84.5 (79.1–89.5)	84.1 (78.3–88.2)	n.s.[a]
Time BL-FU (days)	117.0 (56.0–150.0)	113.0 (77.0–165.0)	N/A	n.s.[b]
Merged longitudinal $CD62L^+$ (% $CD4^+$)	84.5 (80.5–89.5)	81.8 (75.1–86.9)	N/A	n.s.[b]
$CD62L^+$ (% $CD4^+$) last FU	85.4 (79.3–92.4)	82.2 (73.2–87.8)	N/A	n.s.[b]

BL: baseline; CIS: clinically isolated syndrome; FU: follow up; MS: multiple sclerosis; *n*: number of subjects; N/A: not applicable; n.s.: not significant.
FU time and longitudinal CD62L expression (merged and at last FU) are given for patients who did not change their DMT regarding natalizumab or fingolimod during FU. Merged longitudinal CD62L expression is the combined data of all longitudinal measurements. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed by Kruskal-Wallis test[a] or Mann-Whitney *U* test[b].

Table 3. CD62L expression of study subjects per DMT subgroup at baseline and follow up.

	NTZ $n_{BL}=26$ / $n_{FU}=24$	FTY $n_{BL}=20$ / $n_{FU}=13$	DMF $n_{BL}=15$ / $n_{FU}=5$	IFN/GA $n_{BL}=90/30$ / $n_{FU}=21/7$	No DMT $n_{BL}=53$ / $n_{FU}=21$	Controls $n_{BL}=51$	p-value	Differences
$CD62L^+$ (% $CD4^+$) BL	80.2 (72.7–82.5)*	54.7 (44.0–63.1)*	92.3 (89.7–97.7)**	85.6 (80.6–88.9)	85.9 (81.9–90.5)	84.1 (78.3–88.2)	<0.001[a]	NTZ*, FTY*, DMF**
Time BL-FU (days)	168.5 (130.0–176.5)	96.0 (86.0–113.0)	86.0 (85.0–118.0)	115.5 (80.5–162.0)	63.0 (39.0–120.0)	N/A	<0.001[a]	NTZ > No DMT NTZ > FTY
Merged longitudinal $CD62L^+$ (% $CD4^+$)	80.2 (74.5–83.1)	56.5 (46.5–66.3)	92.4 (90.4–97.3)	86.8 (84.1–90.2)	86.1 (80.5–90.7)	N/A	<0.001[a]	NTZ*, FTY* (incl. NTZ)
$CD62L^+$ (% $CD4^+$) last FU	77.6 (73.4–82.1)	52.1 (46.2–63.4)	94.2 (92.5–97.3)	86.2 (85.1–90.0)	85.2 (80.4–90.2)	N/A	<0.001[a]	FTY < DMF / IFN/GA / no DMT NTZ < DMF / IFN/GA

BL: baseline; DMT: disease modifying therapy; DMF: dimethyl fumarate; FTY: fingolimod; FU: follow up; IFN/GA: interferon beta/glatiramer acetate; n: number of subjects; N/A: not applicable; NTZ: natalizumab.
FU time and longitudinal CD62L expression (merged and at last FU) are given for patients who did not change their DMT regarding NTZ or FTY during FU. Merged longitudinal CD62L expression is the combined data of all longitudinal measurements. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed by Kruskal-Wallis test[a] with Dunn's post-hoc test.
*Patients had significantly decreased CD62L expression compared to all other subgroups (except other * group).
**Patients had significantly increased CD62L expression compared to all other subgroups.

Group comparisons

Cross-sectional measurements of CD62L expression of $CD3^+CD4^+$ PBMCs at baseline were comparable between CIS, MS and controls (Table 2). Comparison of subgroups of patients receiving different types of DMT at the time of the first measurement showed that CD62L expression was significantly decreased in natalizumab- (median 80.2, IQR 72.7–82.5% $CD4^+$) and fingolimod-treated patients (median 54.7, IQR 44.0–63.1% $CD4^+$) compared to all other patient subgroups and controls. CD62L expression was significantly increased with dimethyl fumarate (median 92.3, IQR 89.7–97.7% $CD4^+$) compared to the use of other DMTs (interferon beta/glatiramer acetate, $n = 90/30$; median 85.6, IQR 80.6–88.9% $CD4^+$; no difference in CD62L expression was found between both DMTs), no therapy ($n = 53$; median 85.9, IQR 81.9–90.5% $CD4^+$) and controls (median 84.1, IQR 78.3–88.2% $CD4^+$) (multi-comparison model $p < 0.001$) (Figure 1a).

Longitudinal CD62L expression

CD62L expression showed no significant temporal dynamics in longitudinal samples considering all patients, and in patients without switching DMT

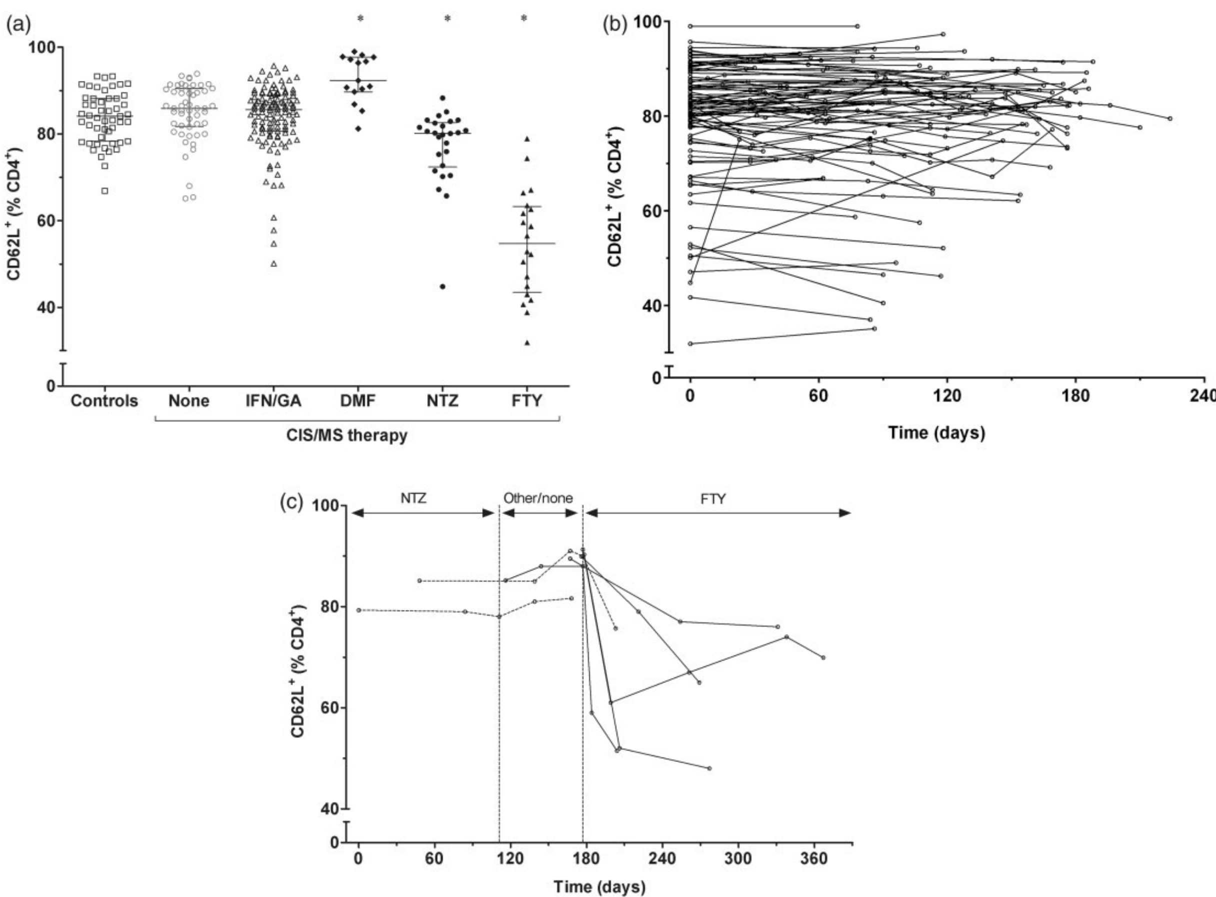

Figure 1. CD62L expression of $CD3^+CD4^+$ PBMCs in CIS/MS patients receiving different types of DMTs and healthy controls. (a) CD62L expression was significantly decreased with the use of natalizumab ($n = 26$) and fingolimod ($n = 20$), and significantly increased with the use of dimethyl fumarate ($n = 15$) compared to all other DMT subgroups and healthy controls (multi-comparison model $p < 0.001$). The data shown refer to baseline measurements. (b) Longitudinal measurements showed no overall alteration of CD62L expression in patients with stable DMT usage ($n = 91$; time interval median 113, IQR 77–163 days). (c) Effects of natalizumab and fingolimod on CD62L expression were visible in longitudinal PBMC samples of patients upon natalizumab withdrawal ($n = 2$, dotted lines; natalizumab treatment until 5.9 and 6.8 years) or after fingolimod initiation ($n = 6$, other/none DMT-treated before). In one patient, CD62L expression was measured when switching from natalizumab to fingolimod with a 2-month interim period.

CIS: clinically isolated syndrome; DMF: dimethyl fumarate; DMTs: disease-modifying therapies; FTY: fingolimod; IFN/GA: interferon beta/glatiramer acetate; IQR: interquartile range; MS: multiple sclerosis; NTZ: natalizumab; PBMCs: peripheral blood mononuclear cells.

Significance ($p < 0.05$) was analysed by Kruskal-Wallis test with Dunn's post-hoc test, or Wilcoxon signed-rank test.

during the sampling interval (Table 2 and 3, Figure 1b). Effects of DMT on PBMC CD62L expression were visible in longitudinal samples of patients who did switch from natalizumab to fingolimod therapy (Figure 1c). CD62L expression was increased upon natalizumab withdrawal in two patients (natalizumab treated until 5.9 and 6.8 years, respectively). A rapid and considerable decrease in CD62L expression was visible in six patients upon fingolimod initiation.

Associations with demographic and clinical data

No gender or age-related effects on CD62L expression were seen. Spearman correlations were not significant for baseline CD62L expression with age (CIS: $r = -0.060$, $p = 0.684$; MS: $r = 0.069$, $p = 0.348$; controls: $r = -0.243$, $p = 0.085$), age at disease onset (CIS: $r = -0.032$, $p = 0.831$; MS: $r = -0.054$, $p = 0.461$), or disease duration (CIS: $r = -0.122$, $p = 0.409$; MS: $r = 0.023$, $p = 0.754$). Mann-Whitney *U* test showed that CD62L expression was equal between males and females for CIS ($p = 0.602$), MS ($p = 0.198$) and controls ($p = 0.263$). Moreover, Kruskal-Wallis test showed that CIS, MS and control subjects had equal CD62L expression in both males ($p = 0.830$) and females ($p = 0.620$).

We also did not find any significant correlations of CD62L expression with other clinical data, i.e. therapy duration, EDSS at time of sampling and during follow up, and the annualised relapse rate.

None of the patients included developed PML during the study follow-up time. Considering only natalizumab-treated patients, there was no significant difference between JC virus-antibody seronegative ($n = 15$) and seropositive ($n = 11$) patients regarding CD62L expression at the time of the first measurement. A total of six natalizumab treated patients did change their therapy during the total clinical follow-up period (total treatment time natalizumab median 5.8, IQR 5.3–6.9 years; change to dimethyl fumarate $n = 1$, fingolimod $n = 1$, no DMT $n = 4$). Patients who switched from natalizumab during follow up had similar CD62L expression at baseline compared to patients who stayed on natalizumab.

Discussion

In this study we investigated the CD62L expression of $CD3^+CD4^+$ PBMCs in freshly collected whole blood during the use of various DMTs in MS, using direct FACS flow cytometry. We show that the CD62L expression of fresh $CD3^+CD4^+$ PBMCs is decreased with natalizumab and even more with fingolimod treatment, whereas increased levels are found in dimethyl fumarate-treated patients when compared to patients treated with other/no DMTs and healthy controls.

The direction of changes in CD62L expression with natalizumab and fingolimod is comparable to previous results on cryopreserved $CD4^+$ PBMCs.[3,4] Treatment with fingolimod causes a selective retention of predominantly $CD4^+$, and subsidiarily $CD8^+$, naive T cells and central memory T cells (both $CD62L^+$) in the lymphoid tissues, without affecting effector memory T cells ($CD62L^-$) or inducing lymphocyte destruction.[5,14,15] As a result, the overall count of peripheral blood lymphocytes is reduced under fingolimod, in particular that of $CD62L^+$ lymphocytes. Our results on dimethyl fumarate are in line with previous findings, showing that expanded $CD4^+CD62L^+$ expression was found in fresh blood samples of fumarate-treated MS patients compared to non-treated patients and healthy controls.[16] It is thought that this immunophenotypical shift consists of a reduction in the number of effector memory T cells ($CD62L^-$) and a relative increase of naive T cells ($CD62L^+$). The underlying mechanisms leading to alterations in CD62L expression under various DMTs are debated and further research into lymphocyte differentiation and redistribution is needed to better understand the information conveyed by this marker.

Results from a small subcohort of patients who switched their treatment during the time interval of longitudinal sampling show that CD62L expression increases after natalizumab withdrawal, and decreases rapidly upon fingolimod initiation. This indicates the hampering effect of these DMTs on CD62L expression of $CD3^+CD4^+$ PBMCs, and the ability for CD62L recovery after DMT withdrawal. Similar results were shown in a recent study on $CD4^+$ and $CD8^+$ PBMCs in freshly collected blood samples (processed within 6 hours of collection, subsequent analysis within 12 hours).[17] Our longitudinal data further demonstrate temporal stability of CD62L expression in patients who were untreated or did not change their DMT use during multiple sampling. Until now, the invariability of CD62L expression was merely indicated for long-term natalizumab treatment in MS.[4,17]

It has been suggested that CD62L expression of PBMCs might be used as a risk-stratification marker for PML.[3,4,6–8] PML is usually a fatal

opportunistic infection of the CNS caused by the JC virus, which appears most frequently in natalizumab-treated patients,[18] although cases have also been described during treatment with fingolimod[1] and dimethyl fumarate.[19] The association between CD62L and PML is not completely understood; it has been suggested that the general loss of T cells (lymphocytopenia), or the differential effect on T cell subsets by DMTs could be the most important risk factor.[1,19] Some studies showed decreased CD62L expression in pre-PML compared to non-PML natalizumab-treated patients when using cryopreserved PBMCs.[3,6,8] However, these data could not be confirmed in another study[10] and in a sub-cohort using freshly isolated PBMCs.[6] This discrepancy could be caused by assay variability due to pre-analytical procedures, i.e. the freeze/thaw procedure,[1,6,10] or the sample storage protocol handled prior to the cell surface assessment.[8] Here we solely analysed freshly collected blood samples within 1 hour of sampling to prevent any potential pre-analytical bias.

Overall, we could demonstrate feasibility to detect significant alterations in CD62L expression of $CD3^+CD4^+$ PBMCs with various DMTs when measured in fresh blood samples, without laborious pre-analytical steps. We found CD62L to be temporally stable, but differentially regulated with the use of various DMTs. The clinical significance of these findings is not yet clear. Future research is warranted to investigate if CD62L, when immediately measured, may serve as a biomarker for risk stratification of DMT side effects in MS, and as a possible marker for treatment response or disease activity/progression over a longer time. Flow cytometry is a widely accessible method, and the protocol proposed here could readily be implemented in clinical practice.

Conflict of Interests

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S Fuchs serves on scientific advisory boards and/or has received speaker honoraria from Biogen Idec, Novartis, Genzyme, Merck, Roche and Teva Pharmaceutical Industries.

F Fazekas serves on scientific advisory boards for Biogen Idec, Sanofi Genzyme, Merck, Novartis, and Teva ratiopharm; serves on the editorial boards of the European Stroke Journal, Multiple Sclerosis Journal, Neurology, the Polish Journal of Neurology and Neurosurgery, and the Swiss Archives of Neurology and Psychiatry; provides consulting services for Actelion, Medday, Parexel and Teva Ratiopharm; and has received speaker honoraria from Merck, Genzyme-Sanofi and Teva Ratiopharm.

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