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Expression of melanoma cell adhesion molecule-1 (MCAM-1) in natalizumab-treated multiple sclerosis

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ABSTRACT

The objectives were to study the expression of very late antigen (VLA)-4, melanoma cell adhesion molecule-1 (MCAM-1) and activated leukocyte cell adhesion molecule (ALCAM) on CD4+ T cells during natalizumab treatment and to investigate the association with disease activity.

We find that subgroups of autoreactive T cells are retained in peripheral blood, in particular MOG-reactive CD4+ T cells expressing MCAM-1. The expression of MCAM-1 or ALCAM on CD4+ T cells was, however, not clearly associated with disease activity (clinical or MRI) during natalizumab treatment. We confirm upregulation of MCAM-1 on CD4+ T cells during natalizumab treatment while VLA-4 is downregulated.

1. Introduction

Activation of autoreactive CD4+ T cells and migration of activated immune cells across the blood-brain barrier (BBB) are central parts of the pathogenesis in multiple sclerosis (MS). Focal infiltrates formed by these immune cells are involved in inflammation, myelin loss and axonal damage in the central nervous system (CNS) (Sospedra and Martin, 2005). Distinct CD4+ T cells have a high pathogenic potential in MS, and especially T helper type 17 (T_H17) cells are more frequent in peripheral blood and CSF of MS patients compared with controls (Matusevicius et al., 1999; Frisullo et al., 2008; Brucklacher-Waldert et al., 2009; Durelli et al., 2009; Li et al., 2011; Hedegaard et al., 2008). Interactions between endothelial cells and lymphocytes are crucial for transmigration of immune cells into the CNS parenchyma (Yednock et al., 1992; Ransohoff and Engelhardt, 2012). Natalizumab, a monoclonal antibody targeting the α4 integrin subunit of very late antigen-4 (VLA-4) on lymphocytes, inhibits the binding of VLA-4 to vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Miller et al., 2003; Polman et al., 2006). The binding of VLA-4 to VCAM-1 mediates the adhesion of lymphocytes to the endothelial cells and subsequent diapedesis across the BBB (Baron et al., 1993; Yednock et al., 1992). Thus, anti-VLA-4 treatment decreases the CSF lymphocyte count (Stuve et al., 2006b) and reduces the relapse rate by approximately two thirds in relapsing-remitting MS (RRMS) (Polman et al., 2006). However, some patients continue to have disease activity. Therefore, it is important to identify alternative routes for leukocyte recruitment to the CNS as blocking of such routes may lead to the development of even more efficacious therapies.

Melanoma cell adhesion molecule (MCAM-1 or CD146) and activated leukocyte cell adhesion molecule (ALCAM or CD166) are candidate molecules promoting migration of lymphocytes across the BBB (Larochelle et al., 2012; Schneider-Hohendorf et al., 2014; Cayrol et al., 2008; Lyck et al., 2016). Targeting these molecules in animal models has been shown to reduce the migration of Th17 and Th1 cells, respectively, across the BBB (Lyck et al., 2016; Larochelle et al., 2012). Indeed, animal studies suggest that blocking VLA-4 does not impair the recruitment of Th17 cells to the CNS (Glatigny et al., 2011), and a recent study suggested that the recruitment of Th17 cells in long-term natalizumab-treated patients may depend on MCAM-1 rather than VLA-4 (Schneider-Hohendorf et al., 2014).

We investigated if stable and unstable patients treated with natalizumab showed differences in their biological profile. We examined the expression of MCAM-1, ALCAM and VLA-4 on CD4+ T cells during natalizumab treatment and investigated whether expression of MCAM-1 and ALCAM was associated with disease activity in natalizumab-treated RRMS patients. Further, to assure that patients with disease activity on natalizumab were not misdiagnosed we measured anti-aquaporin 4 (AQP4) IgG and anti-myelin oligodendrocyte glycoprotein (MOG) IgG. Serum neurofilament light (sNfL) levels were measured as a biomarker of neuroaxonal damage associated with disease activity.

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2. Methods

2.1. Ethics

The study was approved by the local Ethics Committee (KF-01314009). Written informed consent was obtained from all participants.

2.2. Patients

We included RRMS patients untreated for three months or more before blood sampling ($n = 20$); stable RRMS patients treated with natalizumab for at least two years (n = 20); and unstable RRMS patients treated for at least one year with natalizumab with breakthrough disease activity ($n = 20$). In addition, we studied paired CSF and blood cells from four untreated and four natalizumab-treated RRMS patients and blood cells from 37 healthy control subjects.

All natalizumab-treated patients were seen by a neurologist at treatment start, after three and six months and six-monthly thereafter. MRI was performed at baseline and thereafter annually or more frequently depending on the presence and titer of John Cunningham (JC) virus antibodies (Mcguigan et al., 2016). Smoking information was obtained from a lifestyle and environmental factor questionnaire developed at the Karolinska Institute, Stockholm, Sweden, translated into Danish language, and used with permission.

Stable patients had no evidence of disease activity on natalizumab treatment, i.e., no relapses, gadolinium-enhancing or new T2 lesions during the observation period. Unstable patients had two or more relapses in the preceding two years of natalizumab treatment. If they were treated less than two years they still had at least 2 relapses during natalizumab treatment. A relapse was defined as neurological symptoms or signs lasting for at least 24 h in the absence of fever or infections, and preceded by a four weeks stable period. Patients with persisting anti-natalizumab antibodies were excluded.

2.3. Blood and CSF samples

Venous blood samples were collected in BD Vacutainer EDTA tubes (BD Bioscience, Denmark). Samples were obtained at least three months after the latest relapse and/or steroid treatment for a relapse, except in one patient, in whom the time from latest steroid treatment to blood sample was 1.5 month. CSF cells were obtained from 12 ml of CSF collected for routine diagnosis, assessment of disease activity, or JC virus antibody analysis. CSF samples were collected in polypropylene tubes on an ice bath and centrifuged within 30 min and resuspended in FACS/PBS (PBS/2% FBS/0.02% NaN_3). All samples were collected between 8 and 10 am.

2.4. Clinical data

Clinical data were prospectively collected, obtained from medical records and consisted of sex, age at sampling date, expanded disability status scale (EDSS) score, number of relapses, steroid treatments, treatment duration, and date of last natalizumab infusion.

2.5. Flow cytometry analysis of freshly isolated cells

Within one hour from sampling, blood was diluted with cold PBS/2 mM EDTA and peripheral blood mononuclear cells (PBMC) were separated on Lymphoprep (Axis-Shield, Norway). PBMC and CSF cells were washed in FACS/PBS and FcR Blocking Reagent (Miltenyi Biotec, Germany) was added to prevent non-specific binding of antibodies. The cells were then stained with fluorochrome-conjugated antibodies against CD3 (APC, UCHT1, Biolegend), CD4 (APC-AF750, S3.5, Life Technologies), CD49d (BV605, 9F10, Biolegend), MCAM-1 (BV421, P1H12, Biolegend) and ALCAM (PE, 3A6, Biolegend) and isotype control mIgG1 (PerCP-CY5.5, Biolegend). Isotype controls were used to define CD49d negative CD4+ T cells. A FACS Canto II 8-color flow cytometer (BD Biosciences, USA) was used for data acquisition and FlowJo (Tree star, USA) for data analysis.

2.6. Flow cytometry analysis of T cell activation

PBMC were stained with carboxyflourescein diacetate succinimidyl ester (CFSE; Molecular Probes, Denmark) for 2.5 min at room temperature, washed in RPMI/2,5% human AB-serum (Invitrogen, USA) and cryopreserved. The cells were thawed at 37 degrees and washed twice in RPMI with 5% fetal bovine serum (FBS; ThermoFisher, USA). $0{,}5 \times 10^6$ cells in RPMI/5% human AB-serum (Sigma-Aldrich, USA)/PenStrep were added to each well in a 96 wells flat bottom culture plate. The human autoantigens myelin basic protein (MBP; 30 μg/ml; HyTest, Finland) or MOG (10 μg/ml;AnaSpecINC, USA), the positive control heat-killed *Candida albicans* (CA; 5×10^6 /ml; InVivogen, USA) or no antigen (negative control) were added to the cells in 96 wells flat bottom culture plates (Greiner Bio-one, Germany). The cells were incubated for seven days at 37 °C/5% CO_2. After 4 days in culture fresh medium was added. After seven days cells were harvested, washed twice in FACS/PBS and stained for 25 min on ice with fluorochrome-conjugated antibodies against CD3 (APC, UCHT1, Biolegend), CD4 (APC-AF750, S3.5, Biolegend), MCAM-1 (BV421, P1H12, 1Biolegend) and ALCAM (PE, 3A6, Biolegend). After incubation cells were washed with FACS/PBS and analyzed by flow cytometry as described above. ALCAM and MCAM-1 positive cells were defined from the non-proliferating cell population and gates were applied to the corresponding proliferating population.

2.7. Neurofilament light chain protein

Concentrations of neurofilament light in serum were measured in duplicate by single molecule array (Simoa) technology (Quanterix, Billerica, USA), according to manufacturer's instructions. A maximum coefficient of variance of 20% of duplicate measurements was accepted.

2.8. Detection of anti-aquaporin-4 (AQP4) IgG and anti-MOG IgG

Serum samples from 22 patients with relapses during natalizumab treatment were available for anti-AQP4 and anti-MOG analyses. 19 samples were from the group of unstable patients and three were from the natalizumab-treated patients with paired blood and CSF samples. All samples were tested for anti-AQP4 IgG and anti-MOG IgG, using fixed cell-based assays employing AQP4- and MOG-transfected HEK293-cells, respectively (Euroimmun Ag, Lübeck, Germany). Screening was performed in a dilution of 1:10, as recommended by the manufacturer.

2.9. Statistics

Paired and unpaired samples *t*-tests were used for analysis of the paired blood and CSF samples. One-way analysis of covariance (ANCOVA) was used to compare data between healthy controls, untreated, stable and natalizumab-treated patients. Untransformed data were used if residuals were normally distributed; otherwise, log-transformed data were used to obtain normally distributed residuals. Sex, age and smoking status were used as covariates as these were previously reported to be associated with relapses in RRMS (Bove and Chitnis, 2014; Tremlett et al., 2008; Petersen et al., 2018, 2019). Bonferroni correction was applied to correct for multiple comparisons. A p-value $< .05$ was considered statistically significant. Bonferroni-corrected post hoc tests were used for variables that were statistically significant in the ANCOVA; a Bonferroni-corrected p-value $< .05$ was considered statistically significant.

We used IBM SPSS statistics 22 for statistical analyses.

Table 1
Demographic characteristics and serum neurofilament light (sNfL) in the healthy controls (HC), untreated RRMS patients, stable natalizumab-treated patients and unstable natalizumab-treated patients.

	HC	Untreated RRMS	Stable natalizumab	Unstable natalizumab
Sex, m/f	23/14	10/10	7/13	5/15
Age at blood sample, mean (range)	36.9 (21–60)	40.7 (25–59)	40.1 (22–69)	44.6 (22–65)
Treatment duration, years, mean (range)			4.6 (2.4–7.2)	5.0 (1.4–8.1)
EDSS at blood sample, mean (range)		2.8 (1.5–6.5)	2.3 (1–4)	3.8 (0–6)
Smokers/non-smokers	19/18	4/16	9/11	6/14
Last natalizumab infusion to blood sample, days, mean (range)			22 (2–35)	22 (2–36)
First relapse on natalizumab treatment, months, mean (range)				9.8 (1–35)
Patients with sNfL over upper limit[a], (N)		5 (20)	0 (19)	2 (19)

[a] Upper normal limit = $0.302 \times \text{Age}^{0.049}$.

3. Results

3.1. Demographics

Patient characteristics in the four groups are shown in Table 1. All, except one, of the unstable patients had their first relapse on natalizumab within the first two treatment years (Table 1). Characteristics of the eight patients with paired blood and CSF samples are shown in Table 2. No significant effects of the covariates age, sex and smoking status were observed for any of the immunological measures studied (data not shown).

3.2. sNfL, AQP4 and anti-MOG IgG

AQP4 or anti-MOG antibodies were not found in patients with disease activity on natalizumab treatment (data not shown). sNfL levels were normal in stable natalizumab treated patients and elevated levels were found in 2 unstable patients and in 5 untreated patients (Table 1).

3.3. Expression of MCAM-1 and ALCAM on CD4+ T cells after antigen stimulation

Fig. 1 shows the gating of MCAM-1 and ALCAM after antigen stimulation in a representative untreated patient. Under unstimulated conditions (negative control) the percentage of non-proliferating CD4+ T cells expressing MCAM-1 after 7 days of incubation differed significantly between healthy controls, untreated, stable, and unstable natalizumab-treated patients ($p = .024$ with Bonferroni correction) (Fig. 2 and supplementary table). The percentage of unstimulated CD4+ T cells expressing MCAM-1 was higher in stable ($p = .036$) and unstable ($p = .003$) natalizumab-treated patients compared to healthy controls (Fig. 2). Although there was a trend towards unstable patients having a higher frequency of CD4+ T cells expressing MCAM-1 than the stable patients, this difference was not statistically significant.

We also found a significant difference in the percentage of CD4+ T cells proliferating after stimulation with MOG that expressed MCAM-1 between groups (Fig. 3 and supplementary table, $p = .012$ with Bonferroni correction). Stable natalizumab-treated patients had a significantly higher percentage of proliferating CD4+ T cells expressing MCAM-1 after stimulation with MOG than healthy controls ($p = .010$), whereas there was no significant difference between stable and unstable natalizumab-treated patients (Fig. 3).

We observed a nominally significant difference in the percentage of proliferating CD4+ T cells expressing ALCAM after MOG stimulation ($p = .011$), but this was not significant after Bonferroni correction (supplementary table). There were no other significant differences in the percentage of proliferating CD4+ T cells expressing MCAM-1 or ALCAM after stimulation with MBP, MOG or CA (supplementary table).

Table 2
Characteristics of the 4 untreated RRMS patients and the 4 natalizumab-treated patients from whom paired blood and CSF were collected.

	Untreated RRMS	Natalizumab treated
Sex, m/f	0/4	0/4
Age at blood sample, mean (range)	35.5 (26–49)	36 (33–40)
EDSS at blood sample, mean (range)	1.7 (1–3)[a]	3.5 (2–6)
Treatment duration, years, mean (range)		3.8 (1.9–6.9)
Number of relapses during treatment, mean (range)		2 (1–4)

[a] A recent EDSS was available from 3 of the 4 untreated patients.

3.4. Antigen-induced CD4+ T cell proliferation

The percentage of CD4+ T cells proliferating after stimulation with MOG differed significantly between healthy controls, untreated, stable and unstable natalizumab-treated patients ($p < .001$ with Bonferroni correction) and was higher in both natalizumab-treated groups than in healthy controls and untreated RRMS patients (all $p < .05$) (Fig. 4 and supplementary table).

There was no difference in the percentage of CD4+ T cells proliferating after stimulation with MOG in stable or unstable natalizumab-treated patients (Fig. 4 and supplementary table). Neither were there any significant differences in the percentage of CD4+ T cells proliferating after stimulation with MBP (uncorrected $p = .741$), CA (uncorrected $p = .072$) or negative control conditions (uncorrected $p = .305$) (supplementary table).

3.5. Expression of VLA-4, MCAM-1 and ALCAM in peripheral blood and CSF

Fig. 5 shows the gating of VLA-4, MCAM-1 and ALCAM on CD4+ T cells from a representative untreated RRMS patient. In blood and CSF of untreated MS patients most CD4+ T cells expressed VLA-4 (median 84.6% (interquartile range (IQR) = 62.1–90.2%) in blood and median 98.4% (IQR = 85.4–99.2%) in CSF (Fig. 6). MCAM-1 was expressed by a lower percentage of cells in blood (median 6.1%, IQR = 4.1–6.9%) and CSF (median 8.1%, IQR = 7.6–9.2%) (Fig. 6) and was mainly co-expressed with VLA-4 (Fig. 5). Expression of ALCAM was low (3.6% in blood and 4% in CSF), and ALCAM was mainly co-expressed with VLA-4 but not with MCAM-1 (Fig. 5).

In both natalizumab-treated and untreated patients, the percentage of CD4+ T cells in blood and CSF expressing VLA-4 was higher than the percentage of CD4+ T cells expressing MCAM-1. Moreover, the percentage of CD4+ T cells expressing VLA-4 was higher in CSF than in blood in untreated patients (Fig. 6). Furthermore, a higher percentage of CD4+ T cells in CSF from untreated than natalizumab-treated patients expressed VLA-4. Both untreated and natalizumab-treated patients had a higher percentage of CD4+ T cells expressing MCAM-1 in CSF than in blood, and the percentage of MCAM-1 positive CD4+ T cells was even higher in natalizumab-treated than in untreated patients. There was no difference in ALCAM expression in untreated and

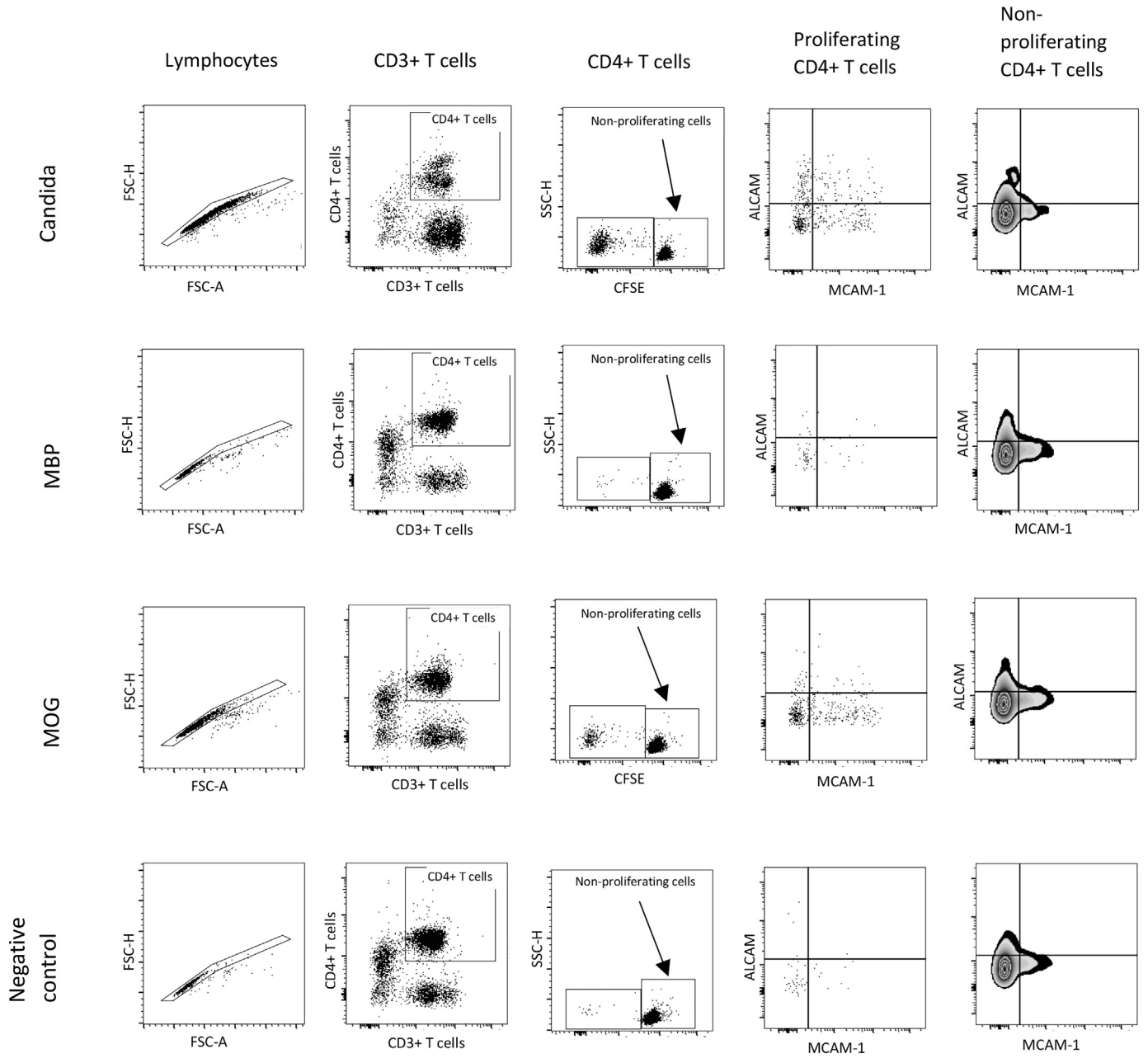

Fig. 1. Percentage of CD4+ T cells in blood from a representative untreated RRMS patient expressing MCAM-1 and ALCAM after stimulation with myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), *Candida albicans* (CA) and no antigen (negative control).
The isotype control was not useful as the background fluorescence of the primary antibody was too strong. Therefore we defined ALCAM and MCAM-1 positive cells from the non-proliferating cell population using the zebra plot and gates were applied to the corresponding proliferating population.

natalizumab-treated patients and no evidence of recruitment of ALCAM-positive CD4+ T cells to the CSF.

4. Discussion

The novel findings in our study were: 1) The percentage of MCAM-1-expressing, unstimulated CD4+ T cells was higher in natalizumab-treated patients than in healthy controls; 2) patients treated with natalizumab had more MOG-reactive CD4+ T cells in blood than untreated patients and healthy controls; 3) patients stable on treatment with natalizumab had a higher percentage of MOG-reactive T cells expressing MCAM-1 than healthy controls. Furthermore, our findings confirm: 1) The percentage of CD4+ T cells expressing VLA-4 was lower in CSF in patients treated with natalizumab than in untreated RRMS patients and 2) the percentage of MCAM-1 expressing CD4+ T cells was higher in CSF than in blood in untreated and natalizumab-treated patients and even higher on CD4+ T cells in CSF from natalizumab-treated patients than in untreated patients.

Lack of treatment response to natalizumab has previously been described in patients with neuromyelitis optica spectrum disorders (NMOSD) (Kleiter et al., 2012). We therefore analyzed AQP4- and anti-MOG IgG in the natalizumab-treated patients with disease activity. None of the patients had anti-AQP4 or anti-MOG antibodies. We used sNfL as a biomarker of neuroaxonal damage. High levels of sNfL have been associated with disease activity in MS patients (Disanto et al., 2017), and higher NfL levels have been found in untreated patients compared to patients treated with natalizumab (Gunnarsson et al., 2011). We found normal sNfL levels in the stable natalizumab treated

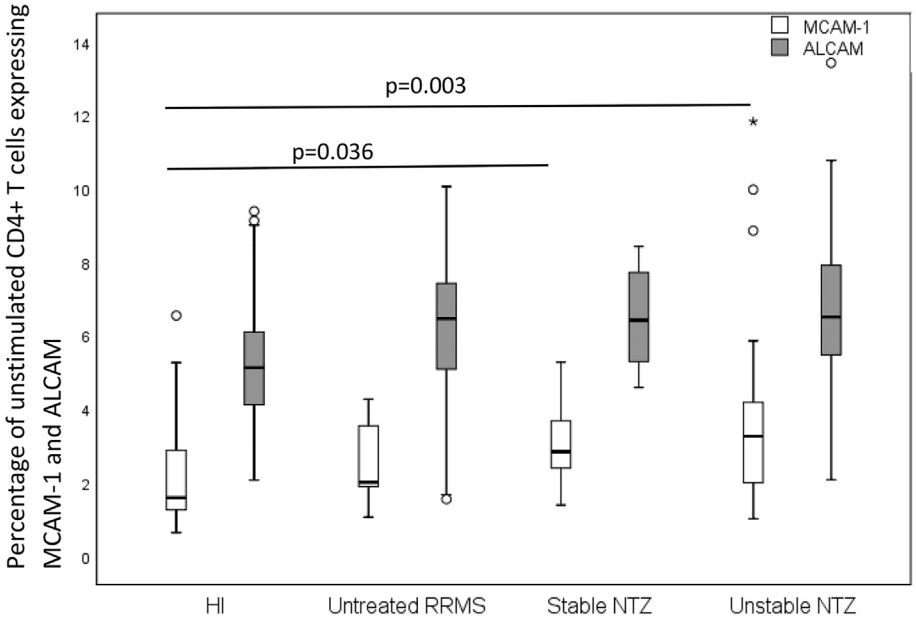

Fig. 2. Percentage of unstimulated, non-proliferating CD4+ T cells expressing MCAM-1 and ALCAM in 37 healthy controls (HC), 19 untreated RRMS patients, 20 stable and 20 clinically active natalizumab-treated patients.

patients which confirms their clinically stable condition. Two unstable and five untreated patients had increased levels. Thus, despite having disease activity, they did not have evidence of ongoing neuroaxonal damage at the time of sampling.

In blood, unstimulated CD4+ T cells had higher expression of MCAM-1 in the two natalizumab-treated groups compared to healthy controls. This may reflect that effector cells expressing MCAM-1 are retained in the circulation in natalizumab-treated patients, but might also reflect a compensatory increase in MCAM-1 expression upon treatment with natalizumab. MCAM-1 contributes to the adhesion of T_H17 cells to the endothelium in the brain (Schneider-Hohendorf et al., 2014). MCAM-1 expression was previously found to be upregulated in inflammatory CNS lesions and blocking of MCAM-1 or depletion of MCAM-1 expressing CD4+ T cells lead to a decrease in migratory cells and disease severity in EAE (Larochelle et al., 2012; Schneider-Hohendorf et al., 2014). We also found that the percentage of MOG-reactive CD4+ T cells expressing MCAM-1 was higher in stable natalizumab-treated patients than in healthy controls, whereas there was no significant difference between clinically active natalizumab-treated patients and healthy controls. Although it is tempting to speculate that this reflects selective retention of pathogenic T cells in patients without disease activity, this must be confirmed in larger studies and studies including analysis of T cell recruitment to the CNS. Furthermore, it should be assessed whether the expression of adhesion molecules is increased in patients with ongoing disease activity since only two out of our twenty patients with on-study relapses had actual increases in sNFL

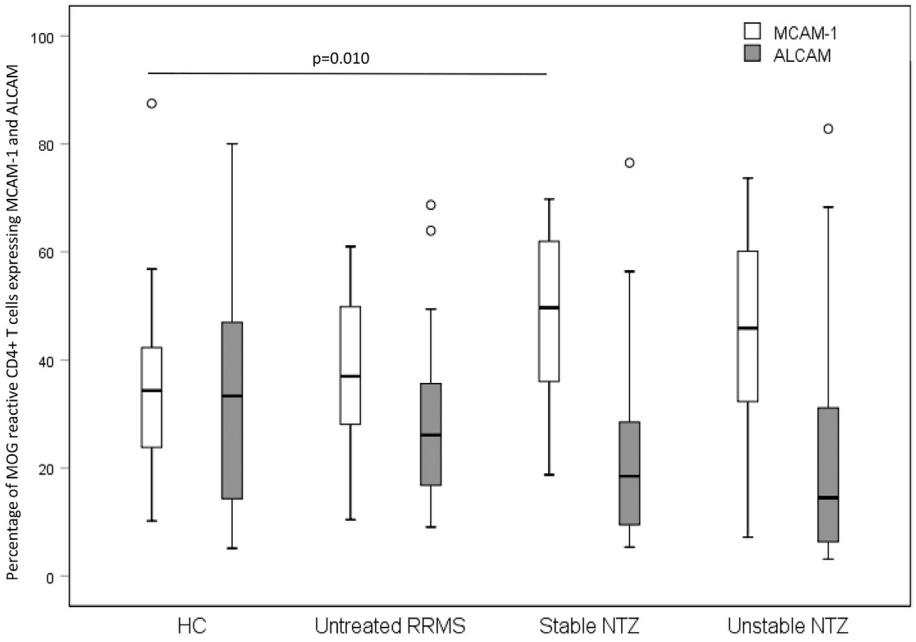

Fig. 3. Percentage of CD4+ T cells expressing MCAM-1 and ALCAM after myelin oligodendrocyte glycoprotein (MOG) stimulation in 36 HC, 19 untreated RRMS patients, 20 stable and 20 clinically active natalizumab-treated patients. All *p*-values are from the ANCOVA with Bonferroni post hoc test.

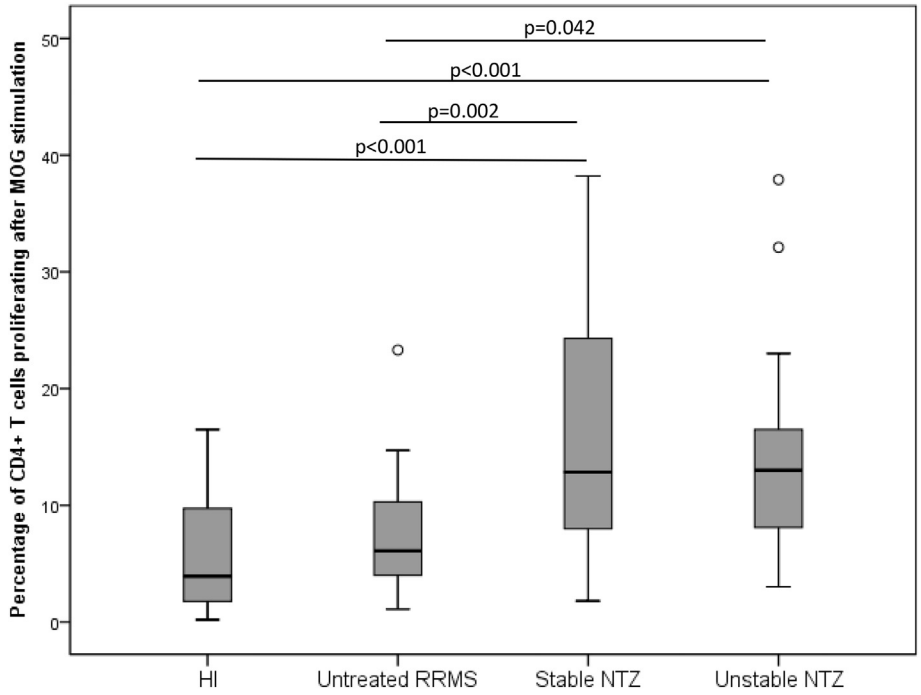

Fig. 4. Percentage of CD4+ T cells proliferating after myelin oligodendrocyte glycoprotein (MOG) stimulation in 36 healthy controls (HC), 19 untreated RRMS patients, 20 stable and 20 clinically active natalizumab-treated patients. *P*-values are from the ANCOVA with Bonferroni post hoc test.

at the time of blood sampling in the present study.

Treatment with natalizumab results in an increased pool of circulating immune cells, reflecting retention of memory T cells, and a decrease in the CSF leukocyte count (Bornsen et al., 2012; Khademi et al., 2009; Khademi et al., 2008; Kivisakk et al., 2009; Stuve et al., 2006b; Ramos-Cejudo et al., 2011; Niino et al., 2006). This is consistent with the known role of VCAM-1 and VLA-4 in T cell recruitment to the CNS (Ransohoff and Engelhardt, 2012). Consistent with the notion that treatment with natalizumab results in the retention of pathogenic, autoreactive T cells in the circulation, we found that natalizumab-treated patients showed increased CD4+ T cell reactivity in circulating blood cells to the myelin autoantigen MOG (Fig. 3). This was not due to general retention of antigen-reactive CD4+ T cells or an altered activation status of circulating immune cells since we found no difference in CD4+ T cell reactivity to CA antigen or non-specific proliferation (supplementary table). Neither did we find any difference in the

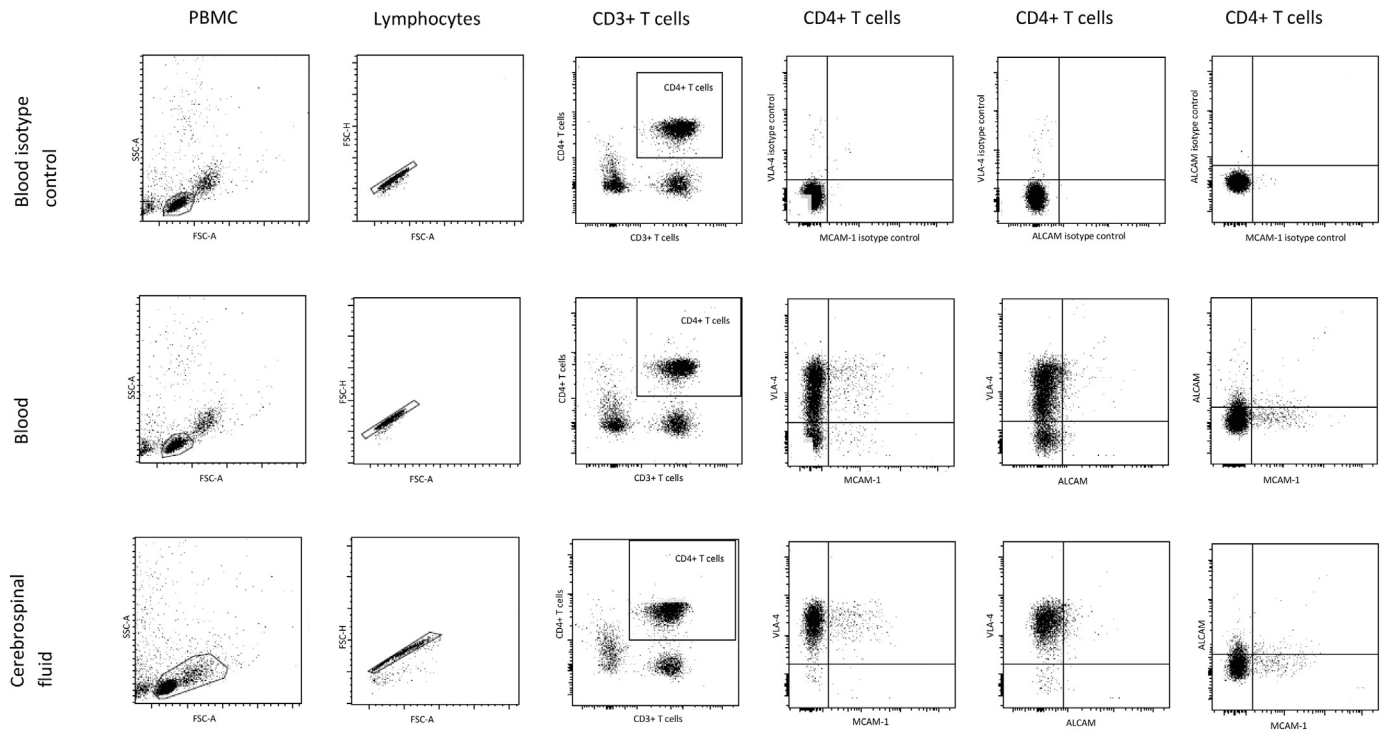

Fig. 5. Percentage of CD4+ T cells in blood and in cerebrospinal fluid from a representative untreated RRMS patient expressing VLA-4, MCAM-1 and ALCAM. Isotype controls were used to define CD49d negative CD4+ T cells.

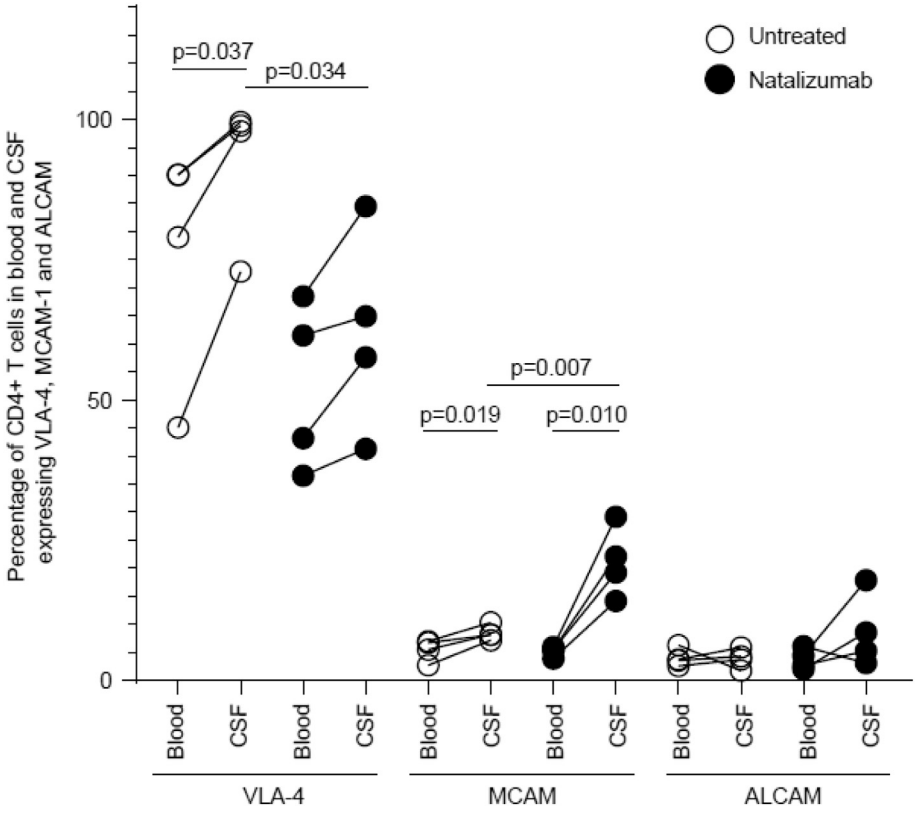

Fig. 6. Percentage of CD4+ T cells expressing VLA-4, MCAM-1 and ALCAM in blood and cerebrospinal fluid (CSF) in four untreated and four natalizumab treated patients. Paired-samples and independent-samples *t*-tests were used for statistical analysis.

reactivity to the myelin autoantigen MBP (supplementary table), which is in accordance with the results of a previous study from our group (Bornsen et al., 2012). The finding is also consistent with the notion that MOG may be a principal pathogenic autoantigen in MS. MOG is a CNS sequestered antigen and thus not expressed in thymus or peripheral organs. It is therefore thought that immunological tolerance to MOG is less well established compared to other CNS antigens (Bruno et al., 2002). Sun et al. studied MOG reactive T cells and found an increased frequency of MOG-reactive T cells in MS patients compared to neurologic controls in both blood and CSF (Sun et al., 1991). Further, experimental autoimmune encephalomyelitis (EAE) induced by MOG results in CNS damage similar to what is observed in MS (Rangachari and Kuchroo, 2013). The retention of an increased pool of MOG-reactive T cells may also at least partly explain the rebound phenomenon observed in some patients upon discontinuation of natalizumab therapy (Sorensen et al., 2014).

The decrease in CSF lymphocyte count in natalizumab-treated patients affects T cells, B cells and monocytes, and is more pronounced for CD4+ T cells than for CD8+ T cells (Stuve et al., 2006a; Ransohoff and Engelhardt, 2012). We could confirm that VLA-4 expression is lower on CD4+ T cells in CSF in natalizumab-treated patients than in untreated patients. We also found a significantly higher percentage of CD4+ T cells expressing MCAM-1 in CSF than in peripheral blood and a significantly higher percentage of CD4+ T cells expressing MCAM-1 in CSF in natalizumab-treated patients compared to untreated patients; as previously suggested this might indicate that MCAM-1 is used for the recruitment of CD4+ T cells after blockade of the VLA-4-VCAM-1 interaction (Schneider-Hohendorf et al., 2014).

The majority of CD4+ T cells in CSF did, however, not express MCAM-1, which suggests that other adhesion molecule pairs such as platelet selectin glycolipid ligand-1 (PSGL-1) and P-selectin, lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), homotypic ALCAM-ALCAM binding, or ALCAM-CD6 binding may also be involved in CD4+ T cell recruitment in natalizumab-treated patients. Although we found only minor expression of ALCAM on freshly isolated blood and CSF CD4+ T cells and ex vivo activated CD4+ T cells in the present study, binding of ALCAM on endothelial cells to CD6 on T cells may, indeed, still be involved in T cell recruitment in natalizumab-treated patients (Li et al., 2017; Cayrol et al., 2008).

In conclusion, we have shown that natalizumab treatment leads to retention of potentially pathogenic MOG-reactive CD4+ T cells in the circulation, and that there may be more prominent retention of MOG-reactive T cells expressing MCAM-1 in stable natalizumab-treated patients.

A limitation of our study is the small cohorts, which might explain why we were not able to demonstrate significant differences between stable and clinically active patients. Future studies of additional adhesion molecules and chemokine receptors on circulating cells as well as studies comparing CSF and blood cells may lead to a better understanding of T cell recruitment in MS patients with disease activity on treatment with natalizumab.

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Author disclosures

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Søndergaard HB has nothing to declare.

Oturai AB has served on scientific advisory boards for Biogen Idec and Genzyme; has received research support from Novartis and Biogen Idec; has received speaker honoraria from Biogen Idec, Novartis and TEVA; and has received support for congress participation from, Merck Serono, Teva, Biogen, Novartis and Genzyme.

Sørensen PS has received personal compensation for serving on scientific advisory boards, steering committees or independent data monitoring boards or speaker honoraria from Biogen, Merck Serono, Novartis, Genzyme, Teva., GlaxoSmithKline, medDay Pharmaceuticals and Forward.

Nilsson AC has nothing to declare.

Börnsen L has nothing to declare.

Sellebjerg S has served on scientific advisory boards, been on the steering committees of clinical trials, served as a consultant, received support for congress participation, received speaker honoraria, or received research support for his laboratory from Biogen, EMD Serono, Genzyme, Lundbeck, Merck Serono, Novartis and Teva.

Appendix A. Supplementary data

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References

Baron, J.L., Madri, J.A., Ruddle, N.H., Hashim, G., Janeway Jr., C.A., 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. J. Exp. Med. 177, 57–68.

Bornsen, L., Christensen, J.R., Ratzer, R., Oturai, A.B., Sorensen, P.S., Sondergaard, H.B., Sellebjerg, F., 2012. Effect of natalizumab on circulating CD4+ T-cells in multiple sclerosis. PLoS One 7, e47578.

Bove, R., Chitnis, T., 2014. The role of gender and sex hormones in determining the onset and outcome of multiple sclerosis. Mult. Scler. 20, 520–526.

Brucklacher-Waldert, V., Stuerner, K., Kolster, M., Wolthausen, J., Tolosa, E., 2009. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. Brain 132, 3329–3341.

Bruno, R., Sabater, L., Sospedra, M., Ferrer-Francesch, X., Escudero, D., Martinez-Caceres, E., Pujol-Borrell, R., 2002. Multiple sclerosis candidate autoantigens except myelin oligodendrocyte glycoprotein are transcribed in human thymus. Eur. J. Immunol. 32, 2737–2747.

Cayrol, R., Wosik, K., Berard, J.L., Dodelet-Devillers, A., Ifergan, I., Kebir, H., Haqqani, A.S., Kreymborg, K., Krug, S., Moumdjian, R., Bouthillier, A., Becher, B., Arbour, N., David, S., Stanimirovic, D., Prat, A., 2008. Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. Nat. Immunol. 9, 137–145.

Disanto, G., Barro, C., Benkert, P., Naegelin, Y., Schadelin, S., Giardiello, A., Zecca, C., Blennow, K., Zetterberg, H., Leppert, D., Kappos, L., Gobbi, C., Kuhle, J., 2017. Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. Ann. Neurol. 81, 857–870.

Durelli, L., Conti, L., Clerico, M., Boselli, D., Contessa, G., Ripellino, P., Ferrero, B., Eid, P., Novelli, F., 2009. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. Ann. Neurol. 65, 499–509.

Frisullo, G., Nociti, V., Iorio, R., Patanella, A.K., Marti, A., Caggiula, M., Mirabella, M., Tonali, P.A., Batocchi, A.P., 2008. IL17 and IFNgamma production by peripheral blood mononuclear cells from clinically isolated syndrome to secondary progressive multiple sclerosis. Cytokine 44, 22–25.

Glatigny, S., Duhen, R., Oukka, M., Bettelli, E., 2011. Cutting edge: loss of alpha4 integrin expression differentially affects the homing of Th1 and Th17 cells. J. Immunol. 187, 6176–6179.

Gunnarsson, M., Malmestrom, C., Axelsson, M., Sundstrom, P., Dahle, C., Vrethem, M., Olsson, T., Piehl, F., Norgren, N., Rosengren, L., Svenningsson, A., Lycke, J., 2011. Axonal damage in relapsing multiple sclerosis is markedly reduced by natalizumab. Ann. Neurol. 69, 83–89.

Hedegaard, C.J., Krakauer, M., Bendtzen, K., Lund, H., Sellebjerg, F., Nielsen, C.H., 2008. T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. Immunology 125, 161–169.

Khademi, M., Stol, D., Olsson, T., Wallstrom, E., 2008. Induction of systemic TNFalpha in natalizumab-treated multiple sclerosis. Eur. J. Neurol. 15, 309–312.

Khademi, M., Bornsen, L., Rafatnia, F., Andersson, M., Brundin, L., Piehl, F., Sellebjerg, F., Olsson, T., 2009. The effects of natalizumab on inflammatory mediators in multiple sclerosis: prospects for treatment-sensitive biomarkers. Eur. J. Neurol. 16, 528–536.

Kivisakk, P., Healy, B.C., Viglietta, V., Quintana, F.J., Hootstein, M.A., Weiner, H.L., Khoury, S.J., 2009. Natalizumab treatment is associated with peripheral sequestration of proinflammatory T cells. Neurology 72, 1922–1930.

Kleiter, I., Hellwig, K., Berthele, A., Kumpfel, T., Linker, R.A., Hartung, H.P., Paul, F., Aktas, O., 2012. Failure of natalizumab to prevent relapses in neuromyelitis optica. Arch. Neurol. 69, 239–245.

Larochelle, C., Cayrol, R., Kebir, H., Alvarez, J.I., Lecuyer, M.A., Ifergan, I., Viel, E., Bourbonniere, L., Beauseigle, D., Terouz, S., Hachehouche, L., Gendron, S., Poirier, J., Jobin, C., Duquette, P., Flanagan, K., Yednock, T., Arbour, N., Prat, A., 2012. Melanoma cell adhesion molecule identifies encephalitogenic T lymphocytes and promotes their recruitment to the central nervous system. Brain 135, 2906–2924.

Li, Y., Wang, H., Long, Y., Lu, Z., Hu, X., 2011. Increased memory Th17 cells in patients with neuromyelitis optica and multiple sclerosis. J. Neuroimmunol. 234, 155–160.

Li, Y., Singer, N.G., Whitbred, J., Bowen, M.A., Fox, D.A., Lin, F., 2017. CD6 as a potential target for treating multiple sclerosis. Proc. Natl. Acad. Sci. U. S. A. 114, 2687–2692.

Lyck, R., Lecuyer, M.A., Abadier, M., Wyss, C.B., Matti, C., Rosito, M., Enzmann, G., Zeis, T., Michel, L., Garcia, M., B, A., Sallusto, F., Gosselet, F., Deutsch, U., Weiner, J.A., Schaeren-Wiemers, N., Prat, A., Engelhardt, B., 2016. ALCAM (CD166) is involved in extravasation of monocytes rather than T cells across the blood-brain barrier. J Cereb Blood Flow Metab 37, 2894–2909 (271678x16678639).

Matusevicius, D., Kivisakk, P., He, B., Kostulas, N., Ozenci, V., Fredrikson, S., Link, H., 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. Mult. Scler. 5, 101–104.

Mcguigan, C., Craner, M., Guadagno, J., Kapoor, R., Mazibrada, G., Molyneux, P., Nicholas, R., Palace, J., Pearson, O.R., Rog, D., Young, C.A., 2016. Stratification and monitoring of natalizumab-associated progressive multifocal leukoencephalopathy risk: recommendations from an expert group. J. Neurol. Neurosurg. Psychiatry 87, 117–125.

Miller, D.H., Khan, O.A., Sheremata, W.A., Blumhardt, L.D., Rice, G.P., Libonati, M.A., Willmer-Hulme, A.J., Dalton, C.M., Miszkiel, K.A., O'connor, P.W., 2003. A controlled trial of natalizumab for relapsing multiple sclerosis. N. Engl. J. Med. 348, 15–23.

Niino, M., Bodner, C., Simard, M.L., Alatab, S., Gano, D., Kim, H.J., Trigueiro, M., Racicot, D., Guerette, C., Antel, J.P., Fournier, A., Grand'maison, F., Bar-Or, A., 2006. Natalizumab effects on immune cell responses in multiple sclerosis. Ann. Neurol. 59, 748–754.

Petersen, E.R., A, O., Koch-Henriksen, N., Magyari, M., Sørensen, P.S., Sellebjerg, F., Søndergaard, H.B., 2018. Smoking affects the interferon-beta treatment response in multiple sclerosis. Neurology 90, e593–e600 (Accepted and scheduled to appear in the February 13, 2018 print issue of Neurology®).

Petersen, E.R., Sondergaard, H.B., Laursen, J.H., Olsson, A.G., Bornsen, L., Soelberg Sorensen, P., Sellebjerg, F., Bang Oturai, A., 2019. Smoking is associated with increased disease activity during natalizumab treatment in multiple sclerosis. Mult. Scler. 25, 1298–1305.

Polman, C.H., O'connor, P.W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D.H., Phillips, J.T., Lublin, F.D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M.A., Sandrock, A.W., 2006. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N. Engl. J. Med. 354, 899–910.

Ramos-Cejudo, J., Oreja-Guevara, C., Stark Aroeira, L., Rodriguez De Antonio, L., Chamorro, B., Diez-Tejedor, E., 2011. Treatment with natalizumab in relapsing-remitting multiple sclerosis patients induces changes in inflammatory mechanism. J. Clin. Immunol. 31, 623–631.

Rangachari, M., Kuchroo, V.K., 2013. Using EAE to better understand principles of immune function and autoimmune pathology. J. Autoimmun. 45, 31–39.

Ransohoff, R.M., Engelhardt, B., 2012. The anatomical and cellular basis of immune surveillance in the central nervous system. Nat Rev Immunol 12, 623–635.

Schneider-Hohendorf, T., Rossaint, J., Mohan, H., Boning, D., Breuer, J., Kuhlmann, T., Gross, C.C., Flanagan, K., Sorokin, L., Vestweber, D., Zarbock, A., Schwab, N., Wiendl, H., 2014. VLA-4 blockade promotes differential routes into human CNS involving PSGL-1 rolling of T cells and MCAM-adhesion of TH17 cells. J. Exp. Med. 211, 1833–1846.

Sorensen, P.S., Koch-Henriksen, N., Petersen, T., Ravnborg, M., Oturai, A., Sellebjerg, F., 2014. Recurrence or rebound of clinical relapses after discontinuation of natalizumab therapy in highly active MS patients. J. Neurol. 261, 1170–1177.

Sospedra, M., Martin, R., 2005. Immunology of multiple sclerosis. Annu. Rev. Immunol. 23, 683–747.

Stuve, O., Marra, C.M., Bar-Or, A., Niino, M., Cravens, P.D., Cepok, S., Frohman, E.M., Phillips, J.T., Arendt, G., Jerome, K.R., Cook, L., Grand'maison, F., Hemmer, B., Monson, N.L., Racke, M.K., 2006a. Altered CD4+/CD8+ T-cell ratios in cerebrospinal fluid of natalizumab-treated patients with multiple sclerosis. Arch. Neurol. 63, 1383–1387.

Stuve, O., Marra, C.M., Jerome, K.R., Cook, L., Cravens, P.D., Cepok, S., Frohman, E.M., Phillips, J.T., Arendt, G., Hemmer, B., Monson, N.L., Racke, M.K., 2006b. Immune surveillance in multiple sclerosis patients treated with natalizumab. Ann. Neurol. 59, 743–747.

Sun, J., Link, H., Olsson, T., Xiao, B.G., Andersson, G., Ekre, H.P., Linington, C., Diener, P., 1991. T and B cell responses to myelin-oligodendrocyte glycoprotein in multiple sclerosis. J. Immunol. 146, 1490–1495.

Tremlett, H., Zhao, Y., Joseph, J., Devonshire, V., 2008. Relapses in multiple sclerosis are age- and time-dependent. J. Neurol. Neurosurg. Psychiatry 79, 1368–1374.

Yednock, T.A., Cannon, C., Fritz, L.C., Sanchez-Madrid, F., Steinman, L., Karin, N., 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature 356, 63–66.