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Increased frequency of CD4$^+$ CD25$^+$ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients

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Summary

Naturally occurring CD4$^+$ CD25$^+$ regulatory T cells (nT$_{reg}$) play a major role in controlling autoimmunity by suppressing self-reactive T cells. Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system (CNS), where T cells play a key role in orchestrating tissue damage. While CD4$^+$ CD25$^+$ nT$_{reg}$ have been investigated in peripheral blood from MS patients, little is known about their presence and possible function within the target organ, the CNS. In order to study whether these cells are present in the cerebrospinal fluid (CSF) under pathological conditions, we have analysed the frequency of CD4$^+$ CD25$^+$ nT$_{reg}$ in peripheral blood and CSF from MS patients ($n = 14$), patients with other neurological disorders (OND; $n = 9$) and compared peripheral levels with healthy controls ($n = 40$). We found that the frequency of CD4$^+$ CD25$^+$ forkhead transcription factor 3 (FOXP3)$^+$ nT$_{reg}$ was significantly elevated in the CSF from MS patients (mean CSF = 4·05 ± 1·54% *versus* mean peripheral blood = 2·93 ± 0·94%) but not from patients with other neurological disorders (mean CSF = 3·78 ± 1·26% *versus* mean peripheral blood = 3·74 ± 1·4%). The frequency of nT$_{reg}$ in the periphery did not differ between MS patients and healthy donors; however, nT$_{reg}$ from MS patients showed reduced suppressive capacity.

Keywords: CSF, multiple sclerosis, regulatory T cells

Introduction

Autoimmune diseases result from the immune system's failure to maintain tolerance to self-structures. In multiple sclerosis (MS), the prototype autoimmune inflammatory disorder of the central nervous system, myelin-reactive T helper cells play a pivotal role in orchestrating self-reactive immune responses [1]. Peripheral regulatory mechanisms are necessary to control autoreactive cells which have escaped thymic tolerance. CD4$^+$ CD25$^+$ nT regulatory cells (nT$_{reg}$) are critical players, exerting their potential via active suppression. Depletion of CD4$^+$ CD25$^+$ nT$_{reg}$ in mice results in autoimmune disease and the transfer of these cells, together with CD4$^+$ CD25$^-$ T cells, can prevent autoimmunity in experimental models [2]. Although there is not yet a specific surface marker for this T cell subset, CD4 CD25$^+$ nT$_{reg}$ cells can be characterized on the basis of their high expression of CD25 (in contrast to the intermediate expression in recently activated T cells), their memory phenotype (CD45RO) and the intracellular expression of the forkhead transcription factor 3 (FOXP3) [3].

As opposed to animal models, it is still unclear how nT$_{reg}$ contribute to the development of human autoimmune disease. Autoimmune diabetes was the first example where decreased frequency of peripheral nT$_{reg}$ was reported as a potential indicator for the impaired state of peripheral tolerance to islet antigens [4]. However, available data on the frequency of nT$_{reg}$ in the peripheral blood of MS patients has so far not revealed significant differences [5–7]. At first glance this is not surprising, given the fact that peripheral blood may only reflect insufficiently the pathological situation of the disseminating inflammation within the central nervous system (CNS). Because direct investigation of brain tissue in MS patients is difficult, cerebrospinal fluid (CSF) seems to be the best alternative to study the immune elements involved in CNS pathology. We thus addressed this issue by measuring the frequency of CD4$^+$ CD25$^+$ nT$_{reg}$ in blood and CSF of MS patients in comparison to healthy

donors (HD) and patients with other neurological diseases (OND).

Materials and methods

Patients, CSF and blood specimens

Patients were diagnosed according to the criteria of McDonald *et al.* [8] and classified as clinically isolated syndrome (CIS), relapsing remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). All patients from whom CSF was analysed had a clinically isolated syndrome or relapsing remitting disease and were without corticosteroids for at least 2 months. CSF controls included patients with other neurological diseases of non-autoimmune origin such as dementia, normal pressure hydrocephalus and stroke (Table 1). All patients gave informed consent according to a protocol approved by the local ethics committee of the University of Tübingen. Eight to 20 ml of CSF were obtained by lumbar puncture from the patients. At the same time peripheral blood was collected by venous puncture. Forty age- and sex-matched healthy donors were used as controls for the analysis of peripheral blood.

Flow cytometry analysis of peripheral blood and CSF: quantitative analysis of $CD4^+$ $CD25^{bright}$ nT_{reg}

The percentage of $CD4^+$ $CD45RO^+$ cells expressing high levels of CD25 was quantified by flow cytometry on a fluorescence activated cell sorter (FACS) Calibur cytometer (BD Biosciences, Heidelberg, Germany). After lysis of the erythrocytes, 100 µl of peripheral blood were incubated with anti CD4-peridinin-chlorophyll-protein complex (PerCP), anti-CD45RO-fluorescein isothiocyanate (FITC), anti-CD25-phycoerythrin (PE) (BD Biosciences) or with the relevant isotype controls. CSF was processed at 4°C immediately after spinal tap. Cells were centrifuged after collection and resuspended in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) before staining with the corresponding antibodies. A minimum of 6000 cells per staining was required, with at least 1000 events measured.

For analysis, a first gate on a forward scatter/CD4-PerCP dot plot was set up to collect all $CD4^+$ cells excluding the $CD4^{low}$ monocytes. A second dot plot (CD45RO-FITC/CD25-PE) gated on the $CD4^+$ cells selected previously allowed us to visualize the different levels of expression of CD25 in the CD4-positive memory cells ($CD45RO^+$). A quadrant was set on this second dot plot such that the percentage of $CD25^{bright}$ cells in the CD45RO-negative fraction (upper left quadrant) always contained 0·1% of $CD25^{bright}$ cells. The percentage of $CD45RO^+$ $CD25^{bright}$ (upper right quadrant) was given as the frequency of $CD4^+$ $CD25^+$ nT_{reg} in blood or CSF.

Expression of FOXP3

Expression of FOXP3 was assessed by intracellular staining according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Briefly, cells were first surface-stained with CD25-allophycocyanin (APC) (BD Biosciences) and CD4-Pacific blue (Dako, Hamburg, Germany). After washing, cells were resuspended in fixation/permeabilization buffer, incubated for 30 min at room temperature (RT) in the dark and washed. Next, cells were blocked with 2% normal rat serum and subsequently the FOXP3 antibody (PCH101) or the isotype control (rat IgG2a) was added for 30 min incubation at 4 °C. After washing, cells were analysed with the Cyan, using the Summit software (Dako Cytomation).

Isolation of nT_{reg}

nT_{reg} cells were isolated according to the manufacturer's protocol ($CD4^+$ $CD25^+$ regulatory T cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, CD4 T cells were isolated by negative selection, incubated subsequently with CD25 microbeads and enriched by positive selection. To ensure highest purity, $CD4^+$ $CD25^+$ T cells were run over a second column. In all cases purity was > 92%.

Suppression assay for nT_{reg}

Lymphocyte proliferation of responder T cells ($CD4^+$ $CD25^-$ T cells) was assessed in the presence of nT_{reg}. Briefly, 1×10^6 $CD4^+$ $CD25^-$ responder T cells were incubated in 500 µl of PBS containing 10 µM carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes, Karlsruhe, Germany) and blocked subsequently with medium containing 15% FCS. To assess the suppressive nature of nT_{reg}, CFDA-SE-labelled responder T cells (1×10^5 cells per well) were cultured in the presence of increasing numbers of nT_{reg}, irradiated allogeneic PBMCs and 1 µg/ml soluble anti-CD3 monoclonal antibody (OKT3). We measured proliferation on the fourth day of culture by flow cytometry. Suppression was calculated after normalization of the values to a maximum given by the proliferation of responder T cells in the absence of nT_{reg}.

Statistical analysis

Paired and unpaired *t*-tests were used to compare the frequency of $CD25^{bright}$ cells in (i) in peripheral blood $CD4^+$ T cells from patients with MS (including subgroups) and healthy donors, and (ii) between CSF and blood from MS and OND patients. In all cases the *P*-values were calculated two-tailed and considered statistically significant if $P < 0{\cdot}05$.

Results

MS patients and healthy donors have similar frequencies of nT_{reg} in peripheral blood

Thirty-six patients with MS (CIS, $n = 5$; RRMS, $n = 25$; SPMS, $n = 5$; PPMS, $n = 1$; ages 21–62, mean = 40) and 40

Table 1. Characteristics of multiple sclerosis (MS) patients and cerebrospinal fluid (CSF) controls.

No.	Sex	Age (years)	Diagnosis/classification	Acute disease	CSF analysed
HD1	F	24	Healthy donor		
HD2	M	39	Healthy donor		
HD3	M	66	Healthy donor		
MS1	F	26	CIS	Yes	No
MS2	M	34	RRMS	Yes	No
MS3	M	60	SPMS	Yes	No
MS4	M	42	RRMS	No	Yes
MS5	M	37	RRMS	No	Yes
MS6	M	38	RRMS	yes	Yes
MS7	F	40	CIS	Yes	Yes
MS8	M	37	CIS	Yes	Yes
MS9	M	41	CIS	No	Yes
MS10	F	54	SPMS	Yes	Yes
MS11	M	50	RRMS	Yes	Yes
MS12	F	39	RRMS	Yes	Yes
MS13	F	28	RRMS	No	Yes
MS14	F	42	RRMS	Yes	Yes
MS15	M	44	CIS	No	Yes
MS16	F	38	RRMS	No	Yes
MS17	F	37	RRMS	No	Yes
MS18	F	36	RRMS	No	No
MS19	F	47	RRMS	No	No
MS20	M	61	SPMS	No	No
MS21	F	35	SPMS	No	No
MS22	M	44	CIS	Yes	No
MS23	M	36	SPMS	No	No
MS24	F	25	RRMS	No	No
MS25	F	31	RRMS	No	No
MS26	F	33	RRMS	No	No
MS27	F	35	RRMS	No	No
MS28	F	37	RRMS	Yes	No
MS29	F	40	RRMS	No	No
MS30	F	42	RRMS	No	No
MS31	F	50	RRMS	No	No
MS32	F	55	RRMS	No	No
MS33	F	49	RRMS	Yes	No
MS34	M	21	RRMS	No	No
MS35	M	25	RRMS	No	No
MS36	M	26	SPMS	Yes	No
MS37	M	62	PPMS	No	No
MS38	M	38	RRMS	No	No
MS39	M	47	RRMS	Yes	No
OND1	W	24	Stroke		Yes
OND2	M	24	Disturbance eye movements		Yes
OND3	W	33	Stroke		Yes
OND4	M	56	Polyneuropathy		Yes
OND5	M	80	Normal pressure hydrocephalus		Yes
OND6	M	79	Dementia		Yes
OND7	W	66	Dementia		Yes
OND8	M	76	Dementia		Yes
OND9	M	74	Normal pressure hydrocephalus		Yes
OND10	W	24	Myelopathy		Yes

CIS: clinically isolated syndrome; OND: other neurological diseases; PPMS: primary progressive MS; RRMS: relapsing remitting MS, SPMS: secondary progressive MS.

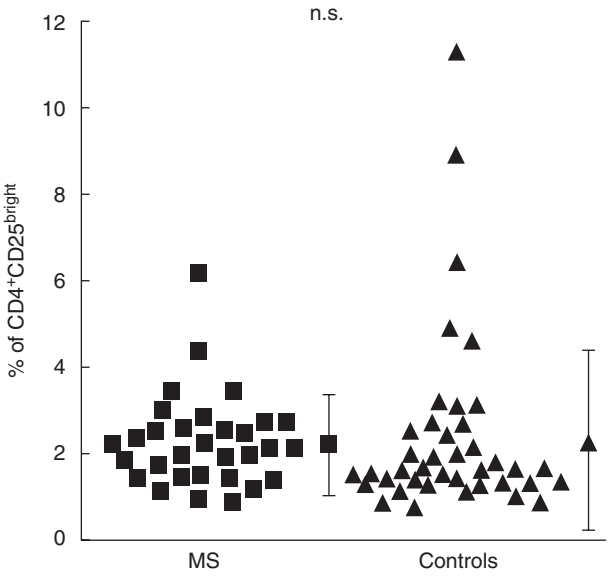

Fig. 1. Comparison of nT_{reg} frequencies in peripheral blood from multiple sclerosis (MS) patients and healthy donors. Percentages of $CD4^+$ $CD25^{bright}$ T cells in peripheral blood of patients with MS ($n = 36$; black squares) in comparison to healthy controls ($n = 40$; black triangles) were assessed by flow cytometry after staining for CD4, CD45RO and CD25. Gates for CD4 cells were set on FSC/CD4 and dot plots to identify the desired subsets. A quadrant was set on a second dot plot (CD45RO/CD25), such that the percentage of $CD25^{bright}$ cells in the CD45RO-negative fraction (upper left quadrant) always contained 0·1% of $CD25^{bright}$ cells. Percentages of $CD45RO^+$ $CD25^{bright}$ T cells (upper right quadrant) were given as the frequency of $CD4^+$ $CD25^+$ nT_{reg}. The mean ± s.d. is shown for each group. The statistical difference is non-significant (n.s.).

age- and sex-matched healthy controls (ages 23–80, mean = 40) were analysed for the frequency of $CD4^+$ $CD25^{bright}$ cells in peripheral blood. In agreement with previous data [5–7], no significant differences were found in the frequency of nT_{reg} between both groups. The average percentage of $CD25^{bright}$ cells in the CD4 compartment was 2·13 ± 1·19% for MS patients and 2·28 ± 2·18% for healthy donors ($P = 0·71$) (Fig. 1).

Furthermore, no significant differences were observed (i) in relation to disease subtype (CIS, RRMS, SPMS or PPMS) or (ii) disease activity (acute relapse *versus* stable disease) (data not shown). Similarly, patients with OND ($n = 9$) did not differ in their peripheral blood nT_{reg} from MS or HD (data not shown).

Suppressive function of $CD4^+$ $CD25^+$ nT_{reg} is impaired in MS patients

Two recent studies have demonstrated an impaired function of $CD4^+$ $CD25^+$ nT_{reg} in MS [6,7], which might serve as an explanation for the loss of tolerance in CNS autoimmunity. To investigate if this was also the case in our patients, we selected a few patients and controls and performed suppression assays in which nT_{reg} were titrated into stimulated responder T cells at different ratios. In the cases analysed ($n = 3$ for MS – acute and untreated – and HD, sex- and age-matched) we also observed a reduced capacity of nT_{reg} from MS patients to suppress proliferation of the corresponding responder population. Suppression by nT_{reg} at a 1 : 1 responder to suppressor ratio ranged between 34 and 41% in MS patients, whereas nT_{reg} from healthy donors showed 68–91% suppression (Fig. 2). Thus, our data in a small series of cases is consistent with the reported reduced suppressive capacity of nT_{reg} in MS patients.

$FOXP3^+$ $CD4^+$ $CD25^{bright}$ nT_{reg} infiltrate the CSF in patients with neuroinflammatory condition

The paucity of T cells in the CNS of MS patients prevented us from performing suppression assays using CSF $CD4^+$ $CD25^+$ nT_{reg}. However, to check if indeed these cells have the requirements to be regulatory, we assessed expression of FOXP3 in CSF samples from patients with neuroinflammatory disease (Fig. 3). FOXP3 is considered the lineage specification marker for nT_{reg}, but until now no specific cell molecule for $CD4^+$ $CD25^{bright}$ nT_{reg} has been described. We therefore performed a combination of surface (CD4 and CD25) and intracellular staining (FOXP3) in both PBMC and CSF from several patients to check if the cells that are $CD25^{bright}$ are also expressing FOXP3. As seen in Fig. 3, in both PBMC and CSF all $CD25^{bright}$ cells are also positive for FOXP3, and thus they can be defined as nT_{reg}. In addition, in both samples a similar percentage of $FOXP3^+$ cells remain CD25 negative, likely to constitute the reservoir of committed regulatory cells that regain CD25 expression and suppressive function upon activation [9].

The frequency of nT_{reg} in the CSF of MS patients is higher than in peripheral blood

We next compared the frequency of nT_{reg} in paired samples of CSF and peripheral blood in 14 patients with MS and nine with OND. Nearly all T cells in the CSF show a memory ($CD45RO^+$) phenotype [10]. Thus, in order to obtain a more relevant comparison between blood and CSF, we analysed the content of $CD25^{bright}$ in the $CD4^+$ $CD45RO^+$ compartment. Interestingly, the percentage of nT_{reg} was elevated significantly in the CSF of MS patients in comparison to their peripheral blood (mean CSF = 4·05 ± 1·54% *versus* mean peripheral blood = 2·93 ± 0·94%, $n = 14$, $P = 0·022$) (Fig. 4). An increase of nT_{reg} in the CSF compartment in comparison to the periphery was noticeable in 11 of the 14 MS cases analysed (79%). This is in contrast to other neurological diseases of non-inflammatory, non-autoimmune origin, where the percentages of $CD4^+$ $CD25^+$ nT_{reg} in CSF remained very similar to blood (mean CSF = 3·78 ± 1·26% *versus* mean peripheral blood =

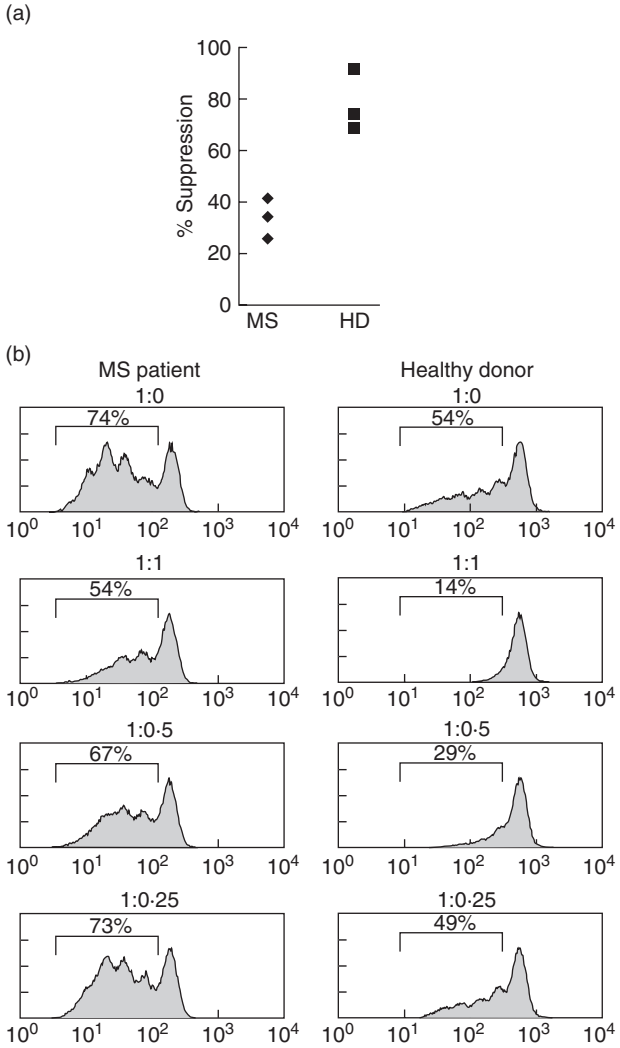

Fig. 2. Impaired suppressive function of nT_{reg} in multiple sclerosis (MS) patients. Suppression assay with $CD4^+$ $CD25^+$ nT_{reg}. $CD4^+$ $CD25^+$ nT_{reg} were titrated into carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)-labelled $CD4^+$ $CD25^-$ responder T cells cultured in the presence of allogeneic peripheral blood mononuclear cells (PBMC) and soluble anti-CD3. Cell proliferation and influence of $CD4^+$ $CD25^+$ nT_{reg} was quantified by fluorescence-activated cell sorter (FACS) analysis on day 4. (a) Graph shows a summary of suppression assays from three MS patients (left) and three healthy donors (HD) (right). Responder ($CD4^+$ $CD25^-$) to suppressor ($CD4^+$ $CD25^+$ nT_{reg}) ratio is 1 : 1. (b) FACS analysis of a representative suppression assay from a MS patient (left) and HD (right) with percentages of proliferation stated in histogram. Responder to suppressor ratios are given on top of each histogram.

3·74 ± 1·48, $n = 9$, $P = 0·92$) (Fig. 4). Of note, in the CSF we could not find significant differences in the frequency of nT_{reg} in MS patients separated according to their disease course. Taken together, our results show that nT_{reg} exist in the CSF, are elevated in MS patients in comparison to peripheral blood, and therefore have the potential to modulate immune responses in the target organ.

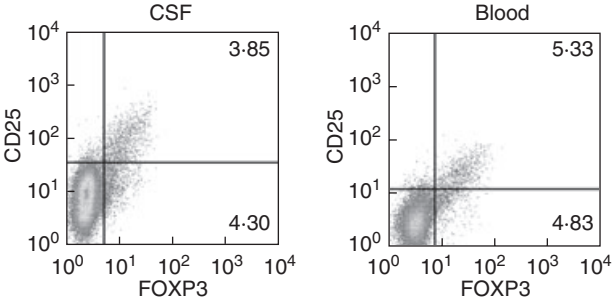

Fig. 3. Identification of CD25 and forkhead transcription factor 3 (FOXP3)-expressing CD4 cells from peripheral blood and cerebrospinal fluid (CSF). Fluorescence activated cell sorter (FACS) staining of a paired CSF/blood sample from a patient with neuroinflammatory disease for surface CD4 and CD25, and intracellular FOXP3. A gate for $CD4^+$ T cells was set on forward scatter/CD4 dot plots to identify the desired subset. In a second dot plot, co-expression of FOXP3 and CD25 was assessed in comparison to the control isotype staining. A representative staining is shown for CSF cells (left) and blood (right).

Discussion

The investigation of nT_{reg} in human autoimmunity has attracted considerable attention. Several studies have recently analysed the frequency and function of $CD4^+$ $CD25^{bright}$ nT_{reg} in multiple sclerosis, the prototype of an autoimmune inflammatory CNS disease [6–7,11,12]. All

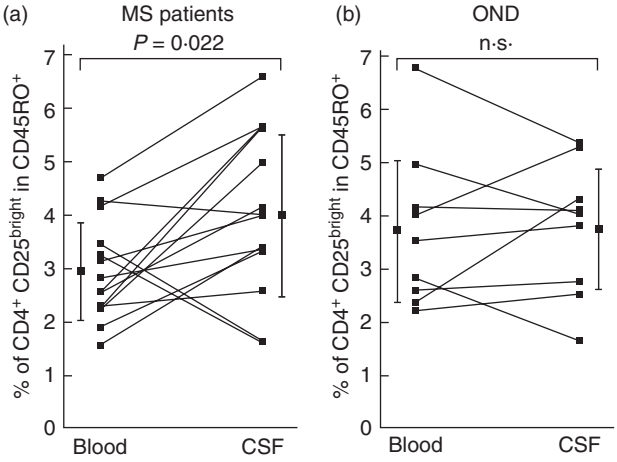

Fig. 4. Comparison of nT_{reg} frequencies in the blood and cerebrospinal fluid (CSF) from multiple sclerosis (MS) patients and other neurological disorders (OND). (a) Percentages of $CD4^+$ $CD25^{bright}$ T cells in the $CD4^+$ $CD45RO^+$ compartment of blood and CSF of MS patients ($n = 14$). Direct comparison of blood *versus* CSF for each analysed individual is represented by connected dots. The difference, indicated by the mean ± s.d., is statistically significant ($P = 0·022$). (b) Percentages of $CD4^+$ $CD25^{bright}$ T cells in the $CD4^+$ $CD45RO^+$ compartment of blood and CSF are represented for patients with OND ($n = 9$). No significant differences were detected. For all groups, flow cymetry analysis for CSF and blood was conducted according to Fig. 1.

these studies, however, assessed peripheral lymphocytes. Assuming that the CSF represents the fluid compartment that is closest to reflect the immunopathogenic situation in MS, this study is the first to demonstrate the presence and enrichment of $FOXP3^+$ $CD4^+$ $CD25^{bright}$ regulatory T cells in close proximity to the autoimmune target CNS.

Similar to the enrichment of functionally active $CD4^+$ $CD25^+$ nT_{reg} in the synovial fluid of inflamed joints that has been observed in rheumatoid arthritis [13,14], our results show that there is an imbalance in the proportion of nT_{reg} in peripheral blood compared to the target organ of inflammation. An increase in nT_{reg} in the CSF was found in 79% of patients with neuroinflammation when compared to their own peripheral compartment, which was accompanied by a modest decrease in the percentage of nT_{reg} in blood.

Besides $FOXP3^+$ $CD25^{high}$ T cells, a similar proportion of $FOXP3^+$ $CD25^{low}$ cells can be found in CSF and blood (see Fig. 3). Studies of $FOXP3^{gfp}$ mice have already pointed to the existence of different populations according to FOXP3 and CD25 expression being the CD25 and FOXP3 double-positive T cells, those with a secure regulatory phenotype [3]. In addition, Zelenay *et al*. reported the presence of $FOXP3^+$ $CD25^{low}$ cells, and have suggested that these cells constitute a reservoir of differentiated regulatory cells that can up-regulate CD25 expression upon activation [9]. Therefore all $CD25^+$ cells that we have considered as regulatory T cells are $FOXP3^+$, and it can therefore be assumed that they represent cells with a truly regulatory phenotype. Our results suggest that nT_{reg} from the periphery are recruited into the CSF; thus we see the slight decrease in the periphery, rather than expanding once some nT_{reg} reach the CSF or the target organ. Furthermore, *de novo* generation of nT_{reg} in the target organ in MS is a possible but unlikely explanation for the observed distribution of nT_{reg}.

It is not known how this enriched population of nT_{reg} in the CSF relates to the lesion pathogenesis in MS. In contrast to animal studies, it is very difficult to assess the 'antigen specificity' of $CD4^+$ $CD25^+$ nT_{reg} and their migration patterns into the CNS in humans. Similarly, functional analysis of nT_{reg} from CSF is virtually impossible, due to the low frequency of CSF cells, as well as the availability of CSF itself.

Our data suggest that the enrichment of nT_{reg} in the CSF is related selectively to the autoimmune inflammatory state in MS, because other neurological diseases of non-autoimmune, non-inflammatory origin (dementia, normal pressure hydrocephalus and stroke) did not show significant differences. It may even seem counterintuitive that, despite the presence (and increase) of regulatory T cells in the CSF, autoimmune inflammatory activity in the CNS occurs. Obviously, in spite of enrichment, nT_{reg} are not sufficient to combat the ongoing inflammation. Insufficiency may either be a matter of quantity or functional capability. Strong arguments voting for the latter hypothesis are studies which show impaired suppressive capacity of $CD4^+$ $CD25^+$ nT_{reg} in MS patients [6,7]. This impairment seems to be limited to RRMS patients, as suppressive function in SPMS patients is unaffected [12]. However, this difference in function of nT_{reg} in different disease courses is not reflected in their frequency. Therefore, our hypothesis would be that nT_{reg} are up-regulated in the CSF of MS patients because they try to combat and down-regulate ongoing chronic (auto)immunity. However, functional impairment of nT_{reg} in MS patients could have contributed (i) to the development of the disease and (ii) the inefficiency in turning down T cell activity towards CNS structures.

The idea of local immunosuppression by functional nT_{reg} affecting disease activity and progression is appealing, both from an immunopathogenetic and a therapeutic view. It remains to be shown whether an increase in the number or function of nT_{reg} and a parallel decrease in number or function of pathogenic T cells could possibly account for the phases of relapses and remissions in MS.

Acknowledgements

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