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T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention

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Interleukin-17-expressing CD4$^+$ T helper 17 (Th17) cells are considered as critical regulators of multiple sclerosis disease activity. However, depending on the species and pro-inflammatory milieu, Th17 cells are functionally heterogeneous, consisting of subpopulations that differentially produce interleukin-17, interferon-gamma and granulocyte macrophage colony-stimulating factor. In the current study, we studied distinct effector phenotypes of human Th17 cells and their correlation with disease activity in multiple sclerosis patients. T helper memory populations single- and double-positive for C-C chemokine receptor 6 (CCR6) and CXC chemokine receptor 3 (CXCR3) were functionally assessed in blood and/or cerebrospinal fluid from a total of 59 patients with clinically isolated syndrome, 35 untreated patients and 24 natalizumab-treated patients with relapsing-remitting multiple sclerosis, and nine patients with end-stage multiple sclerosis. Within the clinically isolated syndrome group, 23 patients had a second attack within 1 year and 26 patients did not experience subsequent attacks during a follow-up of >5 years. Low frequencies of T helper 1 (Th1)-like Th17 (CCR6$^+$CXCR3$^+$), and not Th17 (CCR6$^+$CXCR3$^-$) effector memory populations in blood strongly associated with a rapid diagnosis of clinically definite multiple sclerosis. In cerebrospinal fluid of clinically isolated syndrome and relapsing-remitting multiple sclerosis patients, Th1-like Th17 effector memory cells were abundant and showed increased production of interferon-gamma and granulocyte macrophage colony-stimulating factor compared to paired CCR6$^+$ and CCR6$^-$CD8$^+$ T cell populations and their blood equivalents after short-term culturing. Their local enrichment was confirmed *ex vivo* using cerebrospinal fluid and brain single-cell suspensions. Across all pro-inflammatory T helper cells analysed in relapsing-remitting multiple sclerosis blood, Th1-like Th17 subpopulation T helper 17.1 (Th17.1; CCR6$^+$CXCR3$^+$CCR4$^-$) expressed the highest very late antigen-4 levels and selectively accumulated in natalizumab-treated patients who remained free of clinical relapses. This was not found in patients who experienced relapses during natalizumab treatment. The enhanced potential of Th17.1 cells to infiltrate the central nervous system was supported by their predominance in cerebrospinal fluid of early multiple sclerosis patients and their preferential transmigration across human brain endothelial layers. These findings reveal a dominant contribution of Th1-like Th17 subpopulations, in particular Th17.1 cells, to clinical disease activity and provide a strong rationale for more specific and earlier use of T cell-targeted therapy in multiple sclerosis.

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Abbreviations: CDMS = clinically definite multiple sclerosis; CIS = clinically isolated syndrome; GM-CSF = granulocyte macrophage colony-stimulating factor; RRMS = relapsing-remitting multiple sclerosis

Introduction

Multiple sclerosis is mediated by effector T cells trafficking from the periphery into the CNS to trigger local inflammation, demyelination and neurodegeneration (Dendrou *et al.*, 2015). Although current T cell-directed treatment attenuates disease activity, it often causes serious complications and does not prevent disease progression in multiple sclerosis (Ransohoff *et al.*, 2015). To improve treatment efficacy and risk management, more in-depth insight into human effector T cells during multiple sclerosis onset is warranted. In the earliest clinical presentation of multiple sclerosis, clinically isolated syndrome (CIS), increased peripheral $CD4^+$ T cell activation is linked to the occurrence of a second attack (Corvol *et al.*, 2008). However, substantial knowledge has been gained about specific human T helper (Th) functions, and the exact nature of the pro-inflammatory T helper subsets involved in multiple sclerosis is incompletely understood.

Both Th1 and Th17 cells are known to be encephalitogenic, but use distinct transmigration routes to enter the CNS. In experimental autoimmune encephalomyelitis, Th1 cells preferentially migrate into the spinal cord, while Th17 cells mainly infiltrate the brain (Stromnes *et al.*, 2008). This is facilitated by their differential expression of pro-inflammatory cytokines, chemokine receptors and integrins (Bauer *et al.*, 2009; Reboldi *et al.*, 2009; Larochelle *et al.*, 2011). Interleukin-17 (IL-17) and C-C chemokine receptor 6 (CCR6) are key determinants for Th17 transmigration across the blood–brain barrier (Kebir *et al.*, 2007; Reboldi *et al.*, 2009). IL-17 is generally considered as the signature cytokine produced by CCR6-positive Th17 cells. However, this greatly underestimates Th17 effector function, since subpopulations also (co-)produce interferon-gamma (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF). Next to IL-17, also IFN-γ and GM-CSF are strongly produced by myelin-specific CCR6-positive T helper cells in multiple sclerosis (Cao *et al.*, 2015). Th17 polyfunctionality is differently regulated between species, as reflected by the antagonistic regulation of IL-17 and GM-CSF expression in human compared to murine T helper cells (El-Behi *et al.*, 2011; Noster *et al.*, 2014; Paterka *et al.*, 2016). Particularly GM-CSF produced by T helper cells is implicated as a critical mediator of multiple sclerosis onset (Codarri *et al.*, 2011; Hartmann *et al.*, 2014).

The surface expression of another chemokine receptor, CXCR3, defines Th17 cells with Th1-like features (Acosta-Rodriguez *et al.*, 2007). CCR6 and CXCR3 expression on $CD4^+$ T cells is controlled by transcription factors RORγt and T-bet, respectively, which were originally associated with IL-17/IFN-γ double-production (Acosta-Rodriguez *et al.*, 2007). However, recent findings demonstrate more heterogeneous IL-17, IFN-γ and GM-CSF expression profiles in Th17 cells, depending on the inflammatory milieu (Duhen and Campbell, 2014). Besides CCR6 and CXCR3, also the presence of the α4β1 integrin very late antigen-4 (VLA-4), which is abundant on Th17 cells in multiple sclerosis CSF (Brucklacher-Waldert *et al.*, 2009), determines T cell transmigration capacities. Anti-VLA-4 monoclonal antibody natalizumab is currently one of the most effective therapies in multiple sclerosis, but relapses are still encountered after 1 year in about one-third of treated patients (Polman *et al.*, 2006). Understanding which distinct pro-inflammatory T helper subsets are targeted by natalizumab will help to better predict treatment response in multiple sclerosis (Prosperini *et al.*, 2012).

Here, blood and CSF samples from CIS and both untreated and natalizumab-treated relapsing-remitting multiple sclerosis (RRMS) patients were assessed for distribution, memory phenotype, activation and pro-inflammatory capacity of Th17 subsets. We reveal that IFN-γ-/GM-CSF-producing ($CCR6^+CXCR3^+$), but not IL-17-producing ($CCR6^+CXCR3^-$) Th17 effector cells are key regulators of multiple sclerosis onset. A Th1-like Th17 subpopulation termed Th17.1 ($CCR6^+CXCR3^+CCR4^-$) is selectively targeted by natalizumab in patients with multiple sclerosis who remained free of clinical relapses. This work supports the design and early use of therapeutic strategies against Th17.1 cells to prevent relapses in multiple sclerosis.

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Materials and methods

Patients

Characteristics of patients and controls in the screening cohorts are summarized in Table 1. Main experimental results were confirmed using additional cohorts (Supplementary Table 1). All CIS and RRMS patients as well as controls were included at Erasmus MC (Rotterdam, The Netherlands), which is a national tertiary referral centre for patients with multiple sclerosis (MS Center ErasMS). All primary material was collected between 2007 and 2017.

For blood analyses, we selected 23 patients with CIS who did not experience a second clinical attack for at least 5 years of follow-up (CIS-CIS) and 26 CIS patients who were diagnosed with clinically definite multiple sclerosis (CDMS) within 1 year after CIS (CIS-CDMS) from our prospective cohort. None of these patients were treated with disease-modifying therapies before or at time of sampling. CIS was defined as a first clinical attack of demyelination in the CNS (Miller *et al.*, 2012). CDMS diagnosis was made when a patient experienced two attacks with clinical evidence of two separate lesions according to the Poser criteria (Poser *et al.*, 1983). A relapse was defined as subacute worsening of existing symptoms, or new symptoms after at least 30 days of improvement or stable disease (Schumacher *et al.*, 1965). Fatigue scores were acquired at the time of the first attack using the self-administered Krupp's Fatigue Severity Scale, as shown previously (Runia *et al.*, 2015). Anti-EBNA1 IgG levels were determined in plasma using a well-validated chemiluminescent assay and analyser (Liaison XL; both Diasorin) according to the manufacturers' instructions at our local referral centre for virus diagnostics (Erasmus MC).

Patients with RRMS were diagnosed according to the McDonald 2010 criteria (Polman *et al.*, 2011). Blood T helper cell analyses were performed for 31 treatment-naïve RRMS patients, as well as for 24 RRMS patients before the start and after 6 and 12 months of natalizumab therapy. The median time between the last clinical attack and first administration of natalizumab was 2.8 months [interquartile range (IQR): 1.7–6.3]. Seventeen of these patients (70.8%) were treated with disease-modifying therapy before initiation of natalizumab (14 with IFN-β, one with both dimethylfumarate and fingolimod, one with glatiramer acetate and one with mitoxantron).

CSF with and without paired blood samples was obtained from 14 patients with early-stage multiple sclerosis (ErasMS) and nine with late-stage multiple sclerosis (Netherlands Brain Bank, Amsterdam). In the early-stage multiple sclerosis group, 10 patients were CIS at the time of lumbar puncture and four patients were diagnosed with RRMS within 6 months before sampling. The median time between sampling and the last clinical attack was 2.8 months (IQR: 1.3–5.8). Additional autopsied brain tissues were obtained from five patients with late-stage multiple sclerosis and two non-demented control

Table 1 Characteristics of patients and controls in screening cohorts

Blood, *ex vivo*					
Cohort	**HC**	**CIS-CIS**	**CIS-CDMS**	**RRMS, no treatment[a]**	**RRMS, NAT treatment[a]**
Patients, *n*	19	16	16	18	17[b]
Gender, female, *n* (%)	16 (84)	11 (69)	13 (81)	15 (83)	12 (71)
Age in years, median (IQR)[c]	45 (35–49)	36 (27–40)	33 (28–37)	46 (37–50)	38 (30–46)[d]
Follow-up time in years, median (IQR)	NA	6.8 (6.2–7.3)	4.1 (3.1–5.7)	NA	NA
Disease duration in months, median (IQR)[e]	NA	2.0 (1.3–3.1)	2.0 (1.2–3.0)	120 (48–193)	92 (48–160)[d]
⩾9 lesions on T_2-weighted images at baseline, *n* (%)	NA	3 (19)	10 (63)	NA	NA
CSF/brain, *ex vivo*				**CSF, TCC**	
Cohort[f]	**Early MS**	**Late MS**	**Late NDC**	**Early MS**	**Late MS**
Patients, *n* (paired blood)	4 (4)	5 (5)	2 (2)	10 (4)	7 (7)[g]
Gender, female *n* (%)	2 (50)	5 (100)	1 (50)	10 (100)	5 (71)
Age in years, median (IQR)[c]	32 (18–41)	62 (44–72)	78 (NA)	33 (23–38)	70 (60–82)
Follow-up time in years, median (IQR)	0.3 (0.3–0.5)	NA	NA	1.5 (0.6–5.5)	NA
Disease duration in months, median (IQR)[e]	3.8 (2.7–5.2)	NA	NA	3.8 (1.0–22.4)	NA
PMD in hours, median (IQR)	NA	8.5 (8.4–9.2)	6.1 (NA)	NA	8.6 (8.3–9.3)
pH CSF, median (IQR)	NA	6.3 (6.3–6.7)	6.5 (NA)	NA	6.5 (6.3–6.7)

[a]RRMS according to the McDonald (2010) criteria.
[b]Fourteen patients were included for in-depth analysis of Th17 subpopulations in blood. For three patients, T helper subsets were only used for analysis of pro-inflammatory cytokine expression.
[c]At the time of sampling.
[d]At the time of pretreatment sampling.
[e]Time from CIS diagnosis to sampling.
[f]Samples obtained from either CIS and RRMS patients ('early') or deceased patients with multiple sclerosis and non-demented control subjects ('late').
[g]Three patients were also used for *ex vivo* CSF/brain T cell analysis.
HC = healthy control; MS = multiple sclerosis; NA = not applicable or available; NAT = natalizumab; NDC = non-demented control; PMD = post-mortem delay; TCC = T cell culture.

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subjects (Netherlands Brain Bank). All study protocols were approved by the medical ethics committee of the Erasmus MC (Rotterdam) and VUmc (Amsterdam, The Netherlands). Written informed consent was obtained from all included patients and controls.

Mononuclear cell isolation from blood, CSF and brain tissue

Blood from patients and matched controls was collected using Vacutainer CPT tubes (BD Biosciences) containing sodium heparin. Peripheral blood mononuclear cells were isolated according to the manufacturer's instructions. After centrifugation, cells were taken up in RPMI1640 (Lonza) containing 40% foetal calf serum and 20% dimethyl sulfoxide (Sigma-Aldrich) and stored in liquid nitrogen until further use. Surplus CSF of patients with early-stage multiple sclerosis was obtained through lumbar puncture for diagnostic purposes. Blood and CSF samples from patients with late-stage multiple sclerosis were acquired post-mortem through heart puncture and ventricle drainage, respectively. Collection tubes with CSF were centrifuged for 10 min at 500*g*. Paired blood and blood from buffy coats was diluted in phosphate-buffered saline (PBS), after which mononuclear cells were isolated by density gradient centrifugation using Ficoll® Paque Plus (GE Healthcare). CSF and blood mononuclear cell fractions were resuspended in RPMI 1640 containing 10% heat inactivated human AB serum (Sanquin) and 1% Pen/Strep (Lonza) and left to rest at 37°C until further use. Brain tissue samples were processed and single-cell suspensions were obtained as described previously (van Nierop *et al.*, 2017).

Short-term CSF and blood T cell cultures

Short-term culturing of CSF-derived T cells was required to obtain sufficient cell numbers for fluorescence-activated cell sorting (FACS) and intracellular cytokine staining of the T helper subsets of interest. CSF and blood T cells were cultured as previously described (van Nierop *et al.*, 2016). In short, mononuclear cell fractions were treated for 13 to 15 days with γ-irradiated feeder cells (10×10^6 peripheral blood mononuclear cells and 10^6 EBV$^-$ B cell lines HAL-02 and RS-411), phytohaemagglutinin-L (1 ng/ml; Sigma-Aldrich), IL-2 (25 U/ml; Erasmus MC) and IL-15 (12.5 ng/µl; Miltenyi Biotec) in RPMI1640 containing L-glutamine (Lonza), 1% Pen/Strep and 10% heat-inactivated human AB serum. IL-2 and IL-15 were added every 3 to 4 days. Post-mortem CSF samples were restimulated using the same protocol.

Antibodies and flow cytometry

Multicolour flow cytometric analysis was performed using the following fluorochrome-labelled monoclonal anti-human antibodies: CD3 BV785 (SK7), CD8 FITC (SK1), CD45RA APC-H7 (HI100), HLA-DR FITC and BB515 (G46-6), VLA-4 BV711 and APC (9F10), CD45RO PerCP-Cy5.5 (UCHL1), CD25 BV605 and APC-R700 (2A3), CD226 BB515 (DX11), MCAM PerCP-Cy5.5 (P1H12), PSGL-1 APC (KPL-1), GM-CSF BV421 and PE-CF594 (BVD2-21C11) (all BD Biosciences), CD4 BV510 (OKT4), CD38 BV711 and PerCP-Cy5.5 (HIT2), CXCR3 BV421, PE-Cy7 and APC (G025H7), CCR6 PE (G034E3), CCR7 PE-CF594 (150503), CCR4 PE-Cy7 and PE-Dazzle (L291H4), CD161 BV605 (HP-3G10), IFN-γ BV421 and BV711 (4S.B3), and IL-17A APC (BL168) (all BioLegend). Cells were stained for 30 min at 4°C, measured with an LSRII-Fortessa flow cytometer and analysed using FACSDiva 6.1.2 software (both BD Biosciences). Th1, Th17 and Th1-like Th17 cells were defined based on markers CCR6 and CXCR3 with and without the use of CCR4. For analyses without CCR4, total CD4$^+$ T cells were subdivided into CCR6$^-$CXCR3$^+$ (Th1), CCR6$^+$CXCR3$^-$ (Th17) and CCR6$^+$CXCR3$^+$ (Th1-like Th17) subsets. In each T helper subset, the proportion of effector memory (CCR7$^-$CD45RA$^-$) and central memory (CCR7$^-$CD45RA$^+$) cells was analysed. CCR4 was used as a marker to discriminate CCR6$^-$CXCR3$^+$CCR4$^-$ (Th1), CCR6$^+$CXCR3$^-$CCR4$^+$ (Th17), CCR6$^+$CXCR3$^+$CCR4$^-$ (Th17.1) and CCR6$^+$CXCR3$^+$CCR4$^+$ [Th17 double-positive (DP)] subpopulations (Paulissen *et al.*, 2015).

Intracellular cytokine staining

Th1 (CCR6$^-$CXCR3$^+$), Th17 (CCR6$^+$CXCR3$^-$), Th1-like Th17 (CCR6$^+$CXCR3$^+$), as well as CCR6$^-$ and CCR6$^+$ CD8$^+$ T cells were sorted from blood and CSF T cell memory pools (CD3$^+$CD25$^{-/int}$ CD45RO$^+$CD45RA$^-$) using a BD FACSAria™ III cell sorter. Prior to isolation of T helper memory subsets from buffy coats, CD4$^+$ cells were purified from the mononuclear cell fraction using CD4 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 1:2000) and ionomycin (1:500; both Sigma-Aldrich) for 5 h. GolgiStop™ (1:1500; BD Biosciences) was added during the last 2.5 h of stimulation. Stimulated cells were fixed and permeabilized using the BD Cytofix/CytopermTM kit (BD Biosciences) according to the provided protocol, and stained for IFN-γ, GM-CSF and IL-17A within the same tube.

RNA isolation and quantitative PCR

Sorted T cell subsets were washed with PBS and resuspended in RNA lysis solution with 1% 2-ME. Total RNA was extracted using the GenElute™ Total RNA Purification kit (Sigma-Aldrich) and treated with DNase I (Invitrogen). Complementary DNA was synthesized from total RNA using a reaction mix containing Tris-aminomethane (200 mM), KCl (500 mM), $MgCl_2$ (0.2 M; Sigma-Aldrich), DTT (100 mM; Invitrogen), random hexamers (50 µM; Invitrogen), oligo(dT) 15 primer (100 µg/ml; Promega), dNTP mix (10 mM; Promega), RNAsin® (40 U/µl; Promega) and SuperScript™ II (200 U/µl; Invitrogen). After incubation at 42°C for 50 min and inactivation at 99°C for 3 min, cDNA was diluted and stored at −20°C until use. For quantitative PCR, 0.2 µM forward and reverse primer (Sigma-Aldrich), 10 µM probe (Universal Probe Library; Roche Applied Science) and diluted cDNA were added to TaqMan® Universal PCR Master Mix. Target gene expression was measured using optimal primer/probe assays and TaqMan® 7900HT (Applied Biosystems). We used the following thermal cycle protocol: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. CT values were analysed using SDS 2.4.1

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software (Applied Biosystems). Expression levels of target genes were normalized using 18S rRNA as a reference. Primer sequences are provided in Supplementary Table 2.

T cell transmigration assays

$CD4^+CD25^{-/int}$ memory T cells depleted from naïve populations ($CCR7^+CD45RA^+$) were sorted by FACS and added at 2×10^5 cells/well to 3 µm pore size transwell plates (Corning). Migration of Th17 subsets towards medium or CXCL10 (900 ng/ml; R&D Systems) was analysed after 3 h incubation at 37°C using FACS. To assess trans-endothelial migration of Th17 subsets, migration experiments were performed across confluent monolayers of human brain endothelial cells (hCMEC/D3 cell line) (Weksler *et al.*, 2005) on collagen-coated 5 µm pore size Transwell® plate, as previously described (Lopes Pinheiro *et al.*, 2016). In this system, 5×10^5 T helper memory cells were added per well and migration was assessed after 4 h.

Statistical analyses

Statistical analyses were carried out using Graphpad Prism Software, version 5.04. We used the two-tailed Mann-Whitney U-test to compare two independent groups and the Wilcoxon matched-pairs signed rank test to compare samples of the same persons. Correlations were tested using Spearman's rank. A logistic regression model was used to correct for MRI measurements in the multivariate analyses. Experimental data are depicted as mean and standard error of the mean. *P*-values < 0.05 were considered significant.

Results

Low frequencies of Th1-like Th17 and not Th17 effector cells in CIS blood associate with rapid multiple sclerosis onset

To search for pro-inflammatory T helper subsets that are critically involved in early diagnosis of CDMS, we used peripheral blood mononuclear cells at time of CIS from age- and gender-matched patients who remained monophasic for at least 5 years (CIS-CIS, $n = 16$) and from patients who experienced a second attack within 1 year (CIS-CDMS, $n = 16$; Table 1). Flow cytometric analysis of $CD4^+$ T cells showed decreased Th1-like Th17 ($CCR6^+CXCR3^+$) frequencies in the CIS-CDMS group compared to the CIS-CIS group (median: 5.9% versus 11.2%, $P = 0.011$; Fig. 1A). After correction for lesion load on MRI at baseline, using a logistic regression model, the association remains significant (OR: 0.78 per percent increase in Th1-like Th17; $P = 0.026$). In CIS-CDMS, additional reductions in effector memory (EM) to central memory (CM) ratios were found for Th1-like Th17 (mean: 0.30 versus 0.50, $P = 0.005$; Fig. 1B). Similar but less strong reductions were observed for Th1 ($CCR6^-CXCR3^+$; 10.0% versus 12.5%, $P = 0.070$ and mean EM/CM ratio: 0.21 versus 0.29, $P = 0.021$). Frequencies and EM/CM ratios for Th17 ($CCR6^+CXCR3^-$) did not differ between CIS-CIS and CIS-CDMS (Fig. 1A and B). T helper subset distribution in CIS patients was not affected after stratification for methylprednisolone treatment in the last 3 months prior to sampling (data not shown). Finally, Th1-like Th17 EM/CM ratios in CIS blood inversely correlated to anti-EBNA1 IgG titres ($P = 0.013$; Fig. 1C) and fatigue ($P = 0.001$; Fig. 1D), which were reported as independent predictive markers for early CDMS diagnosis (Lunemann *et al.*, 2010; Runia *et al.*, 2015).

Effector populations of highly activated Th1-like Th17 cells are reduced in blood after multiple sclerosis diagnosis

To verify that these selective differences in T helper subsets are associated with multiple sclerosis diagnosis, we explored total frequencies of blood Th1 effector memory and Th1-like Th17 effector memory cells in treatment-naive RRMS patients ($n = 18$, Table 1), and age-/gender-matched healthy controls ($n = 19$). Strongly reduced frequencies were found for both these subsets in RRMS (median: 1.1% and 0.7%) compared to CIS-CIS (1.9%, $P < 0.001$ and 2.8%, $P < 0.0001$, respectively) and healthy controls (2.8%, $P < 0.001$ and 3.3%, $P < 0.0001$, respectively; Fig. 2A). These frequencies did not significantly differ between the RRMS and CIS-CDMS (1.0% and 1.3%, respectively) group. In RRMS, a significant proportion of blood Th1-like Th17 cells was positive for both CD38 and HLA-DR (Fig. 2B), indicating a highly activated phenotype after multiple sclerosis diagnosis. This was not seen for Th1 cells (Fig. 2B). These data suggest that Th1-like Th17 effector cells are selectively activated in the periphery and recruited to the CNS during multiple sclerosis onset.

Predominant expression of T-bet, RORγt, IFN-γ and GM-CSF, but not IL-17A by activated blood Th1-like Th17 cells

Human $CCR6^+$ $CD4^+$ T cells are not only strong producers of IL-17, but also express IFN-γ and GM-CSF (Cao *et al.*, 2015; Paulissen *et al.*, 2015). To explore how these pro-inflammatory cytokines are co-regulated in our phenotypically defined Th1-like Th17 ($CCR6^+CXCR3^+$) cells, T-bet and RORγt, as well as IFN-γ, GM-CSF and IL-17A expression was compared to paired Th1 ($CCR6^-CXCR3^+$) and Th17 ($CCR6^+CXCR3^-$) populations from healthy blood donors. Th1-like Th17 expressed both *TBX21*/T-bet and *RORC*/RORγt mRNA at higher levels than Th1 ($P = 0.016$ and $P = 0.004$) and Th17 ($P = 0.008$ and $P = 0.039$, respectively; Fig. 3A). *IFNG*/IFN-γ mRNA levels were similar between Th1 and Th1-like Th17, while *CSF2*/GM-CSF mRNA

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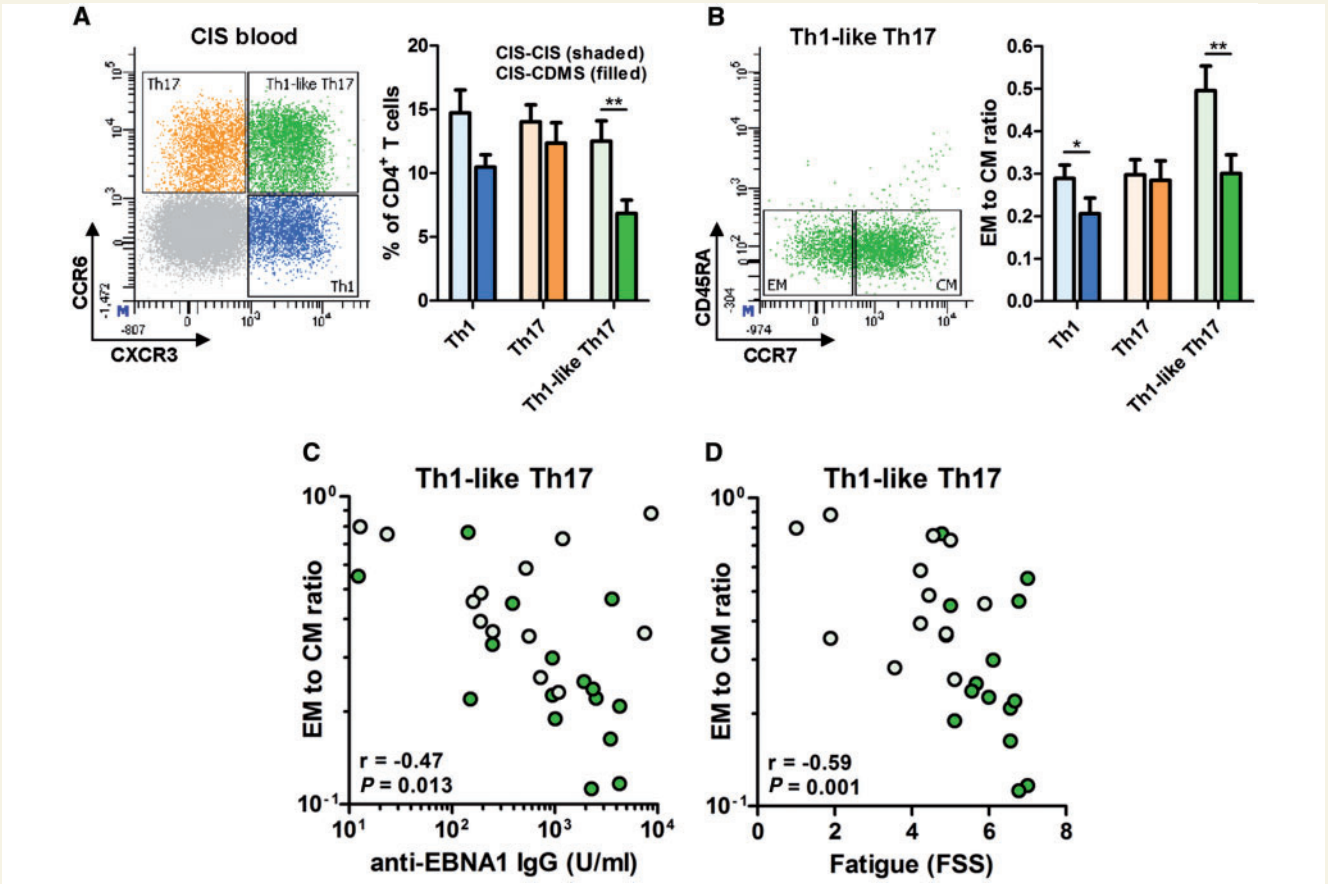

Figure 1 Reduction of Th1-like Th17 effector cells in the blood of CIS patients with short time to CDMS. CIS patients were selected based on blood sampling within 4 months after diagnosis and time between CIS and CDMS. 'CIS-CDMS' patients were diagnosed with CDMS within 1 year ($n = 16$; filled bars), while 'CIS-CIS' patients were not diagnosed with CDMS for at least 5 years ($n = 16$; shaded bars). $CD4^+$ T cells in the blood were compared for (**A**) Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$) and Th1-like Th17 ($CCR6^+CXCR3^+$) cell distribution, as well as (**B**) effector memory (EM; $CCR7^-CD45RA^-$) to central memory (CM; $CCR7^+CD45RA^-$) cell ratios within each of these subsets, as determined by flow cytometry. Th1-like Th17 effector to central memory cell ratios were correlated to reported predictors of early CIS to CDMS transition, anti-EBNA1 IgG blood titre (**C**) and fatigue severity scale (FSS; **D**). $^*P < 0.05$; $^{**}P < 0.01$.

levels in Th1-like Th17 were higher than in Th1 ($P = 0.008$) and Th17 ($P = 0.020$). Th1-like Th17 cells only moderately expressed *IL17A* mRNA (Fig. 3B). Differences in IFN-γ, GM-CSF and IL-17A expression were verified at the protein level (Fig. 3C). The percentage of GM-CSF-positive cells was 2- to 3-fold higher in Th1-like Th17 than in Th1 and Th17. IL-17A-positive cells were ~4-fold less present in Th1-like Th17 compared to Th17. At the single-cell level, IFN-γ was mainly co-expressed with GM-CSF and not with IL-17A in Th1-like Th17 cells (Fig. 3D). These cytokine profiles were the same for T helper subsets from CIS and RRMS blood (data not shown). *TNF* expression was comparable between Th1, Th17 and Th1-like Th17 subsets (Fig. 3B). CD226 was higher, while CD25 and FoxP3 were lower expressed by Th1-like Th17 as compared to Th17 (Fig. 3A and Supplementary Table 3), which supported their pro-inflammatory potential (Lozano *et al.*, 2013; Gagliani *et al.*, 2015). Th1-like Th17 cells also showed sustained CD161 expression (Supplementary Table 3), reflecting an ex-Th17 phenotype (Annunziato *et al.*, 2013).

Pro-inflammatory Th1-like Th17 cells are abundant in CSF T cell cultures of multiple sclerosis patients

To explore the local pro-inflammatory capacity of Th1-like Th17 cells in early multiple sclerosis, CSF ($n = 10$) and paired blood ($n = 4$) T cell subsets from early-stage multiple sclerosis patients (Table 1) were analysed after short-term culturing. Th1 and Th1-like Th17 were the main populations in the CSF $CD4^+$ T cell pool (Fig. 4A). Proportions of Th1-like Th17 were higher than those of Th1 ($P = 0.020$) and their equivalents in blood (2- to 3-fold increase; Fig. 4B). Similar results were obtained with EM/CM ratios, which were high for both subsets but most prominent in Th1-like Th17 cells in CSF (Fig. 4C). Within the total CSF T cell pool, Th1-like Th17 cells were enriched and co-produced more IFN-γ and GM-CSF

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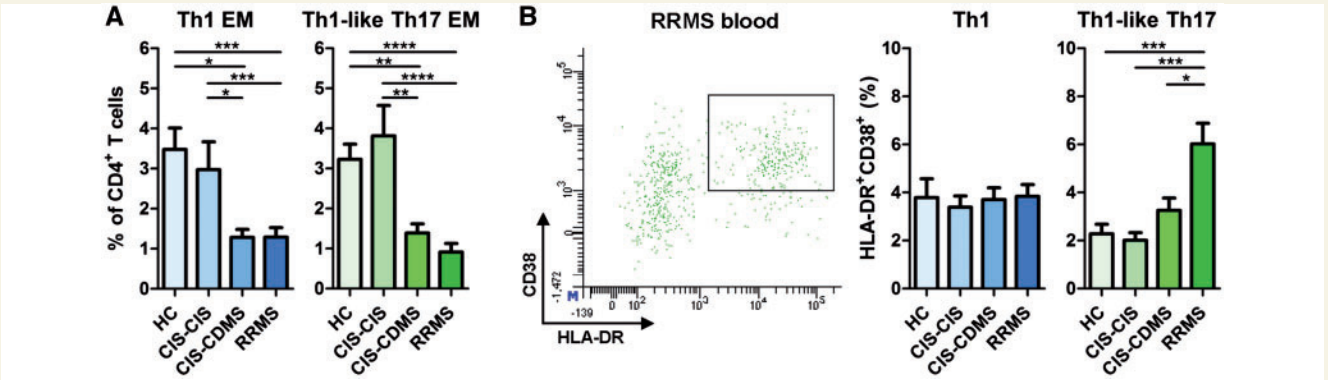

Figure 2 Th1-like Th17 effector cells are highly activated and less present in the blood after multiple sclerosis diagnosis. (**A**) Th1 and Th1-like Th17 effector memory (EM) frequencies in CD4$^+$ T cells from CIS-CIS (n = 14) and CIS-CDMS (n = 16) as well as RRMS (n = 18) and both age- and gender-matched healthy control (HC; n = 19) blood. (**B**) Highly activated fractions of blood Th1 and Th1-like Th17 cells in CIS-CIS (n = 14), CIS-CDMS (n = 16) and RRMS (n = 18) patients as well as HC (n = 19), as defined by co-expression of late T cell activation markers HLA-DR and CD38. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

compared to CCR6$^-$ and CCR6$^+$ CD8$^+$ T cells, and paired blood counterparts (Fig. 4D–G). The percentage of IFN-γ-positive cells was increased in CSF Th1-like Th17 versus Th1 (Fig. 4E). The enrichment of Th1-like Th17 in CSF compared to blood (Fig. 4A and B) was also found in T cell cultures from late-stage multiple sclerosis patients (n = 7, P = 0.016; Table 1 and Fig. 4H), suggesting that Th1-like Th17 recruitment to the CNS also occurs at later stages of the disease.

Ex vivo Th1-like Th17 cells are enriched in the CNS and accumulate in the blood after natalizumab treatment

To confirm their recruitment to the CNS, we compared *ex vivo* Th1 and Th1-like Th17 frequencies in single-cell suspensions of 10 brain tissues and paired CSF and blood samples of five patients with late-stage multiple sclerosis (Table 1, Fig. 5A and B). Th1 and Th1-like Th17 cells were over-represented, while Th17 cells were hardly seen in multiple sclerosis brain tissues and CSF, in contrast to blood. The enrichment of Th1-like Th17 was also found in CSF, but was less in brain tissues from two non-demented controls (Fig. 5B), suggesting that enhanced infiltration of Th1-like Th17 cells into the brain parenchyma is associated with multiple sclerosis (Kebir *et al.*, 2009; Kunis *et al.*, 2013; Johnson *et al.*, 2016).

In addition to chemokine receptors and pro-inflammatory cytokines (Reboldi *et al.*, 2009; Larochelle *et al.*, 2011), adhesion molecules play a key role in migration of peripheral T helper cells into the CNS, including VLA-4, MCAM and PSGL-1 (Schneider-Hohendorf *et al.*, 2014). Interestingly, VLA-4, but not MCAM or PSGL-1, was the most abundant on Th1-like Th17 cells (Supplementary Table 3). In patients with RRMS (n = 14), blood Th1-like Th17 proportions were elevated after 6 months of treatment with natalizumab (anti-VLA-4 monoclonal antibody; median pre- versus post-treatment: 7.7% versus 10.4%, P = 0.006; Fig. 5C and Table 1). Th1-like Th17 cells did not show differences in EM/CM ratio (data not shown), but their activation state (see also Fig. 2B) was significantly reduced after natalizumab therapy (Fig. 5D). Th1-like Th17 showed increased capacity to produce IFN-γ and GM-CSF in post- versus pretreatment blood samples (Fig. 5E and F). These results show that the effects of natalizumab in multiple sclerosis are associated with an accumulation of Th1-like Th17 cells in the blood.

Targeting of VLA-4^{high} Th17.1 cells by natalizumab in multiple sclerosis patients who respond to treatment

To assess the selectivity of natalizumab effects on pro-inflammatory T helper populations in multiple sclerosis patients, CCR4 was included as a surface marker in our flow cytometric panels for subdivision of Th1-like Th17 into recently described pathogenic Th17.1 (IFN-γ^{high}GM-CSF^{high}IL-17^{low}) and Th17 DP (IFN-γ^{low}GM-CSF^{low}IL-17^{int}) subpopulations (Ramesh *et al.*, 2014; Paulissen *et al.*, 2015). Th17.1 (CCR6$^+$CXCR3$^+$CCR4$^-$) frequencies were significantly increased in RRMS blood samples after both 6 and 12 months of treatment (median: 5.3% and 6.1%) versus pretreatment (3.7%; n = 14, both P = 0.0002; Table 1; Fig. 6A and B). No significant differences were found in Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$) and Th17 DP (CCR6$^+$CXCR3$^+$CCR4$^+$) cells (Fig. 6A and B). Importantly, this accumulation of Th17.1 was most pronounced in natalizumab-treated patients who were free of clinical relapses (n = 9; pretreatment, 3.8% versus 6 months post-treatment, 6.5% and 12 months post-treatment, 6.8%; P = 0.008 and P = 0.004, respectively). As compared to patients who had relapses during treatment (n = 5;

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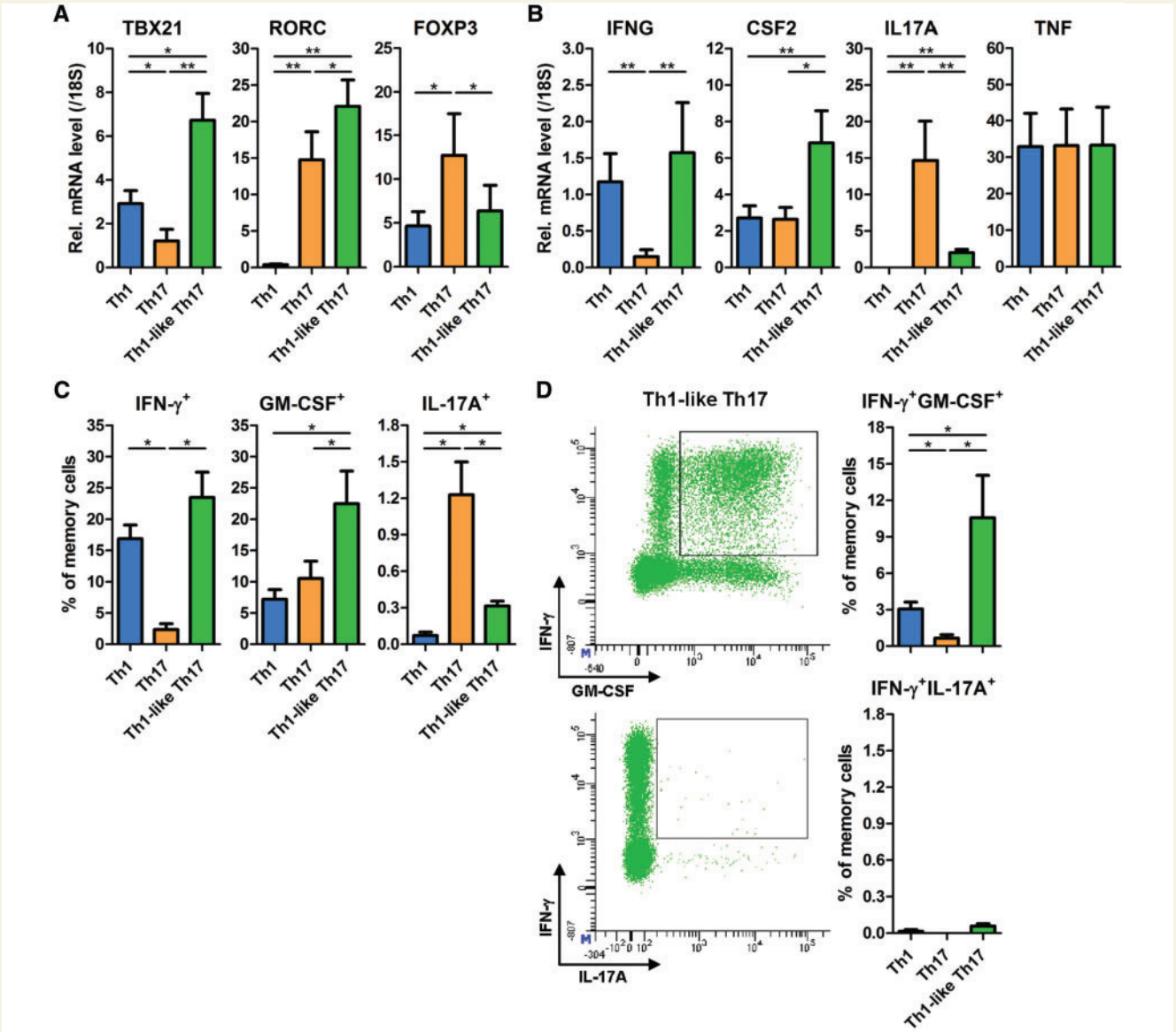

Figure 3 Blood Th1-like Th17 cells predominantly express T-bet and RORγt, and are high IFN-γ and GM-CSF, but low IL-17A producers. Buffy coats from nine healthy blood donors were used to assess gene expression of *TBX21*, *RORC* and *FOXP3* (**A**), as well as *IFNG*, *CSF2*, *IL17A* and *TNF* (**B**) in sorted Th1 ($CCR6^{-}CXCR3^{+}$), Th17 ($CCR6^{+}CXCR3^{-}$) and Th1-like Th17 ($CCR6^{+}CXCR3^{+}$) memory populations. Cells were stimulated with PMA and ionomycin and mRNA levels were measured using quantitative PCR. (**C**) Flow cytometric analysis of intracellular IFN-γ, GM-CSF and IL-17A expression in PMA- and ionomycin-stimulated Th1, Th17 and Th1-like Th17 memory cells from the same blood donors ($n = 7$). (**D**) Representative dot plots and quantification of co-expression of IFN-γ with GM-CSF and IL-17A in Th1-like Th17 memory cells ($n = 7$). $^{*}P < 0.05$; $^{**}P < 0.01$.

pretreatment, 3.2% versus 6 months post-treatment, 4.0% and 12 months post-treatment, 4.1%; Fig. 6C). The accumulation of Th17.1 cells in the blood of clinical responders was validated using a second cohort (Supplementary Table 1 and Supplementary Fig. 1A).

In pretreated RRMS blood, VLA-4 surface expression on Th17.1 (mean MFI: 2603) was the highest of all pro-inflammatory T helper subsets analysed, including Th1 (MFI: 1328, $P = 0.001$), Th17 (MFI: 1255, $P = 0.0001$) and Th17 DP (MFI: 2038, $P = 0.002$; Fig. 6D and Supplementary Fig. 1B). After natalizumab treatment, VLA-4 was downregulated on all subsets analysed, but this was most prominent for Th17.1 (mean reduction: 6 months post-treatment, 56%, 12 months post-treatment, 58%), as compared to Th1 (6 months post-treatment, 52%, $P = 0.038$; 12 months post-treatment, 54%, $P = 0.005$), Th17 (6 months post-treatment, 37%, $P = 0.003$; 12 months post-treatment, 38%, $P = 0.0009$) and Th17 DP (6 months post-treatment, 49%, $P = 0.002$; 12 months post-treatment, 50%, $P = 0.002$; Fig. 6E). This

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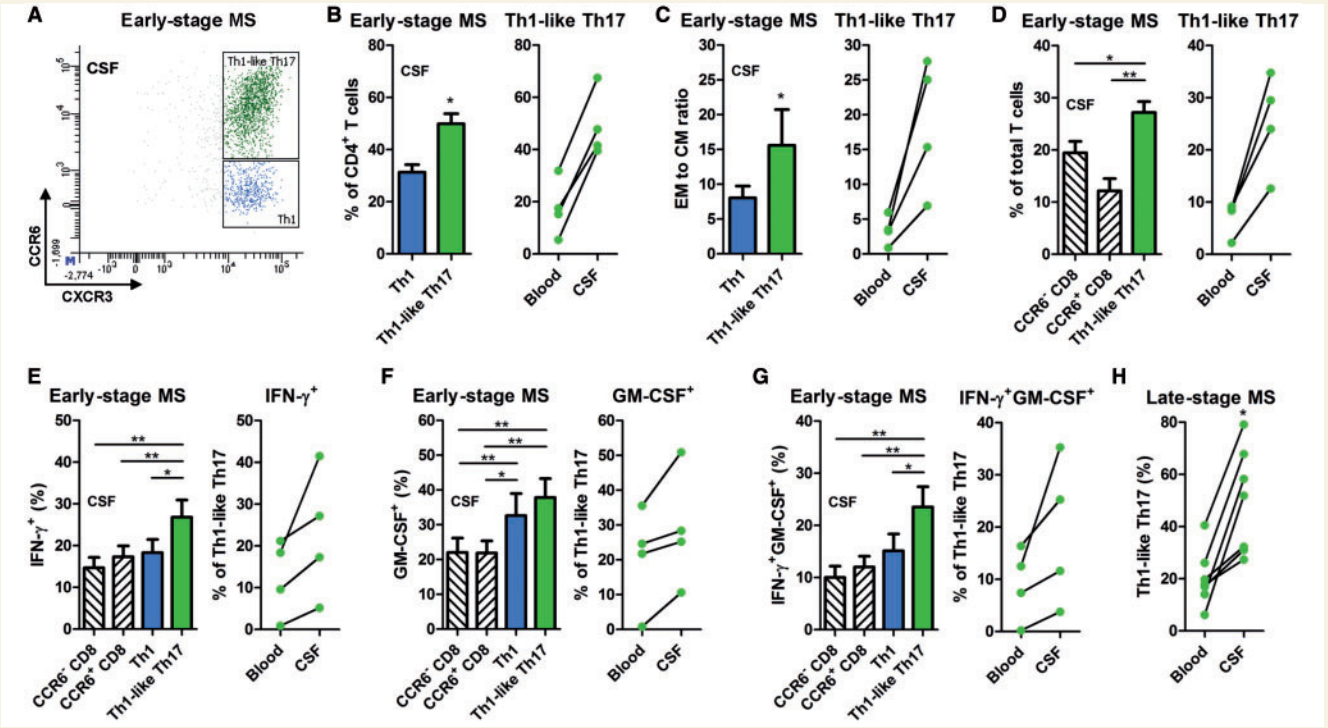

Figure 4 Prevalence of pro-inflammatory Th1-like Th17 cells in CIS and multiple sclerosis CSF compared to blood T cell cultures. (**A–D**) Presence of Th1-like Th17 ($CCR6^+CXCR3^+$) subsets in short-term CSF T cell cultures from 10 early-stage multiple sclerosis patients (CIS, $n = 7$; RRMS, $n = 3$). CSF Th1-like Th17 were compared to Th1 cells and their equivalents in blood for percentages in the total $CD4^+$ T cell pool (**A** and **B**) and for effector memory (EM) to central memory (CM) ratios (**C**) from the same patients. Similar analyses were performed for CSF Th1-like Th17 and both $CCR6^-$ and $CCR6^+$ $CD8^+$ T cell subsets within the total T cell pool (**D**). These T cell subsets were separated, stimulated with PMA and ionomycin and assessed for intracellular expression of (**E**) IFN-γ, (**F**) GM-CSF and (**G**) IFN-γ with GM-CSF. For each analysis, Th1-like Th17 subsets were compared between paired CSF and blood T cell cultures. (**H**) Th1-like Th17 frequencies in T cell cultures from paired blood and CSF of late-stage multiple sclerosis patients ($n = 7$). *$P < 0.05$; **$P < 0.01$.

indicates that Th17.1 cells are preferentially targeted by natalizumab treatment, preventing their transmigration into the CNS of multiple sclerosis patients.

Pathogenic Th17.1 cells have a superior capacity to transmigrate into the CNS in early multiple sclerosis

To study the CNS transmigration potential of Th17.1 in multiple sclerosis further, we performed different *in vitro* transwell migration assays using total T helper memory cell fractions. Th17.1 was the main Th17 subpopulation migrating across transwell filters towards inflammatory mediator CXCL10 (Sorensen *et al.*, 2001) (Fig. 7A and B). No migration was observed towards medium only (data not shown). Particularly Th17.1 cells did show spontaneous transmigration across human brain endothelial layers (hCMEC/D3), which was enhanced under CXCL10-attracting conditions (Fig. 7C and D).

In addition, *ex vivo* flow cytometric analysis revealed an enrichment of Th17.1 versus Th17 and Th17 DP cells in CSF versus paired blood samples from four patients with early-stage multiple sclerosis (three CIS and one RRMS; Table 1, Fig. 7E and F). Consistently, lowered Th17.1 frequencies were found in the blood from 26 CIS-CDMS versus 21 CIS-CIS patients ($P = 0.019$), as well as 13 RRMS patients versus 12 matched healthy controls ($P = 0.031$; Fig. 7G and Supplementary Table 1). Both Th17.1 and Th17 DP cells were abundant in CSF compared to blood from late-stage multiple sclerosis patients (Supplementary Fig. 2).

Finally, to confirm that Th17.1 is a distinct pro-inflammatory Th17 subset, we evaluated the expression of key regulators of Th17 differentiation and pathogenicity. Along with VLA-4 (see also Fig. 6D), CD161, CD226, *ABCB1* (MDR1), *IL23R*, *STAT4*, *FCMR* (TOSO) and *GZMB* (granzyme B; all upregulated), as well as CD25 and *BATF* (downregulated) were discriminative markers for Th17.1 (Supplementary Table 3 and Supplementary Fig. 3). The abundant expression of T-bet, RORγt, IFN-γ and GM-CSF in Th17.1 cells (Supplementary Fig. 4) confirmed the pronounced Th1 features of this Th17 subset (Paulissen *et al.*, 2015).

Collectively, these data demonstrate the propensity of Th17.1 cells to recruit to the CNS and mediate disease activity in early multiple sclerosis.

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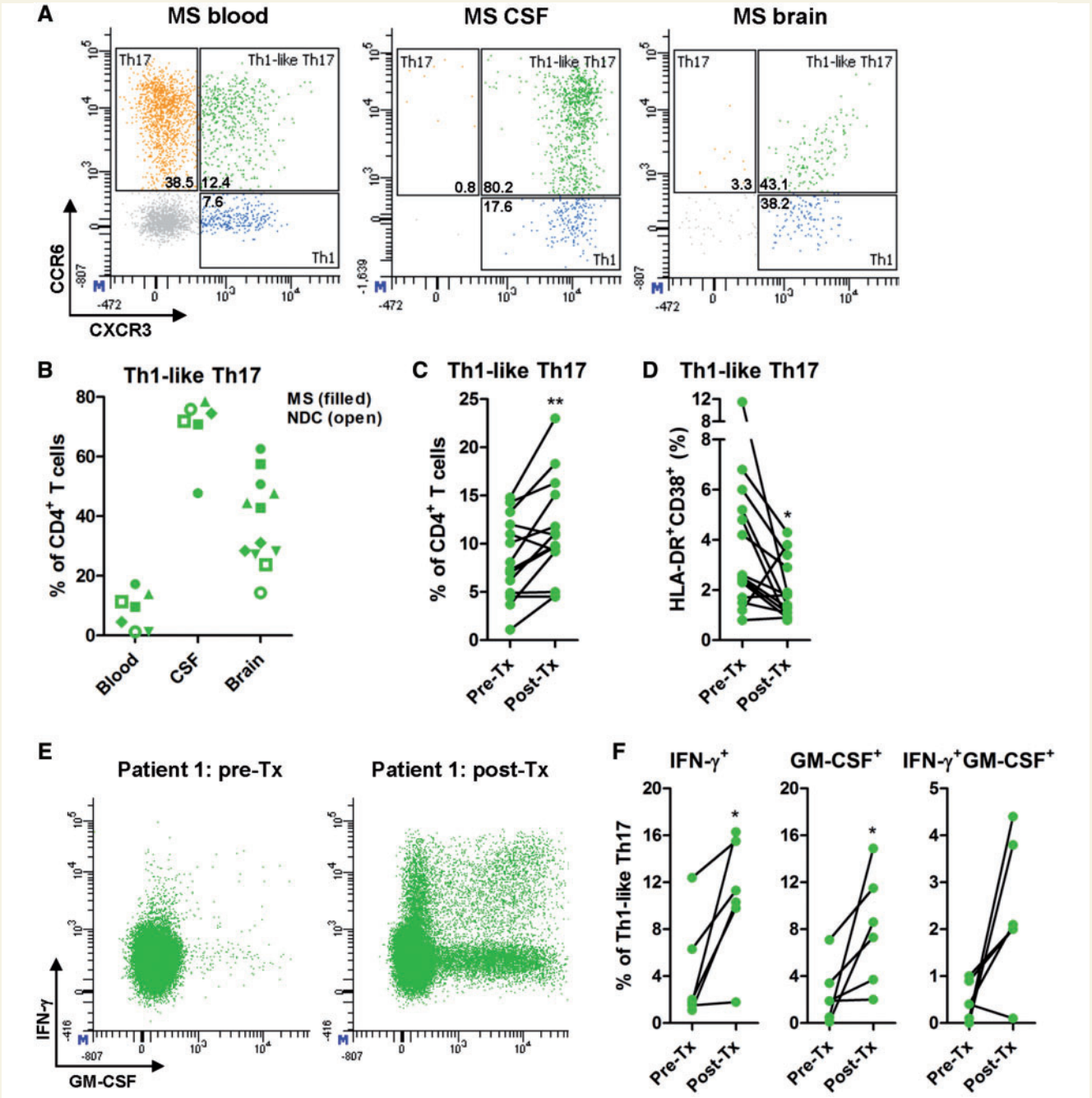

Figure 5 Th1-like Th17 recruitment to the CNS and targeting by natalizumab in multiple sclerosis patients. (**A**) Presence of Th1 ($CCR6^{-}CXCR3^{+}$), Th17 ($CCR6^{+}CXCR3^{-}$) and Th1-like Th17 ($CCR6^{+}CXCR3^{+}$) cells in single-cell suspensions from brain tissue, CSF and blood of a patient with multiple sclerosis, as determined by FACS. (**B**) Th1-like Th17 frequencies in 10 brain tissues and paired CSF and blood samples from five different multiple sclerosis patients (filled shapes). Similar analyses were performed for two non-demented control subjects (NDC; open shapes). Each shape represents a different donor. For Th1-like Th17 cells in multiple sclerosis blood, frequencies ($n = 14$; **C**), activation ($n = 14$; **D**) as well as pro-inflammatory capacities ($n = 6$; **E** and **F**) were determined before and 6 months after natalizumab treatment. T cell activation was assessed by surface expression of both HLA-DR and CD38. To determine their pro-inflammatory capacity, Th1-like Th17 memory cells were isolated from pre- and post-treatment blood, stimulated with PMA and ionomycin, and stained for intracellular IFN-γ and GM-CSF. $^{*}P < 0.05$; $^{**}P < 0.01$.

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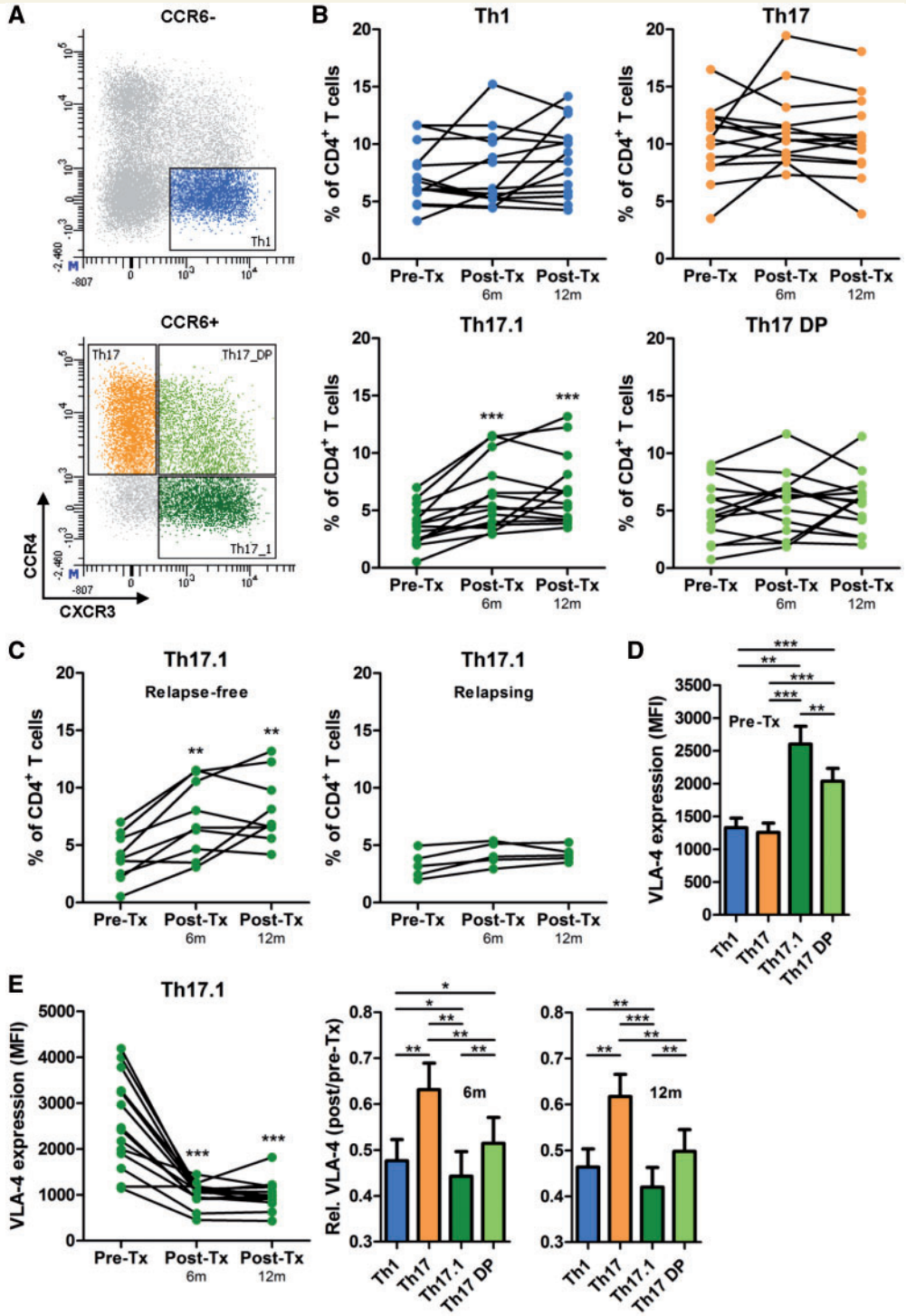

Figure 6 Selective accumulation of Th17.1 cells in natalizumab-treated multiple sclerosis patients who do not experience clinical relapses. Using CCR4 as an additional marker, Th1-like Th17 cells were subdivided into Th17.1 ($CCR6^+CXCR3^+CCR4^-$) and Th17 DP ($CCR6^+CXCR3^+CCR4^+$) subsets and analysed in natalizumab-treated RRMS patients by flow cytometry (**A**). Th1 ($CCR6^-CXCR3^+CCR4^-$), Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17.1 and Th17 DP cells were monitored in pre- and both 6 and 12 months post-treatment blood samples ($n = 14$; **B**). Th17.1 proportions were separately evaluated in relapse-free ($n = 9$) and relapsing ($n = 5$) treatment groups (**C**). VLA-4 surface expression levels were determined on these T helper subpopulations before (**D**) and both 6 and 12 months after (**E**) natalizumab treatment. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. MFI = mean fluorescent intensity; Tx = treatment.

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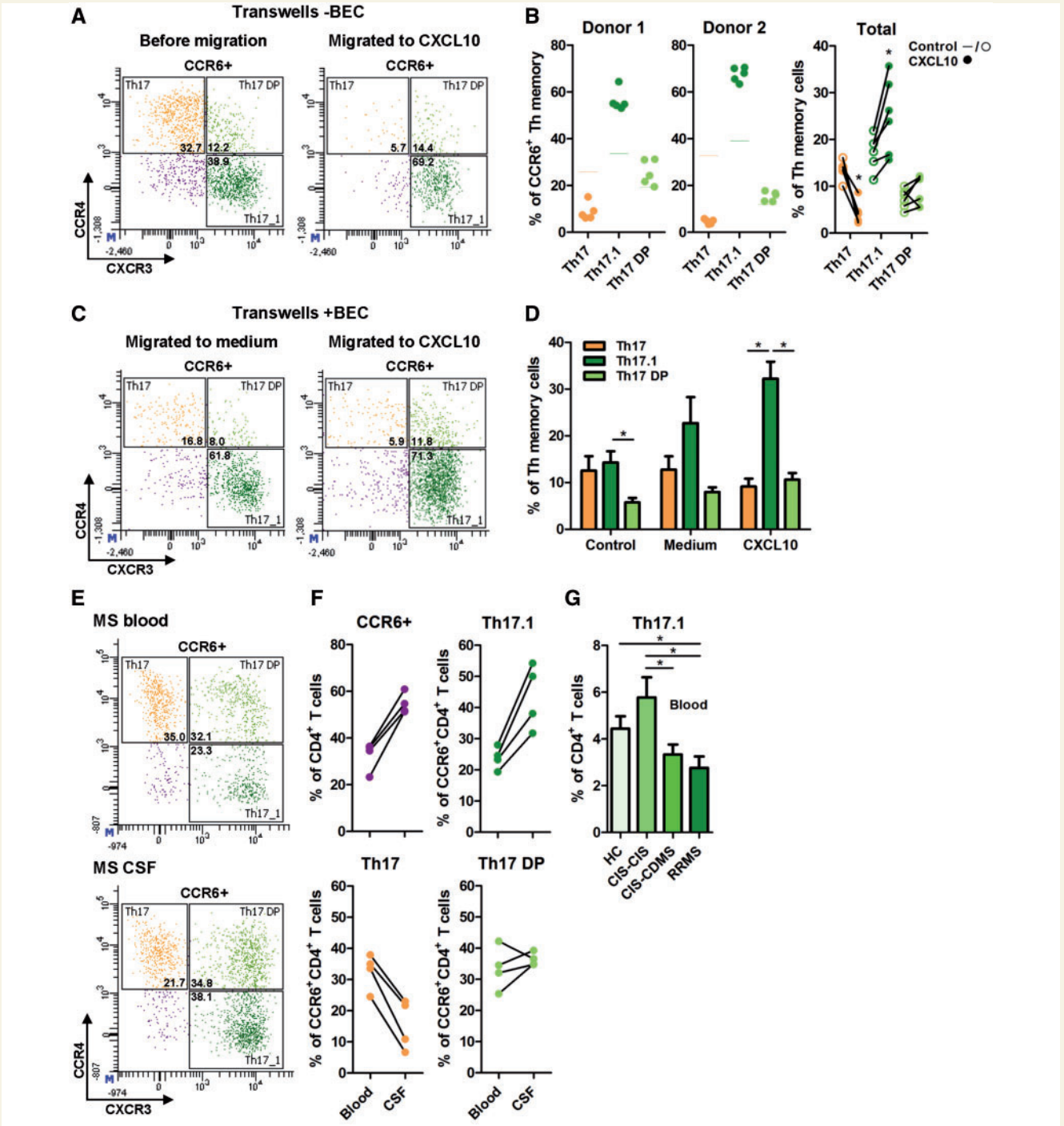

Figure 7 Enhanced CNS transmigration potential of Th17.1 cells and their recruitment to CSF in early multiple sclerosis. Total T helper memory cells were sorted from healthy blood and used to assess the *in vitro* transmigration capacities of Th17, Th17.1 and Th17 DP cells across transwell membranes (**A** and **B**; $n = 6$) and monolayers of human brain endothelial cells (BEC; **C** and **D**, $n = 4$) towards CXCL10. Each experiment was performed in quintuplicate. Th17 subset distribution was assessed before ('control') and after migration towards medium or CXCL10 using FACS. (**E** and **F**) *Ex vivo* Th17, Th17.1 and Th17 DP frequencies of CCR6$^+$ T helper cells in paired CSF and blood from four early multiple sclerosis patients. (**G**) The presence of Th17.1 cells in blood samples from 21 CIS-CIS, 26 CIS-CDMS and 13 treatment-naïve RRMS patients, as well as 12 healthy controls (HC), as determined by FACS. *$P < 0.05$.

Discussion

In this study, we demonstrate that IFN-γ- and GM-CSF-expressing Th1-like Th17 (CCR6$^+$CXCR3$^+$) cells are selectively associated with early disease activity in patients with multiple sclerosis. During disease onset, highly activated and effector memory Th1-like Th17 cells are markedly reduced in the peripheral blood and represents the

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main pro-inflammatory T-cell population within CSF. This local recruitment seemed to be preferentially targeted by natalizumab treatment to prevent subsequent multiple sclerosis relapses, since a Th1-like Th17 subpopulation termed Th17.1, and no other T helper subsets, predominantly accumulated in the blood of relapse-free patients. The current work provides in-depth insights into the pro-inflammatory capacity of distinct CCR6$^+$ T helper subpopulations during the course of multiple sclerosis (Cao *et al.*, 2015), and offers new possibilities to fine-tune currently approved T cell directed treatment for patients with multiple sclerosis.

The use of both CCR6 and CXCR3 as discriminating markers for Th17 cells does not only reflect their pro-inflammatory state, but also their capability to migrate into local inflammatory sites. Previous studies on Th17 cells in experimental autoimmune encephalomyelitis and multiple sclerosis primarily focused on single expression of CCR6 (Cao *et al.*, 2015), or IL-17, which is increased in blood and is further upregulated in CSF during a relapse (Brucklacher-Waldert *et al.*, 2009). Here, we demonstrated that additional expression of CXCR3 subdivides human CCR6$^+$ Th17 into high (CXCR3$^-$) and low (CXCR3$^+$) producers of IL-17A. In these IL-17^{low} producers, which were over-represented in early-stage multiple sclerosis CSF compared to blood T cell cultures, GM-CSF is the major pro-inflammatory cytokine expressed together with IFN-γ. This is likely caused by their elevated levels of T-bet, and not RORγt, as previously reported for human T helper cells (Noster *et al.*, 2014). The association of Th1-like Th17 (T-bet-dependent) and not Th17 (RORγt-dependent) with a short time to CDMS diagnosis is supported by the expression of T-bet, and not RORγt in CD4$^+$ T cells during rapid multiple sclerosis onset (Basdeo *et al.*, 2016). Th1-like Th17 cells were also highly activated after multiple sclerosis diagnosis, which links to the important role of CD4$^+$ T cell activation in CIS progression (Corvol *et al.*, 2008). This suggests that during multiple sclerosis disease onset, the loss of T regulatory function (Kleinewietfeld and Hafler, 2014) results in the activation of peripheral Th1-like Th17 subsets, which infiltrate the CNS to mediate local inflammation. Indeed, memory T helper cells of relapsing multiple sclerosis patients were more capable of differentiating into Th1-like Th17 cells, albeit co-producing IFN-γ and IL-17 (Kebir *et al.*, 2009). These T helper cells were cultured in the presence of IL-23, prompting RORγt and subsequently IL-17 expression (Noster *et al.*, 2014). In our CCR6- and CXCR3-based approach, we defined pro-inflammatory cytokine profiles of Th17 and Th1-like Th17 populations directly from the blood. This could explain why we identified IFN-γ/GM-CSF- and not IL-17-producing Th17 cells as the most pro-inflammatory subset in early multiple sclerosis, and also agrees with the minimal influence of IL-17 and strong impact of GM-CSF on experimental autoimmune encephalomyelitis induction (Ponomarev *et al.*, 2007; Haak *et al.*, 2009; Codarri *et al.*, 2011). For proper analysis of cytokine production by Th1-like Th17 cells in CSF, we had to add IL-2 to short-term T cell cultures, inducing GM-CSF expression (Noster *et al.*, 2014). Th1-like Th17 subsets co-produced more IFN-γ and GM-CSF than other T cell subsets in CSF and their counterparts in blood. Our finding that pro-inflammatory Th1-like Th17 and especially Th17.1 cells were highly enriched in CSF of patients with early-stage multiple sclerosis is in line with their reduced frequencies in the blood (this study), and the increased CSF CD4 to CD14 ratios in CIS patients with a short time to CDMS (Nemecek *et al.*, 2016). Consistent with *in situ* observations in multiple sclerosis brain tissue (Kebir *et al.*, 2009), a small fraction of blood and CSF Th1-like Th17 and Th17.1 cells did co-produce IFN-γ and IL-17, but this was considerably less than their co-production of IFN-γ and GM-CSF. Besides Th17.1, Th17 DP (IL-17^{int}) cells were also enriched in CSF of patients with late-stage multiple sclerosis, suggesting that local IL-17 production is mainly involved in disease progression. Nevertheless, the predominance of Th1-like Th17 cells in multiple sclerosis CSF and brain tissues as observed in this study corresponds to more recent findings that CNS inflammation in multiple sclerosis is largely mediated by infiltrating IFN-γ- and not IL-17-producing T helper cells (Kunis *et al.*, 2013; Johnson *et al.*, 2016; Khaibullin *et al.*, 2017).

Th1-like Th17 cells contain several features promoting their selective intrusion into the CNS, although local Th17 plasticity cannot be completely ruled out (Korn and Kallies, 2017). Th1-like Th17 cells produce high levels of IFN-γ, triggering CXCL10 expression by endothelial cells to favour CXCR3-mediated migration into the CNS (Kunis *et al.*, 2013; Paroni *et al.*, 2017), and thereby multiple sclerosis disease activity (Mahad *et al.*, 2002), which is supported by our *in vitro* and *ex vivo* transmigration results. Additional expression of GM-CSF by this subset may further dysregulate the blood–brain barrier, as described for monocytes (Vogel *et al.*, 2015). Prior to their extravasation, Th17 cells make use of distinct molecules involved in the rolling on and adhesion to endothelial cells, which are activated by pro-inflammatory cytokines and chemokines (Engelhardt and Ransohoff, 2012; Larochelle *et al.*, 2012; Schneider-Hohendorf *et al.*, 2014). One of these molecules is the α4β1-integrin VLA-4, which is targeted by natalizumab to cause a strong reduction of lymphocytes in multiple sclerosis CSF (Stuve *et al.*, 2006). In addition to previous work (Kivisakk *et al.*, 2009), we now show that only a particular Th1-like Th17 subpopulation termed Th17.1 accumulates in the blood from multiple sclerosis patients who clinically respond to natalizumab treatment. These selective effects may thus be useful for predicting freedom from multiple sclerosis activity (Prosperini *et al.*, 2012), and understanding the potential lethal multiple sclerosis rebounds that occur in patients who have to stop this treatment due to increased risk of progressive multifocal leukoencephalopathy (PML) (Sorensen *et al.*, 2014; Larochelle *et al.*, 2017). Multiple sclerosis rebounds are characterized by a rapid influx of

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pro-inflammatory cells into the CNS to cause excessive inflammation, potentially resulting in PML-immune reconstitution inflammatory syndrome (IRIS) (Tan *et al.*, 2011). Although not proven yet, the marked accumulation of Th17.1 in natalizumab-treated multiple sclerosis blood puts forward their transmigration into the CNS as a critical process during these complications. Out of all pro-inflammatory T helper subsets defined by CCR6, CXCR3 and CCR4, Th17.1 revealed the strongest VLA-4 surface expression levels in multiple sclerosis blood, which explains their restricted targeting by natalizumab. Consistent with our results, VLA-4 levels were found to be higher on Th17 than on Th1 cells in multiple sclerosis patients, probably mediating their trafficking into the CNS (Brucklacher-Waldert *et al.*, 2009). However, when we compared individual Th17 subpopulations, i.e. CCR6$^+$ Th17 (CXCR3$^-$CCR4$^+$; IL-17^{high}), Th17 DP (CXCR3$^+$CCR4$^+$; IL-17^{dim}) and Th17.1 (CXCR3$^+$CCR4$^-$; IL-17^{low}) (Paulissen *et al.*, 2015), VLA-4 surface expression seemed to be inversely associated with their ability to produce IL-17, as also described for mice (Rothhammer *et al.*, 2011). The predominant expression of VLA-4 on Th17.1 cells closely parallels the dependence of IFN-γ- and not IL-17-producing T helper cells on this integrin for their entry into the CNS during experimental autoimmune encephalomyelitis (Baron *et al.*, 1993; Rothhammer *et al.*, 2011). However, adhesion molecules other than VLA-4 must be taken into account for alternative transmigration routes of pro-inflammatory Th17 cells as well (Engelhardt and Ransohoff, 2012), especially considering the rebound effects after natalizumab discontinuation in multiple sclerosis.

This cross-sectional study exemplifies that a more refined evaluation of chemokine surface receptors, pro-inflammatory cytokines and adhesion molecules is warranted to better understand the contribution of human Th1 and Th17 to multiple sclerosis and other autoimmune and neuroinflammatory diseases. Based on CCR6/CXCR3, IFN-γ/GM-CSF and VLA-4 expression, we identify Th1-like Th17 as a clinically relevant CD4$^+$ T cell population during disease onset and treatment in multiple sclerosis patients. Future work on the localization and antigen specificity of these subsets in human brain lesions will be critical to determine their local impact on myelin and axonal loss in multiple sclerosis. The prominent association of Th1-like Th17 cells, in particular Th17.1, with multiple sclerosis activity suggests the possibility for more specific T cell-targeted therapies, and pleads for further assessment of the use of natalizumab earlier in the disease course of multiple sclerosis (Nicholas *et al.*, 2014).

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Supplementary material

Supplementary material is available at *Brain* online.

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