

Recognition of viral and self-antigens by T_H1 and T_H1/T_H17 central memory cells in patients with multiple sclerosis reveals distinct roles in immune surveillance and relapses

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Background: Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that is caused by autoreactive T cells and associated with viral infections. However, the phenotype of pathogenic T cells in peripheral blood remains to be defined, and how viruses promote MS is debated.

Objective: We aimed to identify and characterize potentially pathogenic autoreactive T cells, as well as protective antiviral T cells, in patients with MS.

Methods: We analyzed CD4⁺ helper T-cell subsets from peripheral blood or cerebrospinal fluid for cytokine production, gene expression, plasticity, homing potentials, and their reactivity to self-antigens and viral antigens in healthy subjects and patients with MS. Moreover, we monitored their frequencies in untreated and fingolimod- or natalizumab-treated patients with MS.

Results: T_H1/T_H17 central memory (T_H1/T_H17_{CM}) cells were selectively increased in peripheral blood of patients with relapsing-remitting MS with a high disease score. T_H1/T_H17_{CM} cells were closely related to conventional T_H17 cells but had more pathogenic features. In particular, they could shuttle between lymph nodes and the CNS and produced encephalitogenic cytokines. The cerebrospinal fluid of patients with active MS was enriched for CXCL10 and contained mainly CXCR3-expressing T_H1 and T_H1/T_H17 subsets. However, while

T_H1 cells responded consistently to viruses, T_H1/T_H17_{CM} cells reacted strongly with John Cunningham virus in healthy subjects but responded instead to myelin-derived self-antigens in patients with MS. Fingolimod and natalizumab therapies efficiently targeted autoreactive T_H1/T_H17_{CM} cells but also blocked virus-specific T_H1 cells.

Conclusions: We propose that autoreactive T_H1/T_H17_{CM} cells expand in patients with MS and promote relapses after bystander recruitment to the CNS, whereas T_H1 cells perform immune surveillance. Thus the selective targeting of T_H1/T_H17 cells could inhibit relapses without causing John Cunningham virus-dependent progressive multifocal encephalomyelitis. (J Allergy Clin Immunol 2017;■■■■:■■■-■■■.)

Key words: T_H1/T_H17 central memory T cells, autoreactivity, multiple sclerosis, John Cunningham Virus, natalizumab, fingolimod

Multiple sclerosis (MS) is a degenerative autoimmune disease of the central nervous system (CNS). Autoreactive lymphocytes that infiltrate the CNS attack the myelin sheath of neurons, causing relapses and progressive disability. Evidence from genetic association studies in human subjects and from animal models, such as experimental autoimmune encephalomyelitis (EAE), indicate that CD4⁺ T cells play a key pathogenic role.^{1,2} In mice with EAE, different subsets of CD4⁺ T cells can mediate disease, including IFN-γ-producing T-box transcription factor (T-bet)⁺ T_H1 cells and IL-17-producing retinoic acid receptor-related orphan receptor γt (RORγt)⁺ T_H17 cells.^{3,4} T_H1/T_H17 cells that coexpress RORγt and T-bet and coproduce IFN-γ and IL-17 are particularly pathogenic, can be generated with IL-23,⁵⁻⁹ and are present in lesions of patients with MS.¹⁰ Moreover, GM-CSF is required to induce EAE,⁵ and GM-CSF-producing T cells are present in the cerebrospinal fluid (CSF) of patients with MS.¹¹ However, both T_H1 and T_H17 cells can produce GM-CSF.^{3,9,11} Moreover, myelin-reactive T cells are present at comparably low frequencies in healthy subjects and patients with MS,¹² although myelin basic protein (MBP)-specific T cells in patients with MS produce increased amounts of proinflammatory cytokine.¹³ However, the phenotype of pathogenic precursor T cells in the peripheral blood of patients with MS remains to be defined.

MS can be subdivided into relapsing-remitting (RR) and progressive forms.¹⁴ Therapies with fingolimod and natalizumab interfere with lymphocyte migration and efficiently prevent relapses in patients with RR-MS but have severe side effects.¹⁵

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Supported by the Romeo ed Enrica Invernizzi foundation. M.P. and F.C. are supported by the Ministry of Health (Project Code: GR-2011-02352001).

Disclosure of potential conflict of interest: F. Caprioli serves on the board for Takeda and Mundipharma and receives grants from the Italian Ministry of Health; payments for lectures from Abbvie, Takeda, and MSD; and travel support from Alfa-Wasserman, Takeda, Abbvie, and Mundipharma. S. Abrignani receives grant support from Fondazione Invernizzi. J. Geginat receives grant support from the Romeo ed Enrica Invernizzi Foundation and serves as a consultant for Biotest AG. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication July 1, 2016; revised October 18, 2016; accepted for publication November 10, 2016.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2016.11.045>

Abbreviations used

CNS:	Central nervous system
CSF:	Cerebrospinal fluid
DC:	Dendritic cell
EAE:	Experimental autoimmune encephalomyelitis
FACS:	Fluorescence-activated cell sorting
HD:	Healthy donor
IL-7R:	IL-7 receptor
IL-23R:	IL-23 receptor
JCV:	John Cunningham virus
MBP:	Myelin basic protein
MOG:	Myelin oligodendrocyte glycoprotein
MS:	Multiple sclerosis
MSSS:	Multiple sclerosis severity score
PML:	Progressive multifocal encephalomyelitis
ROR γ t:	Retinoic acid receptor–related orphan receptor γ t
RR:	Relapsing-remitting
STAT:	Signal transducer and activator of transcription
T _{CM} :	Central memory T
T-bet:	T-box transcription factor
T _{EM} :	Effector memory T

Fingolimod (FTY20) inhibits sphingosine-1-phosphate-mediated egress of T cells from lymph nodes and thus depletes naive and central memory T (T_{CM}) cells from the circulation.¹⁶ T_{CM} cells are immature memory T cells, express CCR7 and CD62 ligand, home to lymph nodes, and produce IL-21⁹ but only limited amounts of other potentially pathogenic effector cytokines.^{17,18} Conversely, effector memory T (T_{EM}) cells express the inflammatory chemokine receptor CCR5, home to nonlymphoid tissues, and produce high levels of effector cytokines.¹⁷

Natalizumab is a therapeutic antibody that blocks the α 4-integrin and, consequently, CNS homing of lymphocytes.^{15,19} Natalizumab is very efficient at preventing relapses but frequently induces reactivation of John Cunningham virus (JCV),^{20,21} which is normally controlled by CD4⁺ T cells^{22,23} and can lead to fatal progressive multifocal leukoencephalopathy (PML).^{21,24} PML in immune-compromised subjects and the discovery of a lymphatic system in the CNS have challenged the view of the brain as an immune-privileged site²⁵ and suggest that persistent neurotropic viruses are continuously controlled by CNS-homing T cells.^{24,26} Notably, the risk of MS is associated with various neurotropic viruses^{27,28} and in particular with EBV.²⁹ Molecular mimicry was proposed as a possible mechanism because some EBV-specific T cells with a degenerate T-cell receptor (TCR) cross-react with myelin antigens.^{30–32} Nevertheless, a single virus that causes MS was not identified, and the role of viral infections in patients with MS remains enigmatic and debated.^{27–29}

We report here that autoreactive T_H1/T_H17_{CM} cells expand in peripheral blood of patients with RR-MS and accumulate in the CSF on attacks together with virus-specific T_H1 cells, suggesting that these 2 subsets have distinct roles in relapses and immune surveillance.

METHODS**Recruitment, classification, and sample collection for patients with MS**

Fifty-five patients with MS were selected at the MS Center of the Fondazione Ca' Granda, IRCCS Ospedale Policlinico, according to the therapy and disease type (RR or progressive). At the time of analysis, no

patients with MS were undergoing any treatment unless indicated for fingolimod- and natalizumab-treated patients with RR-MS. The Expanded Disability Status Scale was calculated at the time of diagnosis and over time. The multiple sclerosis severity score (MSSS) was used for measuring the progression of the disability and used to stratify patients with MS with mild disease (MSSS < 1) or more severe disease (MSSS > 1). All patients underwent the standard workup for MS, including medical history, physical and neurological examination, screening laboratory tests, and brain magnetic resonance imaging. Diagnoses met the McDonald criteria.³³ Each patient provided informed consent for participating in the study, and their demographic and clinical data are reported in Table E1 in this article's Online Repository at www.jacionline.org.

PBMCs of 20 healthy donors (HDs) from the Policlinico Blood Bank, 14 patients with RR-MS, 10 patients with progressive MS, 8 natalizumab-treated patients with RR-MS, and 13 fingolimod-treated patients with RR-MS were collected and analyzed. When HDs were compared with patients with MS, a cohort of 20 HDs matched for age and sex to patients with RR-MS (see Table E1) were analyzed. All patients with RR-MS who donated blood were in remission. Conversely, CSF samples were obtained from 10 patients with RR-MS shortly after an attack or from 5 nonautoimmune control subjects with dementia. Samples were collected in polypropylene tubes by means of lumbar puncture at the L4/L5 or L3/L4 interspace, centrifuged at 4°C and 2000g, and analyzed immediately. Supernatants were removed and stored at –80°C until use. The study was approved by the Institutional Review Board of the Ospedale Policlinico.

Flow cytometry

Eight-color flow cytometric analysis was performed with a FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ) after staining with fluorochrome-conjugated antibodies. Unless indicated, all antibodies were from eBioscience (San Diego, Calif). For surface marker expression, anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD45RA (HI100), anti-CD25 (BC96), anti-IL-7 receptor (IL-7R; eBioRDR5), anti-CXCR3 (1C6), anti-CCR6 (R6H1), anti-CCR5 (2D7), anti-CCR7 (3D12), anti-CD62 ligand (Dreg 56), anti-CD161 (DX12, BD), anti-CD6 (MEM-98; ImmunoTools, Friesoythe, Germany), anti-CD58 (B-A19, ImmunoTools), anti-CD49d (α 4-integrin, clone 9F10), anti-CD29 (β 1-integrin clone TS2/16) were used. For T-bet intranuclear staining, forkhead box p3 staining buffer set (eBioscience) and anti-T-bet (O4-46, BD Biosciences) were used, according to the manufacturer's recommendations. For intracellular cytokine detection, T cells were incubated for 5 hours in the presence or absence of phorbol 12-myristate 13-acetate (PMA), ionomycin, and brefeldin A (Sigma, St Louis, Mo) in complete RPMI (10% FBS, 1 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, and 1% penicillin/streptomycin). Subsequently, cells were surface labeled with conjugated antibodies. After fixation with 2% paraformaldehyde (Merck, Whitehouse Station, NJ), cells were permeabilized with PBS–0.5% saponin (Sigma) and stained with anti-IL-17 (eBio64DEC17), anti-IL-22 (22URT1), anti-IFN- γ (4S.B3), anti-GM-CSF (BVD2-21C11), anti-Granzyme A (CB9, BD Biosciences), and anti-CD154 (CD40L, clone 24-31) antibodies. Fluorescence-activated cell sorting (FACS) data were analyzed with FlowJo software (TreeStar, Ashland, Ore). For intracellular IL-10 detection, T cells were activated in plate-bound anti-CD3 (2 μ g/mL, BD Biosciences), as previously described³⁴; cells were cultured for 24 hours in the presence of 200 U/mL IL-2, Brefeldin A was added for an additional 6 hours, and cells were analyzed for intracellular IL-10 production after fixation and permeabilization.

IL-23 receptor (IL-23R) expression was determined, as previously described.⁹ Briefly, cells were incubated in culture medium with or without 10 ng/mL IL-23 (R&D Systems, Minneapolis, Minn) for 30 minutes and fixed with Cytofix Fixation Buffer (BD Biosciences), followed by permeabilization with Perm Buffer III (BD Biosciences). Thereafter, cells were stained with anti-phospho-signal transducer and activator of transcription (STAT) 3 (pY705; BD Biosciences), and the percentage of phospho-STAT3⁺ cells was reported as a percentage of IL-23R responsive cells.

RT-PCR

To measure *RORC* mRNA levels, we isolated total RNA using the mirVana Kit (Ambion, Thermo Fisher, Waltham, Mass). mRNA expression levels were assessed by using the TaqMan Gene expression assay (Applied Biosystems, Foster City, Calif) and normalized on 18s rRNA.

Cell purification

All FACS sorting was performed with a FACS Aria III (BD Biosciences), according to the expression of specific surface markers. For characterization of human helper T-cell subsets from PBMCs and CSF, T cells were stained with the following antibodies in combination: anti-CD4, anti-CD45RA, anti-CD25, anti-IL-7R, anti-CCR6, anti-CXCR3, and anti-CCR5. T_H1 cells were sorted as $CD4^+CD45RA^-IL-7R^+CD25^{low}CCR6^-CXCR3^+$ cells and subdivided according to CCR5 expression; T_H1/T_H17 cells were sorted as $CD4^+CD45RA^-IL-7R^+CD25^{low}CCR6^+CXCR3^+$ cells and subdivided according to CCR5 expression; T_H17 cells were sorted as $CD4^+CD45RA^-IL-7R^+CD25^{low}CCR6^+CXCR3^-$ cells; and T_H0 cells were sorted as $CD4^+CD45RA^-IL-7R^+CD25^{low}CCR6^-CXCR3^-CCR5^-$ cells. $CD1c^+$ dendritic cells (DCs) were isolated from PBMCs by means of magnetic enrichment, followed by cell sorting.³⁵ Briefly, cells were incubated with anti-CD1c-fluorescein isothiocyanate (FITC; AD5 8E7; Miltenyi Biotec, Bergisch Gladbach, Germany), nonspecific binding to Fc receptors was prevented with FcR blocking reagent, and cells were magnetically labeled with anti-FITC beads (Miltenyi Biotec) and enriched on columns. The positive fraction was thereafter incubated with lineage markers: anti-CD3 (SK7; BioLegend, San Diego, Calif), anti-CD14 (61D3, BD Biosciences), anti-CD16 (CD16, BD Biosciences), anti-CD19 (HIB19, BD Biosciences), and anti-CD11c (3.9, BD Biosciences). DCs used in all the experiments were sorted as lineage⁺ $CD11c^+CD1c^+$ cells.

In vitro T-cell differentiation

In vitro differentiation of sorted T_H1_{CM} and T_H17 cells was performed at 5000 cells/well in plate-bound anti-CD3 (0.02 μ g/mL, BD Biosciences) and anti-CD28 antibodies (6 μ g/mL, BD Biosciences) in Dulbecco modified Eagle medium (5% human serum, 1 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, 1% penicillin/streptomycin, and 25 mmol/L β -mercaptoethanol).³⁶ Cells were cultured for 4 days under T_H17 -promoting conditions (neutralizing antibodies against IL-12 and IL-4 at 2 μ g/mL [BD Biosciences] and recombinant human TGF- β 1, IL-1 β , IL-6, and IL-23) or under T_H1 -promoting conditions (neutralizing antibody against IL-4 and recombinant human IL-12). All recombinant cytokines (R&D Systems) were added at 10 ng/mL, with the exception of TGF- β 1, which was used at 1 ng/mL. After 4 days, cells were transferred to uncoated plates and cultured for an additional 7 days under the same cytokine conditions and then analyzed for intracellular cytokine (IL-17 and IFN- γ) or CXCR3 and CCR6 surface expression. Because CCR6 up-regulation is transient *in vitro*,³⁷ it was analyzed on day 4, as previously reported.^{9,36}

RNA isolation and gene expression profiling

Total RNA was isolated from FACS-purified T-cell subsets from PBMCs by using the mirVana miRNA Isolation Kit (Ambion), according to the standard protocol. Briefly, lysates were extracted once with acid-phenol chloroform and further purified to yield total RNA. Extracted RNA was quantified with the RiboGreen Quantitation Kit (Molecular Probes, Eugene, Ore) on an Infinite F200 plate reader (Tecan Trading AG, Männedorf, Switzerland). All extracted RNA samples were quality controlled for integrity with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif), and samples with RNA integrity numbers of less than 8 were discarded. Gene expression of whole transcriptome was performed on $CD4^+$ T-cell subsets with Illumina Direct Hybridization Assays (Illumina, San Diego, Calif), according to the standard protocol. For each sample, 80 ng of total RNA was reverse transcribed according to the Illumina TotalPrep RNA Amplification kit (Ambion), and cRNA was generated after 14 hours of *in vitro* transcription. Washing, staining, and hybridization were

performed according to the standard Illumina protocol: briefly, 750 ng of cRNA of each sample in a final volume of 15 μ L were hybridized onto Illumina HumanHT-12 v3 Expression BeadChip arrays. Hybridization and scanning were performed according to the manufacturer's indications on an Illumina iScan System, and data were processed with BeadStudio v.3.

Gene expression analysis

Gene expression arrays were quantile normalized with background subtraction, and average signals were calculated on gene-level data for genes with a detection *P* value of less than .001 in at least 1 of the subsets considered. Normalized data were log₂ transformed and presented as *z* scores. One-way ANOVA (*P* < .001) was used to select gene classifiers for all cell subsets profiled. Unsupervised hierarchical clusters on significant genes were performed with Pearson distance and complete linkage. Statistical tests and hierarchical clustering were performed with MeV software, version 4.5 (MultiExperiment Viewer, Boston, Mass).

Autoreactivity and antigen-specificity assays

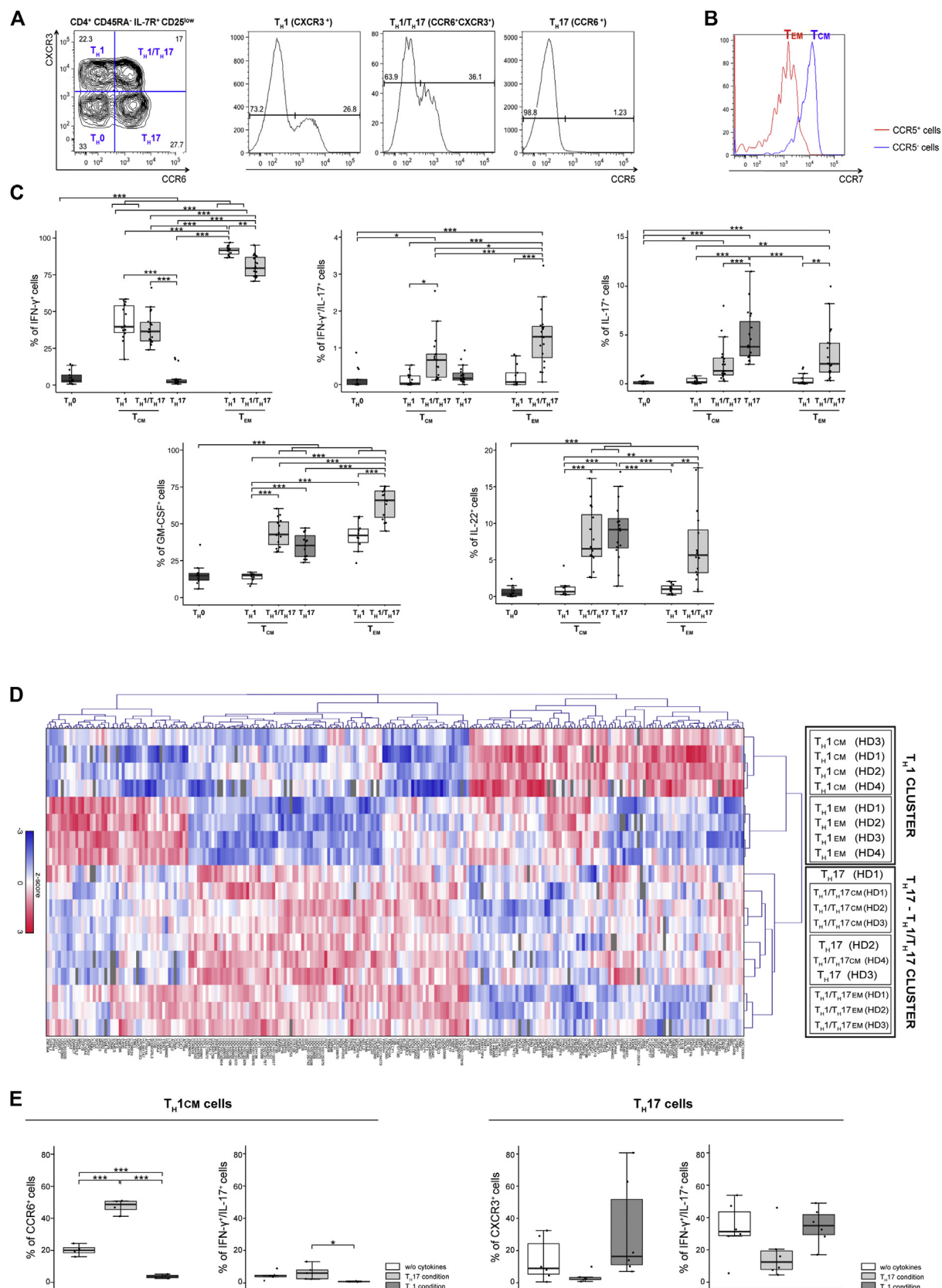
To assess autoreactivity, T-cell subsets (T_H1_{CM} , T_H1/T_H17_{CM} , T_H1/T_H17_{EM} , and T_H17) purified from PBMCs of patients with MS or HDs were Cell Trace labeled (Invitrogen, Carlsbad, Calif) and cocultured with autologous $CD1c^+$ DCs at a 5:1 ratio, as described previously.³⁴ After 5 days, cells were analyzed by using flow cytometry for Cell Trace dye dilution. Antigen-specific proliferation was determined, as previously described.¹⁸ Briefly, T-cell subsets (T_H1_{CM} , T_H1/T_H17_{CM} , and T_H17) isolated from PBMCs of patients with MS or HDs were Cell Trace labeled (Invitrogen) and cocultured with autologous monocytes obtained by using $CD14^+$ selection ($CD14$ Microbeads, Miltenyi Biotec) at a 1:1 ratio in the presence or absence of myelin-derived peptides (PepTivatorMOG and MBP Isoform-I; Miltenyi Biotec) or viral peptides (PepTivatorJCV-VP1 or EBV-Consensus; Miltenyi Biotec). All peptides were used at 0.6 nmol/mL. After 7 days, cells were analyzed by means of flow cytometry for Cell Trace dye dilution. Background proliferation induced by autologous monocytes alone was subtracted to calculate antigen-specific proliferation.

Generation and analysis of T-cell lines from CSF of patients with MS

Fresh CSF-derived mononuclear cells were isolated immediately after CSF collection. Two hundred to 10,000 FACS-purified T_H1 , T_H1/T_H17 , and T_H17 cells were activated in 96-well plates with 10^6 /mL irradiated allogeneic feeder cells, 10^5 /mL irradiated allogeneic EBV-induced lymphoblastoid cell lines, and soluble monoclonal anti-CD3 (0.03 μ g/mL, OKT3; BioLegend) in complete RPMI (10% FCS, 1 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, and 1% penicillin/streptomycin) supplemented with 200 U/mL human recombinant IL-2 (Miltenyi Biotec). T-cell lines were maintained in the same complete medium and were used in functional assays 15 to 20 days after their last stimulation. In addition, CSF-derived T-cell lines were analyzed for cytokine production, surface marker expression, and TCR-V β mRNA expression by using PCR, as described previously.³⁸ T cells were cocultured with autologous $CD14^+$ monocytes isolated from PBMCs, as previously described, at a 5:1 ratio in complete RPMI in the presence or absence of myelin peptides or viral antigens for 4 hours to assess responses of the CSF-derived T-cell lines to self-MHC or antigenic peptides. Brefeldin A (10 μ g/mL) was added for an additional 14 hours of incubation, and thereafter, cells were analyzed for intracellular expression of IL-17, IFN- γ , GM-CSF, and CD40L. In some experiments neutralizing anti-HLA-DR/DP/DQ antibodies (anti-MHC class II, 10 μ g/mL, clone Tu39, BD Biosciences) were added together with peptides.

ELISA

Cell-free supernatants of CSF, stored immediately after collection from patients with MS and those without MS, were analyzed by means of ELISA for CXCL10 (interferon-inducible protein 10, BioLegend), CCL5 (RANTES, BioLegend), CCL20 (R&D Systems), and CCL21 (secondary lymphoid-tissue chemokine, eBioscience), according to the manufacturer's guidelines.



Statistics

Statistical analysis was performed with Prism 5 software (GraphPad Software, La Jolla, Calif). Student *t* tests for unpaired samples or 1-way ANOVA (*post hoc* test: Tukey multiple comparison) were used to evaluate differences.

RESULTS

CCR5[−] T_H1/T_H17 cells are a unique T_{CM} subset that produces high levels of proinflammatory cytokines

Relapses in patients with RR-MS are efficiently reduced by fingolimod, which sequesters T_{CM} cells in lymph nodes,³⁹ suggesting that T_{CM} cells can play a pathogenic role in patients with MS. Moreover, CD4⁺ T cells producing GM-CSF, IFN- γ , and IL-17 have a high pathogenic potential in mice with EAE and are recruited to the CNS of patients with MS. However, T_{CM} cells are known to produce only low levels of effector cytokines, and therefore we first tried to identify human blood T_{CM} cells that could coproduce these proinflammatory cytokines. To this aim, we used a combination of surface markers that directly correlate with expression of effector cytokines⁴⁰ and are involved in CNS homing.^{41,42}

Thus, within the T-helper memory compartment (CD4⁺CD45RA[−]IL-7R⁺CD25^{low}), we used CXCR3 to identify IFN- γ -producing cells (T_H1 cells), CCR6 to identify IL-17-producing cells (T_H17 cells), and coexpression of CXCR3 and CCR6 to identify IFN- γ /IL-17-coproducing cells (T_H1/T_H17 cells; Fig 1, A).⁴⁰ In addition, we used CCR5 to discriminate T_{CM} and T_{EM} cells among T_H1 and T_H1/T_H17 subsets (Fig 1, A). Indeed, CCR5⁺ memory T cells lacked the T_{CM} marker CCR7, whereas CCR5[−] memory T cells expressed high levels of CCR7 (Fig 1, B), as expected.^{17,18} Based on this strategy, we purified and characterized 5 major subsets from the blood of HDs: T_H1_{CM} and T_H1_{EM} cells, T_H1/T_H17_{CM} and T_H1/T_H17_{EM} cells and conventional T_H17 cells. Of note, CCR5⁺ T_H17_{EM} cells were rare and therefore were not analyzed.

After polyclonal *ex vivo* stimulation, T_H1, T_H17, and T_H1/T_H17 subsets displayed characteristic cytokine profiles, whereas control T_H0 cells (defined as CXCR3[−]CCR6[−]CCR5[−]) produced only low amount of effector cytokines (Fig 1, C). Importantly, both T_H1/T_H17_{CM} and T_H1/T_H17_{EM} subsets contained a significant fraction of IFN- γ /IL-17-coproducing cells and secreted high amounts of GM-CSF and IL-22 (Fig 1, C). Furthermore, they expressed both *RORC* and T-bet (see Fig E1, A, in this article's Online Repository at www.jacionline.org), a functional IL-23R (see Fig E1, B), and produced only low levels of the anti-inflammatory cytokine IL-10 (see Fig E1, C), indicating that they had a high pathogenic potential. Notably, although

T_H1/T_H17_{EM} cells produced higher levels of IFN- γ than T_H1/T_H17_{CM} cells, production of IL-17, IL-22, and GM-CSF was comparable in the 2 T_H1/T_H17 subsets (Fig 1, C). Also, conventional T_H17 cells produced IL-17, GM-CSF, and IL-22 and expressed *RORC* (Fig 1, C, and see Fig E1, A). However, in contrast to T_H1/T_H17 cells, they expressed only low levels of T-bet (see Fig E1, A) and IFN- γ but high levels of IL-10 (see Fig 1, C), suggesting that they had only a limited pathogenic potential.⁶ Finally, both T_H1 subsets expressed IFN- γ , GM-CSF and T-bet, but T_H1_{CM} cells expressed only low levels. Moreover, expression of IL-17, IL-22, IL-23R, and *RORC* was hardly detectable in T_H1 cells (Fig 1, C, and see Fig E1, A and B).

We conclude that CCR5[−] T_H1/T_H17 cells are a peculiar subset of T_{CM} cells that produce high amounts of proinflammatory and potentially pathogenic effector cytokines.

T_H1/T_H17_{CM} cells are closely related to conventional T_H17 cells

Because T_H1/T_H17 cells express high levels of IFN- γ , T-bet, and recognize antigens from intracellular pathogens⁴³ they appear to be a subset of T_H1 cells. To define the molecular relationships of T_H1/T_H17 subsets with T_H1 and T_H17 cells, we performed a genome-wide gene expression analysis (Fig 1, D, and see Table E2 in this article's Online Repository at www.jacionline.org). We identified 242 differentially expressed genes whose expression divided the analyzed populations into 2 major clusters, one containing all T_H1 populations and another containing T_H17 cells and both T_H1/T_H17 subsets (Fig 1, D). Notably, in the T_H1 cluster T_H1_{CM} and T_H1_{EM} cells formed 2 distinct subclusters. Conversely, in the T_H17 cluster only T_H1/T_H17_{EM} cells formed a distinct subcluster, whereas T_H17 and T_H1/T_H17_{CM} cells clustered partially together. Among differentially expressed genes, *IFNGR2* and the transcription factor *FOXP1*⁴⁴ were upregulated in T_H1 subsets. Conversely, T_H17 and T_H1/T_H17 subsets expressed higher levels of *CD161*, *HLA-G*, *Bly*s, and *STAT5B*. Furthermore, both T_{EM} subsets expressed higher levels of the MS-associated gene *CD58*,⁴⁵ and genes involved in cytotoxicity, such as eomesodermin, *NKG7*, and granzymes. Differential expression of CD161, Granzyme-A, and CD58 was confirmed at the protein level (see Fig E1, D).

Regarding the generation of T_H1/T_H17 cells, it was demonstrated that T_H17 cells are highly plastic and can differentiate into T_H1/T_H17 cells with appropriate cytokines.^{6,9,46} Also, T_H1_{CM} cells are plastic,⁴⁷ but attempts to induce T_H1/T_H17 cells from human T_H1 cells were thus far unsuccessful. However, under permissive metabolic conditions,³⁶ a combination of T_H17-promoting cytokines^{36,48} strongly induced CCR6 expression on T_H1_{CM} cells, whereas the T_H1-inducing cytokine

FIG 1. T_H1/T_H17_{CM} cells are closely related to conventional T_H17 cells but produce high amounts of proinflammatory cytokines. **A**, Gating strategy to identify T_H1, T_H17, and T_H1/T_H17 cell subsets among antigen-experienced helper T cells according to chemokine receptor expression. **B**, CCR7 expression on gated CCR5⁺ and CCR5[−] memory T cells. One representative donor of 4 is shown. **C**, Production of IFN- γ , IL-17, GM-CSF, and IL-22 of the indicated T-cell subsets was measured in *ex vivo*-stimulated purified T-cell subsets by means of intracellular staining (n = 10-19; 1-way ANOVA). **D**, Heat map and hierarchical clustering of 242 differentially expressed genes in T_H17, T_H1_{CM}, T_H1_{EM}, T_H1/T_H17_{CM}, and T_H1/T_H17_{EM} cell subsets from at least 3 different donors. **E**, Box plots of CCR6 expression on T_H1_{CM} cells and CXCR3 expression on T_H17 cells on *in vitro* anti-CD3/CD28 stimulation in the absence of exogenous cytokines (white box) and under T_H1 (dark gray box) or T_H17 (gray box) conditions. In addition, box plots of the percentage of IFN- γ - and IL-17-coproducing T cells under the same conditions are shown (n = 4-6; 1-way ANOVA). **P* < .05, ***P* < .005, and ****P* < .0005.

IL-12 was inhibitory (Fig 1, E, left panels). Reciprocally, T_H17 cells expressed high levels of CXCR3 under T_H1 conditions, whereas T_H17 conditions were detrimental (Fig 1, E, right panels). Importantly, the acquisition of a T_H1/T_H17 phenotype by T_H1 and T_H17 cells correlated with IFN- γ /IL-17 coexpression. Interestingly, IFN- γ /IL-17-coproducing cells were induced even without exogenous cytokines under these conditions³⁶ but much more efficiently from T_H17 than from T_H1_{CM} cells (Fig 1, E).

In conclusion, the analysis of gene expression and cellular plasticity suggests that T_H1/T_H17_{CM} cells are closely related to T_H17 cells and more distantly to T_H1 cells.

Selective and systemic expansion of T_H1/T_H17_{CM} cells in patients with RR-MS

We then compared the *ex vivo* cytokine-producing capacities of total CD4⁺ T cells in peripheral blood of patients with RR-MS with those of age- and sex-matched HDs and patients with progressive MS (Fig 2, A). Among patients with RR-MS, we further discriminated between those with mild/stable disease (MSSS < 1) and those with more advanced/severe disease (MSSS > 1, see Table E1); the latter are of high clinical relevance because they frequently develop the progressive form, which is difficult to treat. CD4⁺ T cells from peripheral blood of patients with RR-MS with severe disease (RR > 1) produced significantly higher amounts of IFN- γ , IL-17, GM-CSF, and IL-22 compared with healthy subjects (Fig 2, A). Importantly, the enhanced production of IFN- γ , IL-17, and GM-CSF was not observed in patients with progressive MS or patients with RR-MS with mild disease (RR < 1). Conversely, IL-22 production was not significantly different in the analyzed cohorts of MS patients.

We then monitored the frequencies of relevant helper T-cell subsets in the blood of different cohorts of patients with MS to identify the cells responsible for the enhanced cytokine production. Strikingly, patients with RR-MS with severe but not those with progressive or mild disease had significantly higher frequencies of T_H1/T_H17_{CM} cells compared with healthy subjects (Fig 2, B). Conversely, T_H17 cells were largely unaffected, and T_H1 subsets were even reduced. Notably, T_H1/T_H17_{EM} cells were not increased, in contrast to T_H1/T_H17_{CM} cells, but were selectively reduced in patients with RR-MS with mild disease.

In summary, patients with RR-MS with severe disease have systemic and selective expansion of T_H1/T_H17_{CM} cells, and CD4⁺ T-cells in these patients with MS show consequently enhanced production of IFN- γ , IL-17, and GM-CSF.

T_H1/T_H17_{CM} and T_H1_{CM} cells in patients with RR-MS respond selectively to self-antigens and viral antigens

We previously showed that total CCR6⁺ T cells, which contain both T_H17 and T_H1/T_H17 subsets, are autoreactive in healthy individual subjects,³⁴ and therefore we asked whether the increased frequencies of T_H1/T_H17_{CM} cells in patients with RR-MS might be associated with autoreactive T-cell expansion. Thus we analyzed the proliferation of purified T-cell subsets in response to self-MHC displayed on *ex vivo*-isolated autologous CD1c⁺ DCs in the absence of exogenous antigens.³⁴ In healthy subjects T_H17 cells showed the highest autoreactivity, and T_H1_{CM} cells

showed the lowest autoreactivity (Fig 3, A, and see Fig E2, A, in this article's Online Repository at www.jacionline.org). Of note, T_H1/T_H17 subsets were poorly autoreactive in healthy subjects. This weak proliferation observed with autologous DCs reflected low autoreactivity but not reduced proliferative capacities because all T-cell subsets proliferated vigorously with allogeneic DCs (see Fig E2, A). Strikingly, in patients with RR-MS, T_H1/T_H17_{CM} cells were highly autoreactive, whereas T_H1/T_H17_{EM} cells showed hardly any autoreactivity (Fig 3, A). Also, T_H1_{CM} cells were poorly autoreactive in patients with RR-MS, whereas T_H17 cells showed some autoreactivity but were less autoreactive in patients with RR-MS than in healthy subjects (Fig 3, A).

To understand whether T_H1/T_H17_{CM} cells reacted with self-antigens that are implicated in MS, we analyzed their proliferation with autologous monocytes loaded with myelin antigens (ie, MBP and myelin oligodendrocyte glycoprotein [MOG]-derived peptides). Notably, monocytes are much less potent antigen-presenting cells than DCs⁴⁹ and therefore are inefficient to induce T-cell proliferation in the absence of exogenous antigens and allow the assessment of antigen-specific responses,³⁴ including those to myelin self-antigens in patients with MS (see Fig E2, B). In HDs we detected weak proliferation with MOG/MBP peptides of T_H17 and T_H1_{CM} cells, whereas T_H1/T_H17_{CM} cells did not respond (Fig 3, B). In marked contrast, in patients with RR-MS, myelin antigens consistently induced proliferation of T_H1/T_H17_{CM} cells (Fig 3, B, and see Fig E2, B), whereas responses of T_H17 and T_H1_{CM} cells were low and undetectable, respectively.

We also assessed antigen specificities for EBV and JCV because these viruses are risk factors for MS development and therapy, respectively. In healthy subjects both T_H1/T_H17_{CM} and T_H1_{CM} cells proliferated vigorously with JCV-derived peptides (Fig 3, B). In marked contrast, in patients with RR-MS, only T_H1_{CM} cells proliferated with JCV (Fig 3, B, and see Fig E2, B), whereas T_H1/T_H17_{CM} cells did not respond. T_H1_{CM} cells proliferated also with EBV-derived peptides, whereas T_H1/T_H17_{CM} and T_H17 cells responded poorly or not at all.

In conclusion, T_H1/T_H17_{CM} cells are poorly autoreactive in healthy subjects and respond vigorously to JCV. In marked contrast, in patients with RR-MS, they become autoreactive but do not respond to JCV and switch thus from a protective to a potentially pathogenic response. Conversely, T_H1_{CM} cells are in all cases poorly autoreactive and contain virus-specific cells, suggesting that unlike T_H1/T_H17_{CM} cells, they maintain a protective antiviral function in patients with MS.

Myelin-reactive T_H1/T_H17 cells and virus-specific T_H1 cells are enriched in the CSF of patients with active MS

To characterize T cells that could home to the CNS, the site where disease develops, we analyzed CSF-infiltrating T cells, which have very similar characteristics as T cells in the leptomeninges.⁵⁰ The CSF of patients with MS collected briefly after an attack for diagnostic purposes contained significantly higher levels of the CXCR3 ligand CXCL10 compared with control subjects and also contained some CCL21, whereas CCL5 and CCL20 were hardly detectable (Fig 4, A). Consistent with this chemokine pattern, CXCR3-expressing subsets (ie, T_H1/T_H17 and T_H1 cells) were highly abundant in the CSF of patients

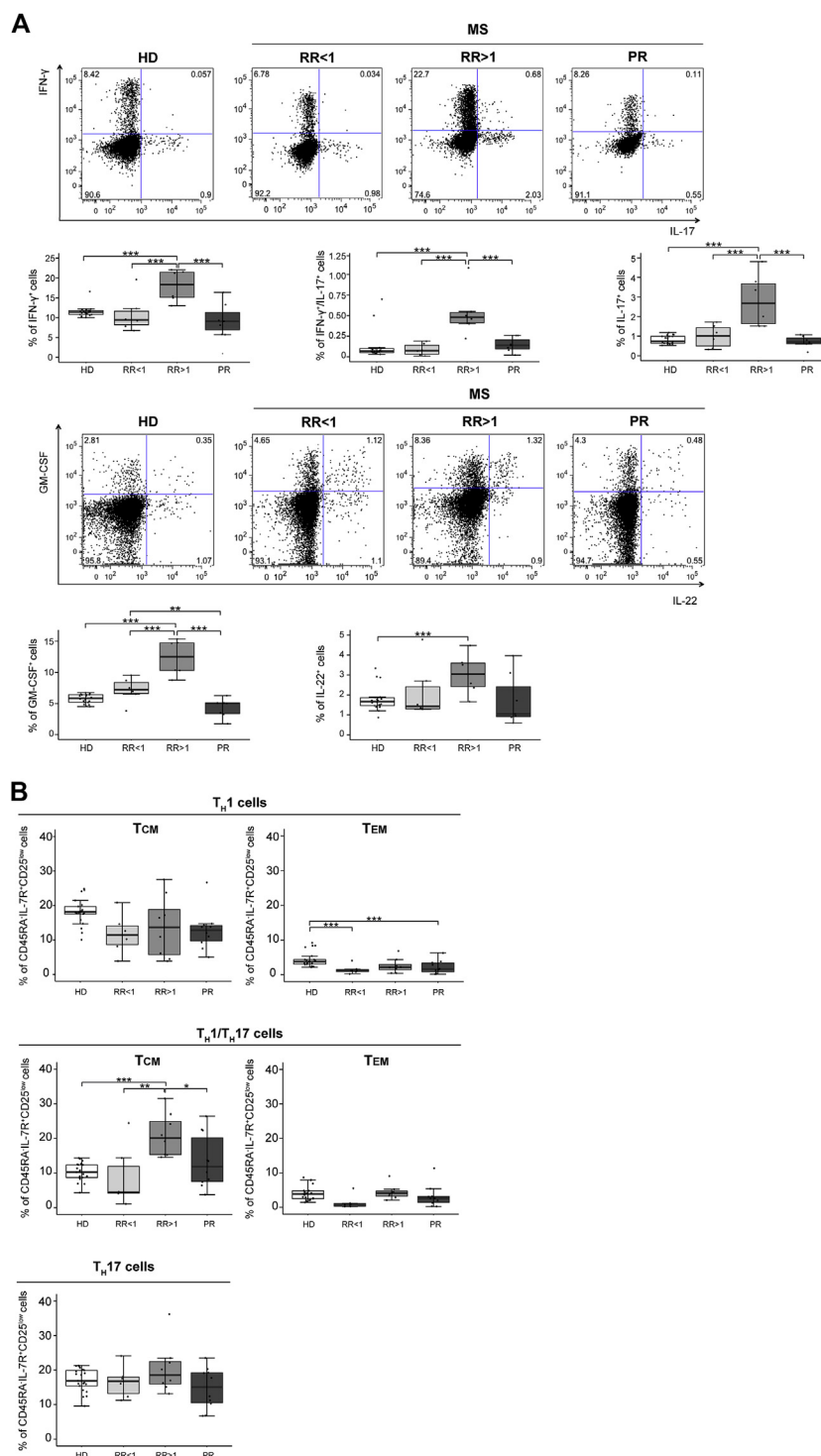


FIG 2. Levels of T_H1/T_H17_{CM} cells and cytokines are selectively increased in patients with RR-MS with severe disease. **A**, Intracellular expression of cytokines in CD4⁺ T cells from peripheral blood in patients with different types of MS (ie, patients with RR-MS with MSSS < 1 [RR < 1, n = 6] or MSSS > 1 [RR > 1, n = 6] and patients with progressive MS [PR; n = 7]) compared with age- and sex-matched HDs (n = 20). Dot plots show intracellular IL-17 versus IFN- γ or GM-CSF versus IL-22 staining in gated CD4⁺ T cells after brief polyclonal stimulation in representative subjects *ex vivo*. Box plots show quantitative flow cytometric analysis of percentages of CD4⁺ T cells that produce IFN- γ and IL-17 alone or in combination (*upper panel*) or GM-CSF or IL-22 (*lower panel*; 1-way ANOVA). **B**, Frequencies of T_H1_{CM}, T_H1_{EM}, T_H1/T_H17_{CM}, T_H1/T_H17_{EM}, and T_H17 cell subsets in HDs (n = 20) and different cohorts of patients with MS (RR < 1, n = 6; RR > 1, n = 8; PR, n = 10), as indicated. Statistical significance was calculated by using 1-way ANOVA between patients with MS and HDs and among different subtypes of MS. **P* < .05, ***P* < .005, and ****P* < .0005.

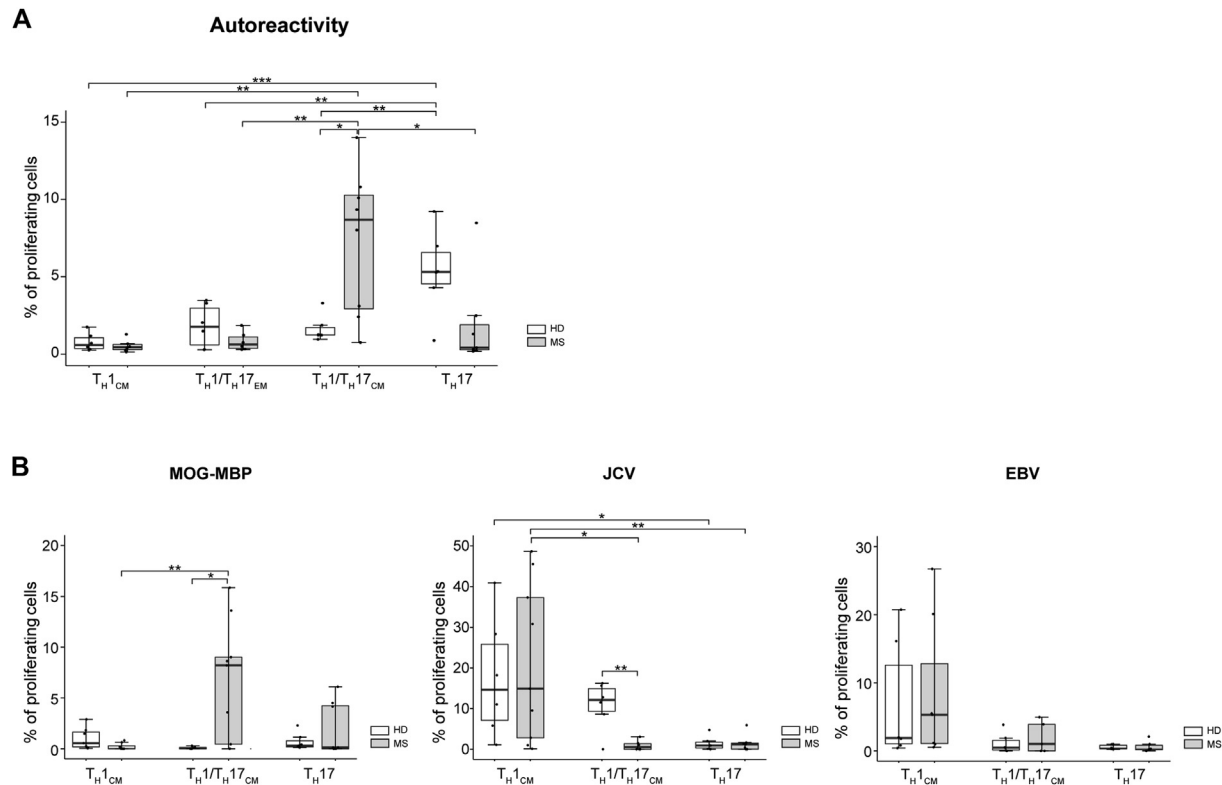


FIG 3. T_H1/T_H17_{CM} cells in patients with MS become highly autoreactive but lose responsiveness to JCV. **A**, Autoreactive proliferation of the indicated helper T-cell subsets with autologous $CD1c^+$ DCs in the absence of exogenous antigens in HDs ($n = 6$, white boxes) and patients with RR-MS ($n = 6-8$, gray boxes). Shown are box plots of divided cells after 5 days and statistical significances (unpaired t test for the comparison of the same subset in HDs vs patients with RR-MS and 1-way ANOVA to compare different T-cell subsets in the same cohort). **B**, Antigen-specific proliferation of the indicated helper T cells with autologous $CD14^+$ monocytes in HDs ($n = 6$) and patients with MS ($n = 5-9$) in the presence of myelin peptides (MOG/MBP, left graph), JCV (VP1), or a mix of immune-dominant EBV-derived peptides (middle and right graphs, respectively). Shown are box plots of divided cells after 7 days induced by specific peptides and statistical significances (analysis as in Fig 3, A). * $P < .05$, ** $P < .005$, and *** $P < .0005$.

with MS (Fig 4, B). Conversely, conventional T_H17 cells, which lack CXCR3 expression, were rare. Moreover, a majority of T_H1 and T_H1/T_H17 cells were $CCR5^-$, suggesting that they were mainly derived from T_{CM} cells (Fig 4, B).

The total number of T cells that can be obtained from the CSF is very low, making a direct *ex vivo* analysis impossible. To overcome this limitation, we polyclonally expanded FACS-purified total T_H1 , T_H1/T_H17 , and T_H17 cell subsets and generated stable T-cell lines. Analysis of the TCR-V β repertoire excluded monoclonal expansions (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Moreover, all CSF-derived T-cell lines expressed high levels of CD40L, IFN- γ , and GM-CSF on brief polyclonal stimulation, whereas IL-17 was produced mainly by T_H17 and T_H1/T_H17 cell lines (see Fig E3, B).

In accordance to what we found in peripheral blood, T_H1/T_H17 cells in the CSF were highly autoreactive because they performed effector functions following contact with autologous monocytes in the absence of exogenous antigens in patients with MS (Fig 4, C). Thus they upregulated CD40L, which is induced on helper T cells on strong TCR stimulation,^{34,51} and produced IFN- γ (Fig 4, C, and see Fig E3, C). Conversely, T_H1 cells were poorly autoreactive in all patients, whereas T_H17 cells were autoreactive in only a single patient. Furthermore, myelin-derived peptides upregulated IFN- γ , IL-17, and GM-CSF production

selectively in T_H1/T_H17 cells, whereas T_H1 cells did not respond (Fig 4, D, and see Fig E3, C). In contrast, JCV and, to a lesser extent, EBV induced IFN- γ and GM-CSF production selectively in T_H1 cells but not in T_H1/T_H17 cells. Responses of T_H17 cells to all these antigens were undetectable. Neutralizing anti-MHC class II antibodies blocked cytokine production and CD40L upregulation by peptide-stimulated T_H1 and T_H1/T_H17 cells, confirming that these effector functions were TCR dependent (see Fig E3, C).

These data show that T_H1 cells and T_H1/T_H17 cells are enriched in the CSF of patients with MS shortly after an attack and that, as observed in peripheral blood, CSF-infiltrating T_H1 cells are specific for viral antigens, whereas CSF-infiltrating T_H1/T_H17 cells are autoreactive and react with myelin antigens. Furthermore, CSF-infiltrating T cells contain T_{CM} -like cells and might be recruited through the CXCL10/CXCR3 axis.^{52,53}

Efficient but unselective targeting of T_H1/T_H17_{CM} cells by therapies that block lymphocyte migration from lymph nodes to the CNS

Fingolimod and natalizumab efficiently reduce relapses in patients with RR-MS by blocking T-cell exit from lymph nodes and extravasation to the CNS, respectively.^{15,16,19} These

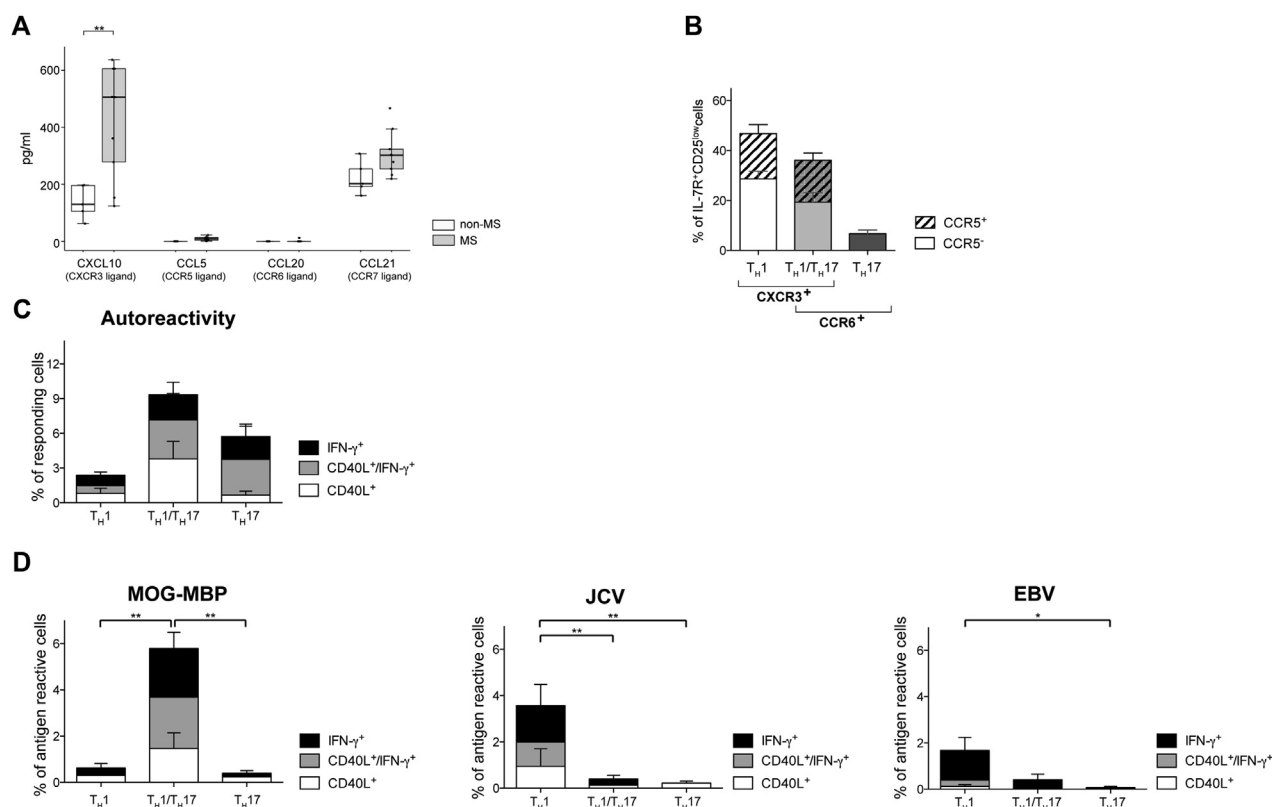


FIG 4. Autoreactive T_H1/T_H17 cells and virus-specific T_H1 cells are enriched in the CSF of patients with active MS. **A**, Chemokines in the CSF of patients with MS shortly after an attack (n = 9) and control subjects without MS (n = 5) were quantified by means of ELISA and represented by using box plots (unpaired *t* test). **B**, Frequencies of T_H1 , T_H1/T_H17 , and T_H17 cell subsets among helper T cells in the CSF of patients with MS (n = 10). **C**, Mean percentage of CD40L⁺, IFN- γ ⁺, and CD40L⁺IFN- γ ⁺ cells among CSF-derived T_H1 , T_H1/T_H17 , and T_H17 cell lines in response to autologous CD14⁺ monocytes in the absence of exogenous antigens were measured to assess autoreactivity (n = 5). **D**, Mean percentages of CD40L⁺, IFN- γ ⁺, and CD40L⁺IFN- γ ⁺ cells induced by antigenic myelin-derived (MOG and MBP, left graph), JCV-derived, or EBV-derived peptides (middle and right graphs, respectively; n = 5). CD40L⁺IFN- γ ⁺ cells induced by monocytes alone were subtracted. In Fig 4, C and D, statistical significance was calculated by using 1-way ANOVA on total percentages of responding cells. **P* < .05 and ***P* < .005.

treatments have not been approved for patients with progressive MS and have severe side effects. Nevertheless, parameters that predict the patient's responsiveness are missing. To address the effects of these therapies on T_H1/T_H17_{CM} cells, we analyzed the effects of natalizumab or fingolimod on the frequencies of different helper T-cell subsets in peripheral blood of treated and untreated patients with RR-MS.

In natalizumab-treated patients the percentage of circulating CD4⁺ T cells was comparable with those in untreated patients (Fig 5, A); among these, the T_H1/T_H17_{CM} subset was enriched, and the T_H1_{CM} subset was reduced (Fig 5, B). The latter was an unexpected finding because T_H1/T_H17 and T_H1 subsets expressed high and comparable levels of the $\alpha 4/\beta 1$ -integrin (Fig 5, C). Interestingly, a relevant fraction of T_H1/T_H17 but also T_H1 and T_H17 cells in both peripheral blood and CSF coexpressed CCR7 and $\alpha 4$ -integrin (see Fig E4, A, in this article's Online Repository at www.jacionline.org), suggesting that these peculiar cells could shuttle between lymph nodes and the CNS and were therefore susceptible to both natalizumab and fingolimod therapies. Consistently, we could detect T_H1/T_H17_{CM} , T_H1_{CM} , and T_H17 cells in human secondary lymphoid organs, and a relevant fraction of them expressed the $\alpha 4/\beta 1$ -integrin (data not shown).

In fingolimod-treated patients the majority of CD4⁺ T cells were depleted from peripheral blood, as expected (Fig 5, A). To quantify the treatment efficacy on different T-cell subsets, we calculated the fold decrease of T-cell subsets in treated versus untreated patients with RR-MS. Numbers of T_H1/T_H17_{CM} cells, but also T_H1_{CM} and T_H17 cells, were strongly reduced in peripheral blood of fingolimod-treated patients with MS (Fig 5, D), whereas T_H1_{EM} and T_H1/T_H17_{EM} cells were less affected, as expected.¹⁶

In summary, circulating T_H1/T_H17_{CM} cells coexpress CCR7 and the $\alpha 4/\beta 1$ -integrin and therefore could be targeted by both fingolimod and natalizumab, suggesting that the high efficacies of these treatments could rely on their common effects on potentially pathogenic autoreactive T_H1/T_H17_{CM} cells. Nevertheless, these treatments are not selective and also target virus-specific T_H1 cells that could perform immune surveillance and play a protective role.

DISCUSSION

MS is a disabling autoimmune disorder driven by autoreactive T cells. We provide strong evidence here that T_H1/T_H17_{CM} cells play a key pathogenic role in MS, whereas T_H1 cells are likely

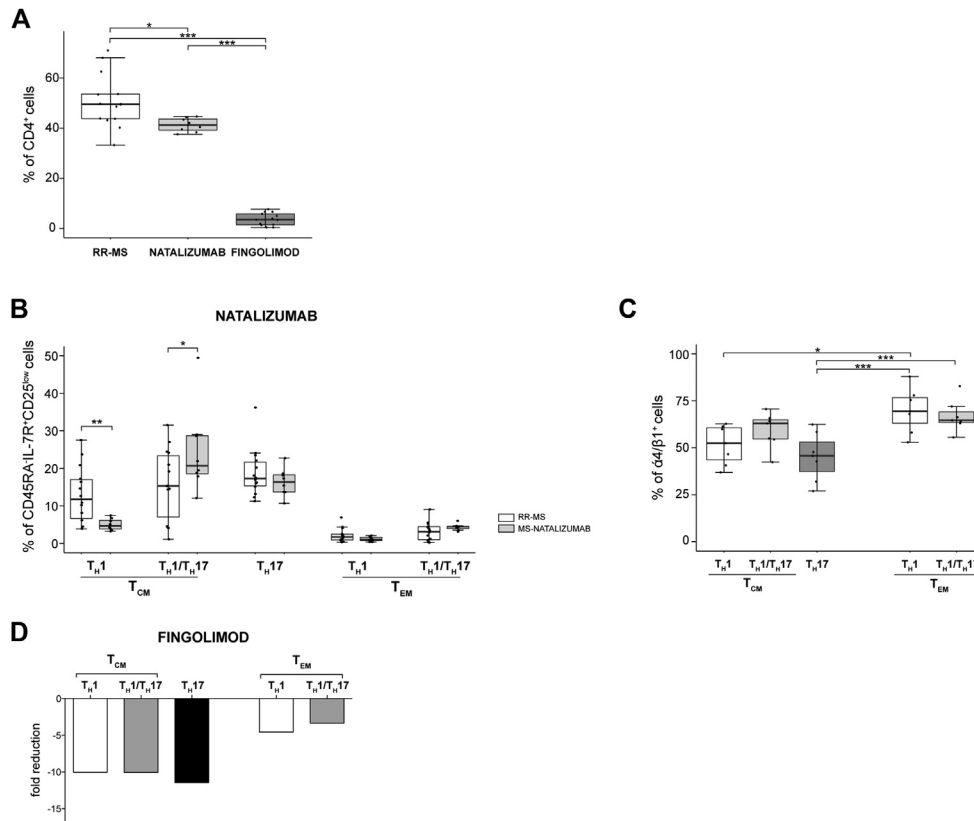


FIG 5. Efficient but unselective targeting of T_H1/T_H17_{CM} cells by fingolimod and natalizumab therapies. **A**, Box plots of total CD4⁺ T cells among lymphocytes in peripheral blood of fingolimod-treated (n = 13) or natalizumab-treated (n = 8) and untreated control patients with RR-MS (n = 14; 1-way ANOVA). **B**, Box plots of T_H1, T_H1/T_H17, and T_H17 cell subsets in natalizumab-treated (n = 8) and untreated (n = 14) patients with RR-MS (unpaired *t* test for the comparison of the same subset in untreated versus treated patients, 1-way ANOVA to compare different T-cell subsets in the same cohort). **C**, Box plots of α4/β1-integrin expression on circulating T_H1 and T_H1/T_H17 cell subsets and T_H17 cells (n = 7, 1-way ANOVA). **D**, Fold reduction of T_H1, T_H1/T_H17, and T_H17 cell subsets in fingolimod-treated patients (n = 13) related to control untreated (n = 14) patients with RR-MS. **P* < .05, ***P* < .005, and ****P* < .0005.

to be critical for antiviral immune surveillance of the CNS.²⁰ These findings advance significantly the understanding of MS pathogenesis and have important clinical implications.

Critical properties that confer a high pathogenic potential to CD4⁺ T cells have been identified in animal models of MS, but the phenotype of pathogenic precursor T cells in the blood of patients with RR-MS remained to be defined. Our data indicate a prominent pathogenic role for T_H1/T_H17_{CM} cells in relapses. This peculiar T-cell subset could migrate from lymph nodes to the CNS, produced pathogenic and MS-associated proinflammatory cytokines, was targeted by therapies that prevent relapses, and proliferated with myelin antigens selectively in patients with MS, strongly suggesting that it contained circulating precursors of pathogenic T cells. T_H1/T_H17 cells in the CSF of patients with MS were activated by nonprofessional antigen-presenting cells in the absence of exogenous antigens, suggesting that they were highly autoreactive, possibly because they express a degenerate TCR⁵⁴ and could thus recognize several self-peptides or self-MHC directly in addition to myelin-derived antigens. Following recruitment to the CNS, T_H1/T_H17_{CM} cells could thus be continuously activated by any MHC class II-expressing cell, upregulate CCR5 and cytokine production, and induce chronic inflammation and extensive damage. In healthy subjects

mainly conventional T_H17 cells were autoreactive, suggesting that pathogenic T_H1/T_H17 cells in patients with MS could be derived from autoreactive T_H17 precursor cells. Consistently, T_H17 cells efficiently generated IL-17/IFN-γ-coproducing T_H1/T_H17 cells on TCR stimulation *in vitro*, and the 2 subsets had a very similar gene expression profile. Also, T_H1 cells are plastic,^{18,47,55} and for the first time, we showed here that human T_H1 cells could be converted to T_H1/T_H17 cells with T_H17-promoting cytokines under permissive metabolic conditions.³⁶ However, T_H1 cells are poorly autoreactive, and this alternative pathway of T_H1/T_H17 cell induction might rather generate polyfunctional effector T cells against intracellular pathogens.^{8,9,43} Indeed, T_H1/T_H17 cells in healthy subjects recognize not only JCV but also influenza virus⁸ and are required to control *Mycobacterium tuberculosis* infections.⁵⁶

Neurotropic viruses are critical risk factors for the development^{27,29} and therapy^{22,23} of MS, but they are not present in standard animal MS models, such as EAE, and how they promote MS is poorly understood.²⁹ We found that patients with MS had a reduced CD4⁺ T-cell response to JCV. In HDs both T_H1 and T_H1/T_H17 cells reacted strongly with JCV. In contrast, patients with MS lacked JCV-specific T_H1/T_H17 cells and had overall reduced frequencies of T_H1 cells. The paucity of conventional

T_H1 cells and lack of polyfunctional, JCV-specific T_H1/T_H17 cells in patients with MS might explain their susceptibility to JCV reactivations after natalizumab treatment.^{22,23} Exaggerated antiviral T-cell responses and JCV itself can induce CNS damage,^{27,29} but the physiologic control of JCV, EBV, and other persistent neurotropic viruses in healthy subjects does not cause immunopathology. Moreover, the activity of virus-specific T cells is limited in time and space by the presentation of viral antigens. These findings suggest that T_H1 cells in patients with MS are critical to contain JCV but are insufficient to induce relapses in the absence of autoreactive T_H1/T_H17 cells.

Natalizumab is one of the most efficient therapies to prevent relapses. It does not deplete pathogenic lymphocytes but blocks their homing to the CNS.¹⁹ Consequently, it leads to the accumulation of activated proinflammatory T cells in the circulation of treated patients with MS.⁵⁷ Consistently, numbers of potentially pathogenic T_H1/T_H17_{CM} cells were selectively increased in peripheral blood of natalizumab-treated patients, whereas on the contrary, numbers of conventional T_H1 cells were further reduced. The imbalance between T_H1 and T_H1/T_H17 cells in patients with MS suggests that T_H1 and T_H1/T_H17 subsets compete for the same homeostatic niche.⁵⁸⁻⁶⁰ Indeed, among $CD4^+$ T cells, only T_H1 and T_H1/T_H17 cells express high levels of T-bet, which induces an IL-15-dependent lifestyle.^{18,58,61} Natalizumab further reduces the ratio of T_H1 to T_H1/T_H17 cells, presumably by favoring interactions of autoreactive T_H1/T_H17_{CM} cells in lymph nodes with DCs, which present self-MHC but could also transpresent IL-15.^{49,62} In addition, natalizumab might limit the access of antiviral T_H1 cells to IL-15-rich tissues.^{58,59}

Viruses were proposed to promote MS through molecular mimicry.^{30,32} Our results support an alternative but not mutually exclusive⁶³ scenario that could explain the permissive role of viruses in patients with MS (see Fig E5 in this article's Online Repository at www.jacionline.org). Healthy subjects are persistently infected with many different viruses, and several of these viruses latently infect the CNS. The reactivation of latent viruses in the CNS leads to type 1 Interferon and CXCL10 production. Virus-specific T_H1 and T_H1/T_H17 cells could then be recruited in a CXCR3-dependent manner to the CNS to control the virus. In patients with MS, this physiologic immune surveillance appears to be disturbed because viral reactivations lead not only to the recruitment of virus-specific T_H1 cells but also that of autoreactive T_H1/T_H17 bystander cells. Although virus-activated T_H1 cells might be important to induce early inflammation, T_H1/T_H17 cells could perpetuate the response after viral control and extend it to uninfected areas where they could even induce *de novo* viral reactivations.²¹ Indeed, latent and lytic EBV is present in brain lesions of patients with MS.^{64,65} In addition, T_H1/T_H17 cells produce IL-17 that is directly neurotoxic⁶⁶ and could enhance permeability of the blood-brain barrier.⁶⁷ Thus in this scenario reactivations of JCV, EBV, or any other persistent neurotropic virus could promote relapses by recruiting autoreactive T_H1/T_H17 cells to the CNS, providing a plausible explanation for the enigmatic associations of MS with several different neurotropic viruses (see Fig E5).^{28,29}

Finally, our findings have several relevant implications for prognosis and therapy of MS. First, the presence of autoreactive T_H1/T_H17 cells in the CSF is a candidate prognostic marker for clinically isolated syndromes that progress to MS. Second, the systemic expansion of autoreactive T_H1/T_H17_{CM} cells is expected to predict the risk of future relapse in patients with RR-MS. Third,

the tissue-homing potentials of autoreactive T_H1/T_H17 cells could predict preferential responsiveness to fingolimod or natalizumab. Fourth, therapies that selectively target T_H1/T_H17 cells but spare T_H1 cells could be highly efficient without causing JCV reactivations and PML. Indeed, in contrast to T_H1 cells, T_H1/T_H17 cells express high levels of CCR6, CD161, and IL-17, which could be targeted in future therapies.^{41,66,68}

Clinical implications: T_H1/T_H17_{CM} cells are a candidate prognostic marker for MS, and future therapies that selectively target these autoreactive T cells might prevent relapses without the risk of PML.

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Supplementary Figure legends

sFig. 1 Characteristics of T-cell subsets from peripheral blood of healthy donors.

(A) T-bet protein expression was measured *ex vivo* by intracellular staining of gated T-cell subsets considering CD4⁺CD45RA⁺ naïve T-cells as negative control (left graph). RORC was assessed by quantitative RT-PCR in purified T-cell subsets (right graph). Mean $\pm$ SEM of at least 4 HD and statistical significances are reported ($P < 0.05$ one-way ANOVA). **(B)** IL-10 production following stimulation with anti-CD3 antibodies was assessed by intracellular staining, mean $\pm$ SEM of at least 4 HD and statistical significances are reported (One-way ANOVA). **(C)** The expression of a functional IL-23R expression was measured by IL-23-induced STAT3 phosphorylation, mean $\pm$ SEM of at least 5 HD and statistical significances are reported (One-way ANOVA). **(D)** Protein expression levels of the selected differentially expressed genes CD161, CD58 and Granzyme A were assessed by cell surface staining of gated T-cell subsets. Mean $\pm$ SEM of at least 5 HD and statistical significances are reported (One-way ANOVA).

sFig. 2: Auto-reactivity and antigen specificity assays of circulating T-cell subsets.

(A) Auto-reactivity assay: Spontaneous proliferation of FACS-purified Th1_{CM}, Th1/17_{CM} and Th17-cells from RR-MS patients and from HD in response to autologous CD1c⁺ DC in the absence of exogenous antigens was assessed after 5 days. Proliferation with allogenic DC was assessed in HD as a positive control. One representative experiment out of 8 is shown. **(B)** Antigen specificity assay: Proliferation with autologous CD14⁺ monocytes of Th1_{CM} cells in the absence or presence of JCV-VP1-peptides (left panel) and of Th1/17_{CM} cells in the absence or presence of MBP and MOG peptides (right panel) on day 7. One representative experiment out of 8 is shown.

sFig. 3: Characteristics of T-cell lines expanded from the CSF of active MS patients.

(A) CSF-derived T-cell lines were analyzed for the expression of 24 TCR V β chains by PCR. The mean number $\pm$ SEM of detected TCR V β chains is shown (n=5). **(B)** CSF-derived T-cell lines were activated with PMA and Ionomycin and the expression of CD40L, IFN- γ , IL-17 and GM-CSF was assessed by intracellular flow cytometry. Shown is the mean percentage $\pm$ SEM of positive cells among Th1, Th1/17 and Th17 subsets (n=5). **(C)** CSF-derived Th1-cells and Th1/17-cells were stimulated with autologous monocytes and respectively JCV or MOG/MBP-derived peptides in the absence or presence of neutralizing anti-HLA-DR,-DQ,-DP antibodies. Expression of CD40L, IFN- γ , IL-17 and GM-CSF was assessed by intracellular staining and flow cytometry. One representative experiment out of 5 is shown.

sFig. 4: Th1_{CM}, Th1/17_{CM} and Th17-cells possess both lymph node and CNS homing potential. **(A)** Representative stainings showing the co-expression of CCR7 and α 4-integrin on the indicated T-helper cells in peripheral blood (upper panels) and in CSF-derived T-cells (lower panel).

sFig 5: Graphical Abstract.

Upper panel: In healthy individuals virus-specific Th1- and Th1/17-cells could be recruited to the CNS upon viral re-activations to control viral replication.

Lower panel: In MS patients viral re-activations could lead not only to recruitment of anti-viral Th1-cells but also to the bystander recruitment of auto-reactive Th1/17-cells, possibly explaining how neurotropic viruses promote relapses.

sTable 1: Characteristics of MS patients and healthy controls.

sTable 2: Differentially expressed genes in Th1, Th17 and Th1/17-subsets.

Supplementary Table 1: Characteristics of MS patients

BLOOD		n	Age*	Gender	EDSS*	MSSS*
<u>HD</u>		20	50.52 ± 1.22	F=100%	-	-
<u>Relapsing-Remitting (RR)</u>	MSSS<1	6	53.83 ± 4.67	F =100%	1.08 ± 0.08	0.55 ± 0.07
	MSSS>1	8	42.00 ± 2.65	F =100%	1.56 ± 0.30	2.08 ± 0.33
<u>Progressive (PR)</u>		10	53.10 ± 2.56	F =70%	5.2 ± 0.37	5.29 ± 0.63
<u>Natalizumab</u>		8	38.88 ± 3.46	F =62.5%	2.88 ± 0.75	3.00 ± 0.72
<u>Fingolimod</u>		13	40.46 ± 2.01	F =69.23%	2.54 ± 0.34	2.51 ± 0.40

Cerebrospinal Fluid (CSF)	n	Age*	Gender	EDSS*	MSSS*
<u>MS</u>	9	33.11 ± 4.29	F =77.78%	-	-
<u>Non-MS</u>	5	47.00 ± 7.05	F =80%	-	-

* Reported as Mean ± SEM

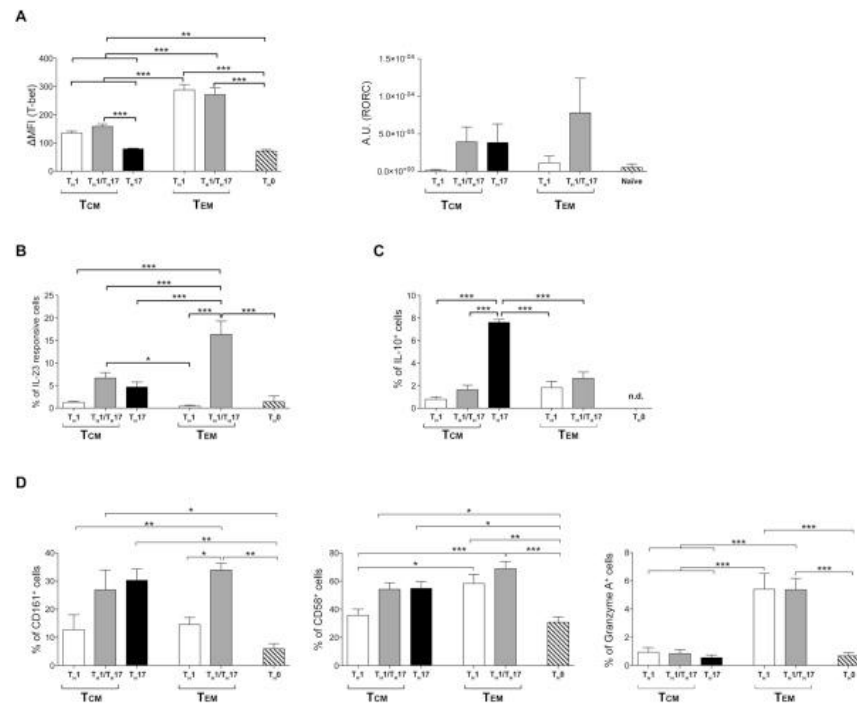


Fig E1.

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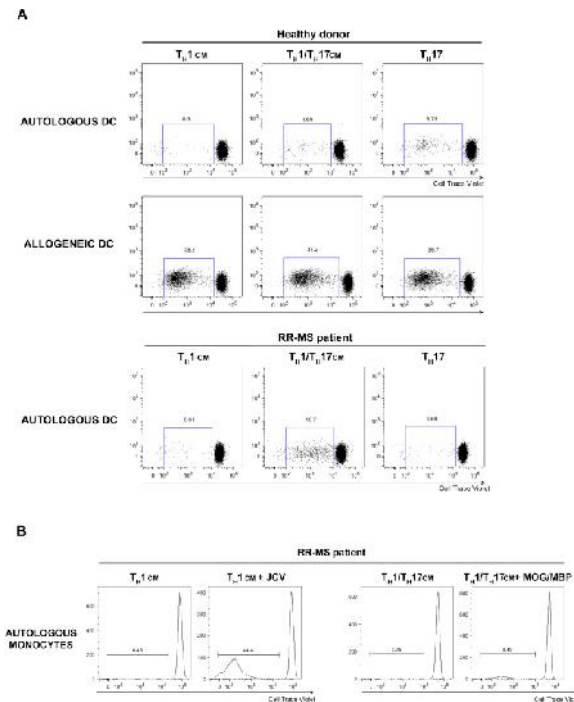


Fig E2.

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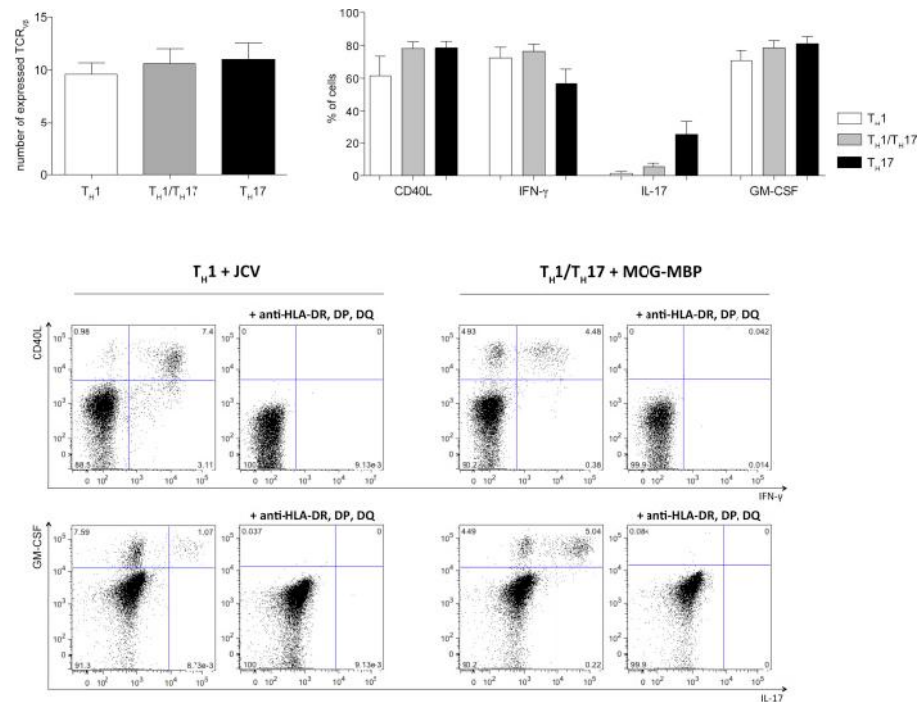


Fig E3.

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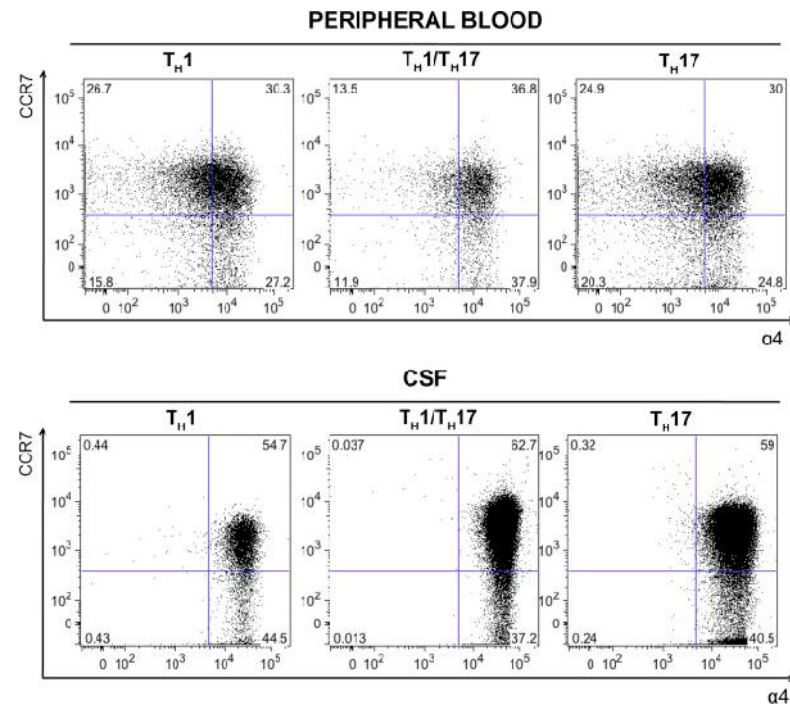


Fig E4.

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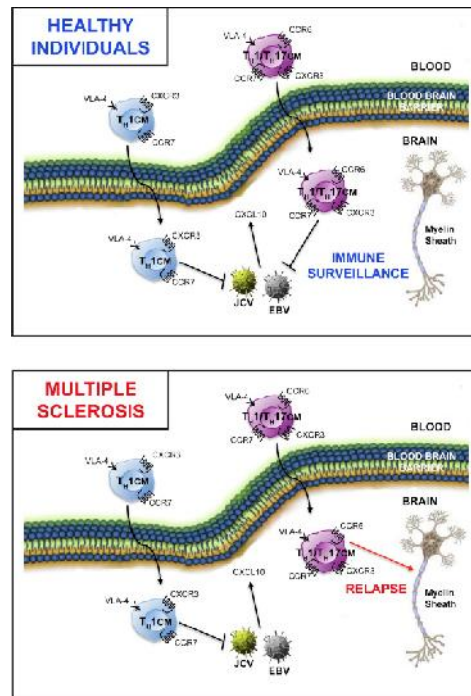


Fig E5.

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Cerebrospinal Fluid (CSF)		n	Age*	Gender	EDSS*	MSSS*
<i>MS</i>		10	32.60 ± 3.87	F =80%	-	-
<i>Non-MS</i>		5	47.00 ± 7.05	F =80%	-	-

* Reported as Mean ± SEM

** Reported as Median (IQR)

Fig E6.

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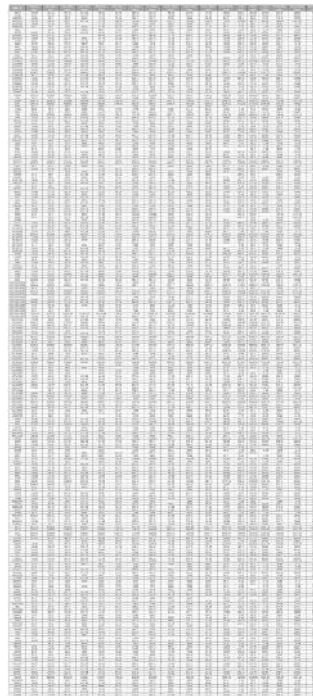


Fig E7.

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