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An intermediate level of CD161 expression defines a novel activated, inflammatory, and pathogenic subset of CD8$^+$ T cells involved in multiple sclerosis

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ABSTRACT

Several lines of evidence support a key role for CD8$^+$ T cells in central nervous system tissue damage of patients with multiple sclerosis. However, the precise phenotype of the circulating CD8$^+$ T cells that may be recruited from the peripheral blood to invade the CNS remains largely undefined to date. It has been suggested that IL-17 secreting CD8 (Tc17) T cells may be involved, and in humans these cells are characterized by the expression of CD161. We focused our study on a unique and recently described subset of CD8 T cells characterized by an intermediate expression of CD161 as its role in neuroinflammation has not been investigated to date. The frequency, phenotype, and function of CD8$^+$ T cells with an intermediate CD161 expression level were characterized *ex-vivo*, *in vitro*, and *in situ* using RNAseq, RT-PCR, flow cytometry, TCR sequencing, and immunohistofluorescence of cells derived from healthy volunteers (n = 61), MS subjects (n = 90), as well as inflammatory (n = 15) and non-inflammatory controls (n = 6). We report here that CD8$^+$CD161^{int} T cells present characteristics of effector cells, up-regulate cell-adhesion molecules and have an increased ability to cross the blood-brain barrier and to secrete IL-17, IFNγ, GM-CSF, and IL-22. We further demonstrate that these cells are recruited and enriched in the CNS of MS subjects where they produce IL-17. In the peripheral blood, RNAseq, RT-PCR, high-throughput TCR repertoire analyses, and flow cytometry confirmed an increased effector and transmigration pattern of these cells in MS patients, with the presence of supernumerary clones compared to healthy controls. Our data demonstrate that intermediate levels of CD161 expression identifies activated and effector CD8$^+$

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T cells with pathogenic properties that are recruited to MS lesions. This suggests that CD161 may represent a biomarker and a valid target for the treatment of neuroinflammation.

Abbreviations list

CSF	Cerebrospinal Fluid
HV	Healthy Volunteer
IBD	Inflammatory Bowel Disease
MAIT	Mucosal Associated Invariant T
NAWM	Normal Appearing White Matter
PBMC	Peripheral Blood Mononuclear Cell
PMA	Phorbol 12-Myristate 13-Acetate
RR	Relapsing Remitting
TCR	T cell receptor

1. Introduction

Multiple sclerosis is a chronic inflammatory disease of the CNS in which $CD8^+$ T cell involvement has increasingly become of interest in recent years. Indeed, compared to $CD4^+$ T cells, these cells predominate in multiple sclerosis lesions through clonal expansions [1,2]. Moreover, their cytotoxic properties suggest that they may directly damage the CNS. However, it remains unclear to date whether a specific subset of $CD8^+$ T cells is involved in CNS lesions. Memory $CD8^+$ T cells have been implicated though [3], as have $CD8^+$ T cells expressing MCAM [4]. The cytokine IL-17 has recently been shown to be involved in the pathophysiology of multiple sclerosis [5], and IL-17-producing $CD8^+$ T cells may represent up to 80% of the $CD8^+$ T cells in active and in chronically active lesions [6], while an antibody targeting IL-17A in multiple sclerosis has been shown to be effective at preventing the occurrence of new active CNS lesions [7]. In humans, at least in the peripheral blood, IL-17 production is specific for CD161-expressing cells [8,9].

CD161 expression in $CD8^+$ T cells defines two specific subpopulations, enriched in memory T cells, displaying a Tc1/Tc17 phenotype and able to secrete proinflammatory cytokines such as TNFα, IFNγ, and IL-17 after stimulation [8–10]. $CD8^+$ T cells with a high level of CD161 expression ($CD8^+CD161^{hi}$) are more than 90% comprised of Mucosal Associated Invariant T (MAIT) cells [11], which have been reported to be involved in the pathophysiology of multiple sclerosis, albeit with a degree of discrepancy between the various studies in this regard [12–17]. Indeed we have recently shown that the frequency of circulating MAIT cells, their phenotype and cytokine secretion pattern is not different between MS patients and healthy controls. Moreover when looking into the CSF or the brain lesions of MS patients, these cells appeared rather scarce suggesting they unlikely contribute to the disease process [15]. However, as we and other have shown the presence of CD161+ T cells in the brain parenchyma of MS patients [15–17], we made the hypothesis that these cells may belong to a $CD8^+$ T cell lineage with an intermediate expression of CD161, which has been used recently to define a specific population of $CD8^+$ T cells. This cell subset has been reported to be a unique population of memory $CD8^+$ T cells, as they have increased effector functions as compared to $CD8^+CD161^{neg}$ and $CD161^{hi}$ cells [18]. Accordingly, they belong to a specific lineage, present already in umbilical cord blood, that exhibits a memory phenotype soon after birth. However, in contrast to MAIT cells, they are not an oligoclonal population [8].

In light of this pattern of effector $CD8^+$ T cells, the presence of T cells expressing CD161 in multiple sclerosis lesions, and the scarcity of MAIT cells in the CNS [15], we hypothesized that $CD8^+CD161^{int}$ T cells could be involved in the pathophysiology of multiple sclerosis. We therefore investigated their potential role in the disease process using RNAseq, Real-Time PCR, flow cytometry, T cell receptor (TCR) sequencing, immunofluorescence histology, and transmigration assays. By showing that these T cells exhibit an activated effector phenotype with migratory potential in multiple sclerosis patients compared to healthy volunteers, our data provide new evidence for their involvement in the disease process. Moreover, these cells are recruited and enriched in the central nervous system of multiple sclerosis patients where they locally and specifically produce IL-17.

2. Materials and methods

2.1. Human samples

Blood samples from multiple sclerosis patients were obtained from the Nantes multiple sclerosis tertiary center together with gender- and age-matched healthy volunteers (HV) and from patients with non-inflammatory disease of the central nervous system. Multiple sclerosis was diagnosed according to the 2010 McDonald Criteria [19]. All of the experiments were performed in parallel on frozen multiple sclerosis and HV samples; with the exception of transmigration experiments, which were performed on fresh blood samples. All-up, 60 patients with relapsing-remitting (RR) multiple sclerosis, 15 with a progressive multiple sclerosis (including eight primary progressive and seven secondary progressive multiple sclerosis), 15 patients with a clinically isolated syndrome, 15 patients with Inflammatory Bowel Disease (IBD, including 13 with Crohn's disease and two with ulcerative colitis), and 61 HV were included in this study. The clinical and demographic characteristics for all of the patients and the HV are summarized in Table 1. The multiple sclerosis patients had not received any disease modifying drugs for at least 6 months prior to the sampling. For the various experiments, groups with the same gender and equivalent ages were used within the described cohort. For the patients presenting a clinically isolated syndrome, that was strongly suggestive of multiple sclerosis (e.g. presence of dissemination in space criteria from the 2010 McDonald criteria on baseline MRI) [19], paired samples of blood and cerebrospinal fluid (CSF) were obtained. Paired blood and CSF samples of six patients with non-inflammatory disease of the central nervous system (e.g. suffering from sudden headaches, or idiopathic intracranial hypertension) were also used for comparison.

CNS samples from 10 multiple sclerosis patients were obtained from the UK multiple sclerosis tissue bank (Imperial College, London, UK), with approval from the Multicenter Research Ethics Committee, (number 08/MRE09/31). Samples of brain and spinal cord from three patients in our local sample collection were also used (Biomedicine Agency identification number PFS13-003). CNS samples were characterized after Luxol Fast Blue, CD3, CD68, and HLA-DR staining on 10 μm serial sections. Lesions were classified as 'active' (e.g. demyelination and homogeneous inflammatory infiltrate), 'chronically active' (e.g. demyelination and inflammatory infiltrate at the edges), or 'normal-appearing white matter'

Table 1
Demographic and disease-related characteristics of patients and controls. MS: Multiple Sclerosis, RR: Relapsing-Remitting, Prog-: Progressive, HV: Healthy Volunteers, NIND: Non-Inflammatory Neurological Diseases, IBD: Inflammatory Bowel Diseases.

	group	n	sex-ratio (F/M)	age at sampling (mean, y)	EDSS Score (median)	D. Duration (mean, y)
MS	CIS	15	3	29.6	1.0	NA
	RR-MS	60	4.5	38.1	2.3	7.3
	Prog-MS	15	1.5	55.2	4.7	10.0
Controls	HV	61	5	36.7	NA	NA
	NIND	6	2	44.6	NA	NA
	IBD	15	2	37.3	NA	10.8

(NAWM) (e.g. no demyelination) with or without inflammatory infiltrate and meningeal infiltrates.

All donors provided written informed consent in compliance with the Declaration of Helsinki and our local university hospital ethics committee.

2.2. $CD8^{+}CD161^{int}$ T cell frequency and phenotype

Peripheral blood mononuclear cells (PBCM) were isolated using a standard Ficoll gradient according to the manufacturer's protocol (Eurobio). $CD8^{+}CD161^{int}$ T cells were studied by multicolor flow cytometry using the following antibodies: CD3-Alexa 700 and V500 (UCHT1), CD4-BD V500 (RPA-T4), CD8-BD V450, PE and PE-Cy7 (RPA-T8), CCR5-FITC (2D7), CCR7-PE-Cy7 (3D12), CD27-PE-Cy7 (1A4CD27), CD28-PE (CD28.2), PSGL-1-PE (KPL-1), CD69-PerCP-Cy5.5 (FN50), IFNγ-Alexa 700 and V450 (B27), GZM-B-Alexa 700 (GB11) from BD biosciences, CD11a-PE (HI111) from Biolegend, PD1-PE (ebioJ105) and IL-17A-e660 (CAP17) from eBiosciences, CD161-FITC, APC and PE-Vio770 (191B8), CD45RA-APC-Vio770 (T6D11) and CD57-FITC (TB03) from Miltenyi. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Yellow stain (Invitrogen) according to the manufacturer's guidelines. For intracellular staining, cells were first stained for viability and extracellular markers, permeabilized and fixed with Perm/Fix reagent (BD Biosciences) before labeling with the appropriate antibodies. Samples were acquired with a LSRII device (BD Bioscience) using FACSDiva™ software (version 6.1.3, BD Biosciences) and analyzed with FlowJo Software (V7.6.5, TreeStar). Application settings and rainbow beads (BD Bioscience) were used to monitor and adjust the laser intensities.

2.3. Stimulation of $CD8^{+}$ T cells

For *in vitro* activation, 18 age- and gender-matched HV and multiple sclerosis patients were compared. Frozen PBMC were used and cultured at 37 °C in supplemented (10% FCS, 1% Penicillin-streptomycin (Invitrogen), 1% glutamine (Sigma)) RPMI medium one day prior to the experiment. The PBMC were then washed twice with PBS and 5×10^5 PBMC were incubated with supplemented RPMI medium at 37 °C for the duration of the experiment. For Phorbol 12-myristate 13 acetate (PMA)/IM stimulation, the PBMC were incubated with PMA (100 ng/ml, Sigma) and Ionomycin (1 μg/ml, Sigma) for 5 h. For CD3/CD28 stimulation, the PBMC were stimulated with anti-CD3 pre-coated wells (1 μg/ml, in-house) and soluble anti-CD28 mAb (1ug/ml, BD Biosciences) over 4 days. For IL-12/IL-18 stimulation, the PMBC were incubated with IL-12 (10 ng/ml, R&D) and IL-18 (100 ng/ml, MBL) over 4 days. For each condition, Brefeldin A (5 μg/ml, Sigma) was added for the last 4 h of the experiment.

2.4. RNA sequencing

For the RNA sequencing studies, $CD8^{+}CD161^{int}$ cells were sorted with BD Aria II cell sorter (BD Biosciences) using the following antibodies: CD3-PE (UCHT1), CD8-FITC (RPA-T8), and CD161-APC (191B8) from BD biosciences. Dead cells were excluded using DAPI staining. After cell sorting, RNA was extracted using a RNeasy Micro Kit according to the manufacturer's guidelines (Qiagen). The 3′ digital gene expression (3′DGE) RNA-sequencing protocol was performed according to Cacchiarelli et al., 2015 [20]. Briefly, the libraries were prepared from 10 ng of total RNA. The mRNA poly(A) tail was tagged with universal adapters, well-specific barcodes, and unique molecular identifiers (UMIs) during template-switching reverse transcriptase. Barcoded cDNAs from multiple samples were then pooled, amplified, and tagmented using a transposon-fragmentation approach which enriches for 3′ ends of the cDNA. A library of 350–800 bp was run on an IlluminaHiSeq 2500 using a TruSeq Rapid SBS Kit.

Read pairs used for analysis met the following criteria: all sixteen bases of the first read had to have quality scores of at least 10, and the first six bases correspond exactly to a designed well-specific barcode. The second reads were aligned to RefSeq human mRNA sequences (hg19) using bwa version 0.7.4 4 with non-default parameter "-l 24". Reads mapping to several positions into the genome were filtered out from the analysis. Digital gene expression (DGE) profiles were generated by counting the number of unique UMIs associated with each RefSeq genes for each sample.

2.5. Microfluidic qPCR analysis on the Fluidigm® Biomark™ HD system

PBMCS from eight untreated RR-multiple sclerosis patients and four age/gender-matched HV were stained with anti-CD3 (UCHT1), anti-CD45RA (T6D11), anti-CD8 (RPA-T8), and anti-CD161 (191B8) antibodies. Dead cells were excluded by DAPI staining. One thousand live $CD3^{+}CD8^{+}CD45RA^{-}CD161^{int}$ cells were sorted into each well of a 96-well plate containing 10 μl of complete RT-PCR mix using a BD FACSAria or BD FACSAria II Cell Sorter (BD Bioscience).

Sorted cells were immediately subjected to a reverse transcription and pre-amplification PCR reaction. The Invitrogen SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific) was used to perform cell lysis, and one step reverse transcription and pre-amplification was performed according to the Fluidigm® Real-Time PCR Analysis user manual. 96 well-selected TaqMan assays (Applied Biosystems, Thermo Fisher Scientific), selected according to their scientific relevance to multiple sclerosis, and were added at a concentration of 18 μM each to ensure gene specific reverse transcription and pre-amplification (a list of the genes is provided in Table S2).

The expression levels of each of the 96 genes of interest were quantified using microfluidic qPCR analysis on the Fluidigm® Biomark™ HD system according to the manufacturer's instructions and expressed as a function of the Ct level. Raw expression data were processed with the Fluidigm® Real-Time PCR Analysis software and analyzed with the Fluidigm® Singular Analysis Toolset in R.

2.6. High-throughput sequencing of the TCR repertoire

For the TCR repertoire studies, ten patients with untreated relapsing-remitting multiple sclerosis and ten age-and gender-matched healthy volunteers were selected. CD161^{int}, CD161^{neg}, and non-mucosal associated invariant T (MAIT) memory CD45RA-^{neg}Va7.2^{neg} CD8^{+} T cells were sorted using FACSAria II to a purity of >90%. Amplification and sequencing of TRBV CDR3 regions was performed using the immunoSEQ platform (Adaptive Biotechnologies, Seattle, WA). ImmunoSEQ combines multiplex PCR with high-throughput sequencing and a sophisticated bioinformatics pipeline for quantitative Vβ CDR3 region analysis.

2.7. Immunofluorescence histology analysis of post-mortem brain samples

Frozen CNS samples from 10 multiple sclerosis patients from the UK multiple sclerosis tissue bank and from three patients in our local tissue bank were used (a total of 42 lesions). Frozen 10 μm sections were thawed, fixed in 4% paraformaldehyde (PFA) (EMS), rinsed in PBS, and incubated for 30 min in blocking solution consisting of PBS with 10% normal goat serum and 0.1% saponin (Sigma-Aldrich), to prevent non-specific staining and to permeabilize the tissue. Sections were stained by overnight incubation at 4 °C with either anti-CD3 (polyclonal, DAKO), anti-CD161 (191B8, Myltenyi), or anti-Vα7.2 (3C10, Biolegend) antibodies diluted in the blocking solution. This first staining was used to screen and select the lesions where no MAIT cells (CD3^{+}CD161^{+}Vα7.2^{+} cells) were observed. Among the 42 lesions, eight lesions without any MAIT cells were selected, and serial sections were stained with anti-CD8 Alexa 647 (RPA-T8, BD), anti-CD161 (191B8, Myltenyi), and anti-IL-17 Alexa 488 (41809, R&D system) antibodies diluted in blocking solution. The corresponding secondary antibody (anti-mouse IgG2a-Alexa 568, Invitrogen) was incubated for 2 h at room temperature in blocking solution. After a 10 min incubation in PBS with 1% DAPI, the slides were mounted using ProLong Antifade Reagent (Invitrogen). Sections from the eight lesions were imaged on an A1R confocal microscope (Nikon Instruments) with a 40× objective. The number of CD161^{int} T cells in the CD8^{+} population was determined on sections containing more than 100 CD8^{+} T cells.

2.8. Transmigration assay

For transmigration assays, we used the hCMEC/D3 human brain endothelial cell line (a gift from P.O. Couraud, INSERM, France), which provides an *in vitro* approximation of the characteristics of the blood-brain barrier [21]. Cells (2.5×10^5) were seeded on a transwell with 8.0 μm-sized pores (Corning) two days prior to the migration experiment in supplemented EMB2 medium (Lonza) according to P.O. Couraud's protocol [21]. The number of cells seeded was adjusted so that, after 2 days of culture, a single-layer barrier was obtained that allowed for migration of less than 5% of Alexa-488-labeled Albumin (Life Technologies) after 6 h, as previously described [15]. In parallel, CD8^{+} and CD8^{-}CD4^{-} T cells were negatively isolated from fresh blood with an AutoMACS cell sorter (Miltenyi) using a CD8^{+} T cell isolation kit (Miltenyi) according to the manufacturer's guidelines. The purified T cells (5×10^5) were suspended in 1 ml of supplemented RPMI and EBM2 (50% v/v each) and added on top of the filter. After 12 h at 37 °C, the cells below the filter (i.e. the "transmigrated fraction") were collected. A control well without filter consisted of the "total fraction" that had not been made to undergo the transmigration experiment. The fractions were counted and stained for flow cytometric analysis with CD3, CD4, CD8, CD161, and LIVE/DEAD Fixable Aqua stain (Invitrogen). The number of transmigrated cells was determined by using CountBright™ Absolute counting beads (Life Technologies). When stated, the barrier cell line was cultured under inflammatory conditions by adding 100 U/ml of TNFα and IFNγ (R&D Systems) for 8 h. To assess the transmigratory ability of each subset of cells, the ratio of the absolute number of transmigrated cells in the subset of cells of interest and the total number of cells in this subset in the control well was determined.

2.9. Statistical analyses

Statistical analyses were performed using PRISM software (V5.03, GraphPad Software). A two-tailed Mann-Whitney test was performed to compare two groups. A p-value <0.05 was assumed to be significant for flow cytometry. A Spearman correlation was used to correlate the frequency of CD8^{+}CD161^{int} with age.

For RNAseq analyses, the comparison of the two groups was performed after normalization of the data in order to remove systematic technical differences between samples and to ensure that technical bias had a minimal impact on the results. After correction for multiple testing, a gene was considered to be significantly different in the two populations when $p < 0.05$. Supplementary information for RNAseq biostatistics is provided in the Supplementary Methods.

R software was used for the qPCR and RNAseq analyses.

3. Results

3.1. The percentage of CD8^{+}CD161^{int} T cells is specifically decreased in the blood and enriched in the CSF and the CNS of multiple sclerosis patients

As shown in Fig. 1A, the CD161 marker identifies three subsets within blood CD8^{+} T cells: the most abundant is the CD161^{neg} population, followed by the populations of cells expressing intermediate and high levels of CD161. We first compared the percentage of blood CD8^{+}CD161^{int} T cells in healthy volunteers (HV) and patients with relapsing-remitting untreated multiple sclerosis. We found a lower abundance of these cells in the blood of multiple sclerosis patients compared to HV (8.07% ± 4.49 vs. 6.58% ± 3.73, respectively, $p < 0.05$) and to IBD patients used as inflammatory controls (10.75% ± 5.82, $p < 0.05$) (Fig. 1B). This suggests that the observed lower frequency is specific to multiple sclerosis patients, while the percentages of CD8^{+}CD161^{high} T cells were not different between the HV and multiple sclerosis patient groups (supplementary Fig. 1A). We observed a positive correlation of CD8^{+}CD161^{int} T cell frequency with age in HV, which was lost in multiple sclerosis patients ($R^2 = 0.34$; $p = 0.0007$ and $R^2 = 0.02$; $p = 0.41$, respectively) (Fig. 1C). In line with these results, a significantly lower frequency of CD8^{+}CD161^{int} T cells was also observed in the blood of progressive (and older) patients as compared to age-matched HV (8.20% ± 3.40 versus 12.18% ± 4.82, respectively, $p < 0.05$) (Fig. 1D).

Using paired samples, for the central compartment we found a specific enrichment of CD8^{+}CD161^{int} T cells in the CSF compared to the blood (median frequency of 9.8% (5.0%–23.4%) in the CSF vs. 6.1% (2.9%–32.7%) in the blood, $p < 0.05$) (Fig. 1E). This enrichment was specific for CD161^{int} T cells as neither the frequency of CD161^{neg} nor the percentage of CD161^{high} CD8^{+} T cells was increased in the CSF as compared to the blood (Fig. S1B and S1C). Importantly, the enrichment of CD8^{+}CD161^{int} cells in the CSF was specific to multiple sclerosis patients, as it was not observed in non-inflammatory neurological controls (median frequency of 7.7% (4.4%–15.9%) in the CSF vs. 7.4% (2.4%–11.8%) in the blood) as shown in Fig. 1E. Interestingly, the CD8^{+}CD161^{int} T cells from the CSF overexpressed CCR5, and this may functionally explain their

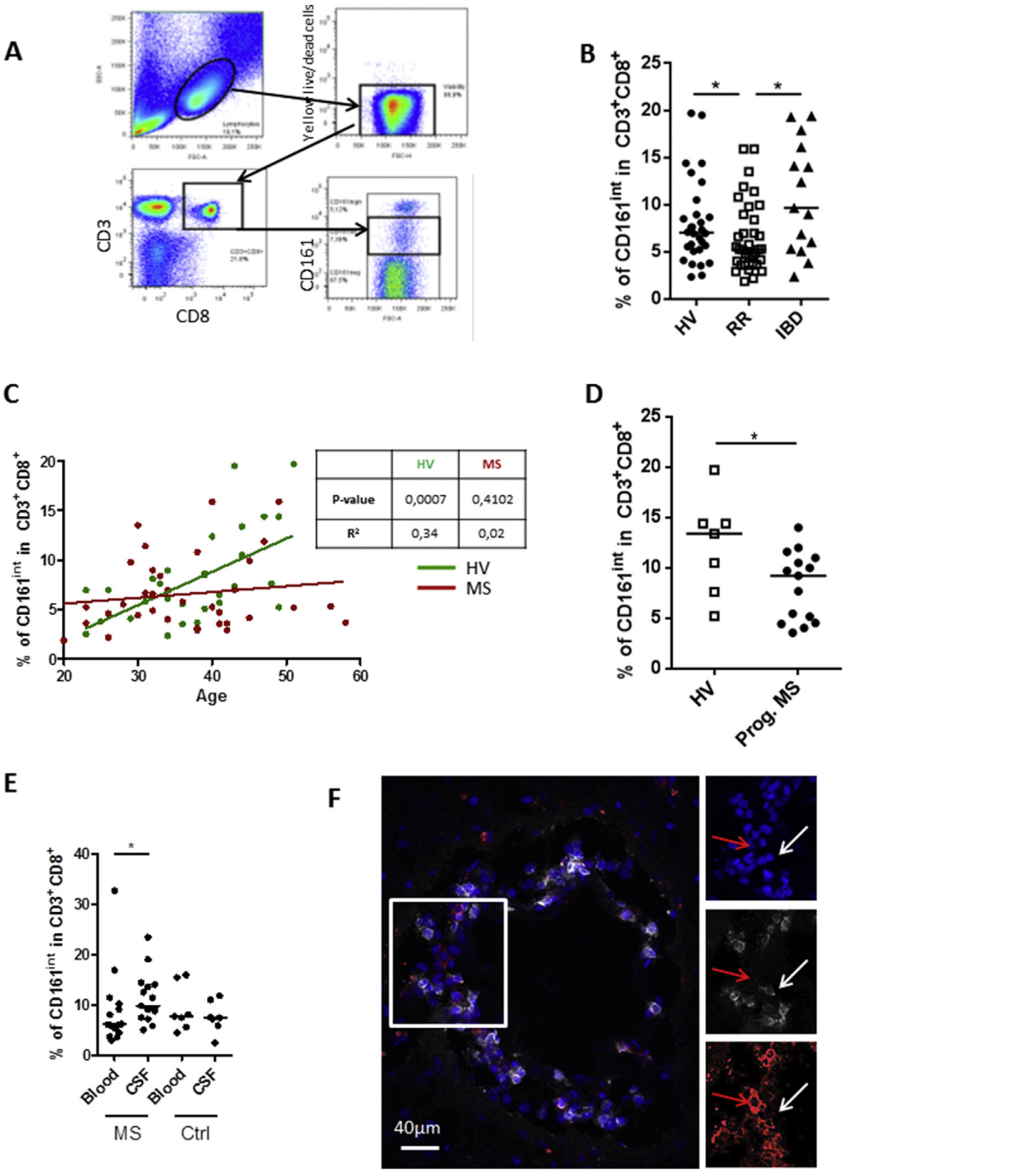

Fig. 1. The frequency of CD8$^+$CD161^{int} T cells is decreased in the peripheral blood and increased in the CSF and CNS of patients with multiple sclerosis. (A) Gating strategy for CD8$^+$ T cells with intermediate CD161 expression levels. (B) Frequency of CD3$^+$CD8$^+$CD161^{int} T cells in untreated relapsing-remitting multiple sclerosis patients (n = 37) versus age- and gender-matched healthy volunteers (n = 29) and patients with inflammatory bowel diseases (n = 15). (C) Correlation between age at sampling and frequency of peripheral CD8$^+$CD161^{int} T cells in multiple sclerosis and HV. (D) Frequency of CD8$^+$CD161^{int} T cells in the blood of patients with progressive multiple sclerosis (n = 15) compared to age-matched HV in the cohort (n = 7). (E) Frequency of CD8$^+$CD161^{int} T cells in the blood and the CSF of CIS (highly suggestive of multiple sclerosis) patients (n = 15) and controls (n = 6). (F) Example of CD8$^+$CD161$^+$ staining in a perivascular infiltrate from a multiple sclerosis lesion. Blue: DAPI, grey: CD8, red: CD161. White arrows indicate CD8$^+$CD161$^+$ cells and red arrow indicates one CD8^{neg}CD161$^+$ cell.

recruitment into the CSF (MFI of 66.3 [53.5–75.0] in the blood vs. 106.3 [82.1–138] in the CSF, $p < 0.05$) (Fig. S1D).

In order to explore the functional relevance of the CD161 marker in the multiple sclerosis lesion process, we also studied the frequency of all CD3$^+$ T cells expressing CD161 in the CNS of multiple sclerosis patients. We found an increased frequency of these cells in the CNS as compared to the blood (22.8% ± 12.5% vs. 16.4% ± 5.1%, $p < 0.01$) (Figure S1E). Since we have previously shown that MAIT cells (expressing CD3, CD161 and Vα7.2) are scarce among these T cells [15], we selected eight lesions where no MAIT cells were seen, so as to estimate the presence of CD8$^+$CD161^{int} cells, as shown in the typical example of a perivascular infiltrate from one MS brain lesion (Fig. 1F). Interestingly, we found similar frequencies as the ones obtained for the CSF (Fig. S1F).

Overall, these data suggest that CD8$^+$CD161^{int} cells are recruited to the central compartment of multiple sclerosis patients.

3.2. $CD8^+CD161^{int}$ T cells display an effector and activated phenotype with enhanced transmigratory abilities

The physiology and characterization of $CD8^+CD161^{int}$ T cells as a unique subset of T cells in healthy individuals is very recent. Using flow cytometry, we characterized these cells in the peripheral blood of healthy donors, confirming and extending the previous results obtained by Fergusson et al. [18]. As shown in Figure S2A, CD161 intermediate expression was found in 8.06 ± 4.48% of circulating $CD3^+CD8^+$T cells (compared to 78.65 ± 8.55% of $CD8^+CD161^{neg}$ and 13.42 ± 11.4% of $CD8^+CD161^{high}$ T cells) from HV. We confirmed that $CD8^+$ T cells with intermediate levels of CD161 overexpress IL-18Rα and CCR6 compared to the $CD161^{neg}$ population (Fig. S2B and S2C) but also CCR2 and CXCR6 (not shown). This population also specifically overexpressed Granzyme B (GZMB) and perforin, as compared to both $CD161^{neg}$ cells (48.3% [24.8%–78.3%] vs. 15.1% [2.5%–49.8%], $p < 0.001$ for GZMB; 25.5% [5.0%–52.0%] vs. 4.8% [0.9%–25.2%], $p < 0.01$ for perforin, respectively) and $CD161^{high}$ cells (9.5% [2.8%–13.8%], $p < 0.001$, for GZMB; 7.9% [2.7%–29.3%], $p < 0.05$ for perforin, respectively) (Fig. S2D and S2E).

As we have recently demonstrated that $CD8^+$ T cells expressing Tbet and CD57 delineate an aggressive subset of T cells involved in kidney graft rejection [20] we also studied these markers specifically in this subset of T cells. We thereby noted a specific enrichment of $CD57^+$/T-bet^+ T cells in this population, as well as of Lag-3^+/PD-1^+ T cells, thus indicating that they have an activated phenotype as compared to both $CD161^{neg}$ and $CD161^{high}$ CD8 T cells (14.69% [3.5%–39.2%] vs. 5.48% [0.7%–29.4%] and 0.13% [0%–0.8%], $p < 0.001$, respectively, for $CD57^+$Tbet$^+$ and 7.4% [3.5%–12.8%] vs. 2.4% [1.2%–3.9%] and 3.3% [0.5%–6.6%], $p < 0.001$ for Lag3^+/PD1^+, respectively) (Fig. 2A, B, and C).

Accordingly, this activated phenotype is correlated with a high potential for cytokine secretion after CD3/CD28 *in vitro* stimulation. Indeed, as has already been reported [18], $CD8^+CD161^{int}$ cells produce more IFNγ, IL-17, and both after CD3/CD28 *in vitro* stimulation as compared to $CD8^+CD161^{neg}$ T cells (Fig. S2D, E, and F). We also found an increased proportion of $CD8^+CD161^{int}$ T cells expressing GM-CSF, IL-22, or both (Fig. 2F, G and H).

We then assessed the homing markers on $CD8^+CD161^{int}$ T cells that may support their migration to tissues. We found that PSGL-1, CD49d (VLA4), CD11a (LFA1) and intermediate CD62L expression (a recently proposed marker for early memory fast-cycling precursor cells [22]) were increased in the $CD161^{int}$ population as compared to both the $CD161^{neg}$ and $CD161^{high}$ populations. Indeed, 22.80% [13.10–31.70] of the $CD8^+CD161^{int}$ T cells vs. 12.70% [2.61–25.10] of the $CD8^+CD161^{neg}$ T cells and 5.20% [1.90–27.50] of the $CD8^+CD161^{high}$ T cells expressed intermediate levels of CD62L ($p < 0.001$ for all comparisons). Similarly the mean fluorescence intensity (MFI) of PSGL1 was 10,660 [7284–14,100] in $CD161^{neg}$ vs. 18,800 [9373–19,100] in $CD161^{int}$ and 12,800 [5931–18,500] in $CD161^{high}CD8^+$ T cells ($p < 0.05$ for all comparisons). The MFI of CD11a was 3320 [2217–4967] in $CD161^{neg}$ vs. 5690 [4051–9734] in $CD161^{int}$ and 6480 [5046–11,700] in $CD161^{high}CD8^+$ T cells ($p < 0.001$, Fig. 3A, B, C, and D). Interestingly, in blood, these cells also overexpress CD103, which is an integrin associated with tissue residency, compared to the $CD161^{neg}$ and $CD161^{high}$ subsets (8.0% [2.3%–16%] vs. 2.3% [1.1%–4%] and 4.5% [1.0%–8.3%], $p < 0.001$ and $p < 0.05$, respectively) (Fig. 3E).

To investigate the ability of the cells to migrate into the CNS, we analyzed their transmigratory potential using a static *in vitro* model of the blood-brain barrier (BBB) using BBB-derived human endothelial cells (hCMEC/D3) [21]. Interestingly, $CD8^+CD161^{int}$ cells transmigrated more than $CD161^{neg}$ and $CD161^{high}CD8^+$ T cells, through both non-inflamed (Fig. 3F) and inflamed BBB (Fig. 3G). Indeed, we found that the $CD161^{int}$ subset was enriched in the transmigrated fraction as compared to both $CD161^{neg}$ and $CD161^{high}$ $CD8^+$T cells (transmigration ratio of 1.6 [0.9–2.2] vs. 0.9 [0.7–1.2] and 0.8 [0.1–2.4], $p < 0.001$ and $p < 0.01$, respectively) for non-inflamed BBB, (1.7 [1.4–2.1] vs. 0.9 [0.9–1.0] and 0.5 [0.2–0.7], $p < 0.001$ for both) for inflamed BBB.

Altogether, these data show that CD8 expressing intermediate levels of CD161 represent a specific activated population, with cytotoxic capacity and the ability to secrete inflammatory cytokines, and that these cells are likely to migrate into the CNS, thus supporting the notion that they are involved in disease development.

3.3. Circulating $CD8^+CD161^{int}$ T cells from multiple sclerosis patients display a specific transcriptomic profile compared to their counterparts from HV

To more fully characterize $CD8^+CD161^{int}$ T cells in multiple sclerosis patients as compared to HV, we studied their transcriptomic profile. First, quantitative PCR was performed on a set of 96 genes chosen for their possible involvement in the pathophysiology of multiple sclerosis (Supplementary Table 1). For this experiment, one thousand $CD8^+CD161^{int}$ T cells were sorted from the blood of eight untreated RR multiple sclerosis patients and four age- and gender-matched HV. $CD8^+CD161^{int}$ T cells from multiple sclerosis patients and HV had different expression profiles, as shown by the unsupervised clustering (Fig. 4A). These differentially expressed genes were mainly effector markers such as IL-12A, CD69, CD107a, CD38 and GZM-A ($p < 0.05$) and migration markers such as CCR7 and VLA-4 ($p < 0.05$) as indicated in Figure S3A, suggesting higher effector and migration abilities in $CD8^+CD161^{int}$ cells in multiple sclerosis patients as compared to HV. These genes were used for PCA analysis, and allowed for a clear separation of the patients and the HV (PCA analysis, Fig. 4B).

To extend the first analysis with qPCR, we decided to analyze the whole transcriptome by RNAseq. $CD8^+CD161^{int}$ T cells were obtained from eight untreated RR-multiple sclerosis patients and twelve age- and gender-matched HV. After normalization of the data using several techniques (Supplementary Methods), RNA sequencing comparisons revealed a significant differential expression of 170 genes between the two cohorts (Volcano plot, Fig. 4C, gene list in Table S2), allowing for efficient discrimination of the two groups of patients and controls (PLSDA analysis, Supplementary Fig. 3B). Most of these genes were related to oxidative phosphorylation, indicating an increased mitochondrial activity in $CD8^+CD161^{int}$ cells from the multiple sclerosis patients compared to their matched controls. Similarly, an increased level of ribosomal activity was found (Fig. S3C).

Moreover, the chemokine-cytokine-receptor interaction pathway was significantly dysregulated in $CD8^+CD161^{int}$ T cells from multiple sclerosis patients compared to HV. The expression of several cytokines/chemokines/receptors was altered. These mRNA with altered expression levels included the chemokine receptors CXCR4, fractalkine-receptor (CX3CR1), CCR1, CCR5, CCR7, CCR9; the chemokines CXCL3, CXCL5, CXCL7, CXCL10, CXCL11, CCL2, CCL7; the cytokines IL-10, IL-23A, IFNγ; and the cytokine receptors IL-2Rα, IL2-Rγ, IL-7Rα, IL9-Rα or IFNγ-R1 and R2 which were overexpressed; while others, including IL-15 or IL-10RA and IL-10RB were downregulated (Figure S4). These results indicate that the multiple sclerosis-derived cells had increased effector and migratory properties.

3.4. Circulating $CD8^+CD161^{int}$ T cells from multiple sclerosis patients display an activated phenotype and increased homing abilities compared to those from HV

In order to extend and understand the transcriptomic data at the

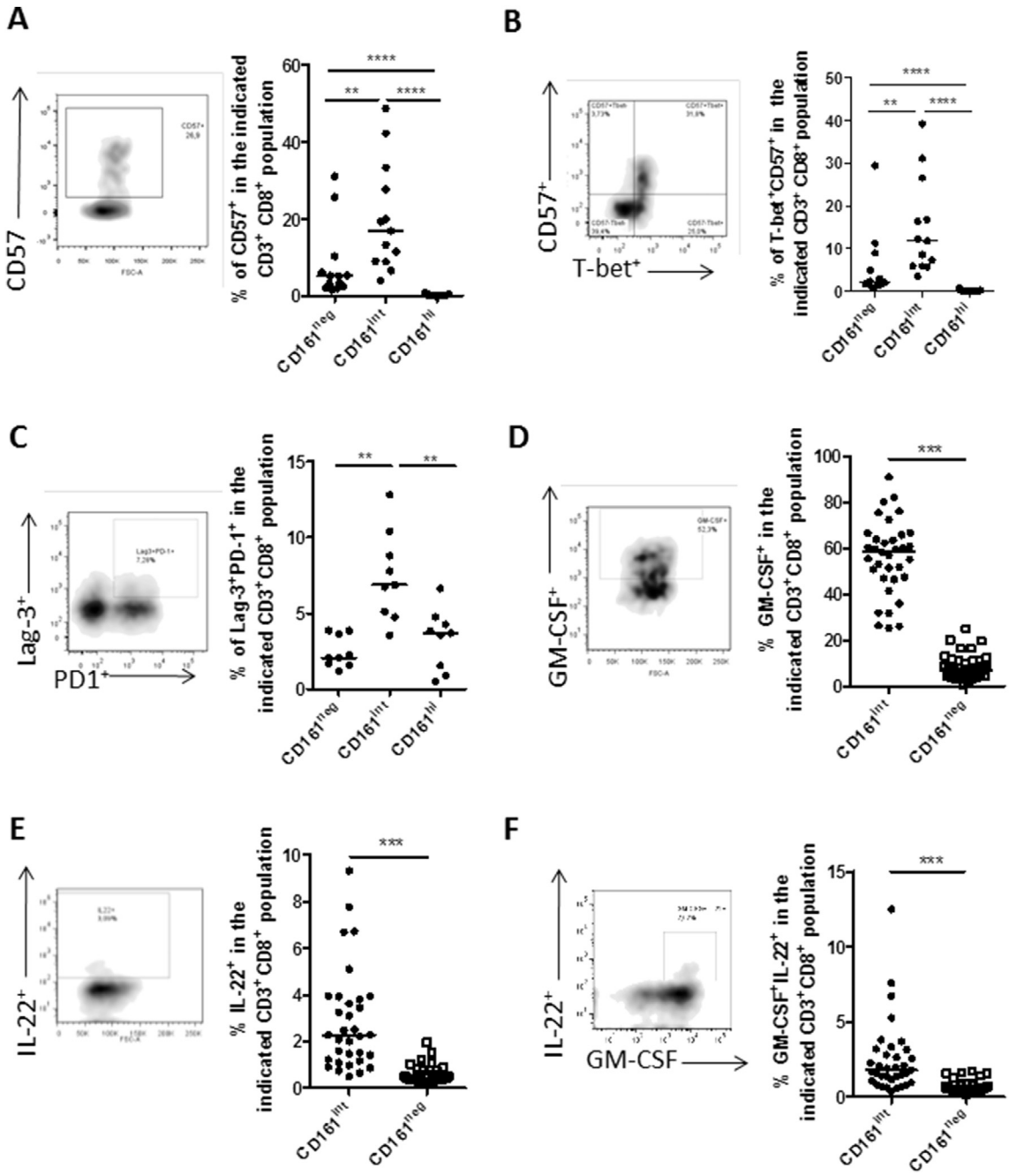

Fig. 2. CD8$^+$CD161^{int}T cells display specific characteristics of effector cells. Frequency of CD3$^+$CD8$^+$T cells expressing CD57 (A), CD57/T-bet (B), Lag-3/PD-1 (C) GM-CSF (D), IL-22 (E) and IL-22/GM-CSF (F) in CD161^{neg}, CD161^{int}, or CD161^{hi} cells in the blood of HV (n = 13, 13, 9, 35, 35 and 35 respectively). Wilcoxon matched-pairs signed rank test, *p < 0.05, **p < 0.01, ***p < 0.001.

phenotypic level, we used flow cytometry to study the expression of several activation markers, and the ability of the cells to secrete different pro-inflammatory cytokines, by comparison of multiple sclerosis patients and HV. First, we noted an increased expression of DNAM-1 (57.4% [11.5%–78.5%] vs. 40.6% [10.8%–60.8%], respectively, p < 0.01) (Fig. 5A), which is a molecule associated with the effector ability of cells, and a decreased expression of PD-1 (51.37% [24.4%–73.3%] vs. 66.36% [45.2%–82.8%], respectively, p < 0.05) in CD8$^+$CD161^{int} T cells from multiple sclerosis patients compared to HV (Fig. 5B). We then explored the ability of the CD8$^+$CD161^{int} T cells to secrete various pro-inflammatory cytokines under several conditions of stimulation: a non-specific stimulation with PMA-ionomycin, a non-TCR-dependent stimulation with IL-12 and IL-18 (as these cells express the IL-18Rα molecule), and a polyclonal stimulation with anti-CD3/CD28. Interestingly, IL-17 was secreted significantly more with the anti-CD3/CD28 stimulation than with the other treatments (Fig. 5C), while GM-CSF, IFNγ, or IL-22 were secreted under all of stimulatory conditions (Figure S5A, B, and C). Despite the fact that the transcriptomic data suggested an increased activation status for blood CD8$^+$CD161^{int} cells in multiple sclerosis patients as compared to HV, this did not impact their secretory capacities in our assay.

As we found that CD8$^+$CD161^{int} T cells from multiple sclerosis patients were recruited to the CSF and the CNS lesions, we also

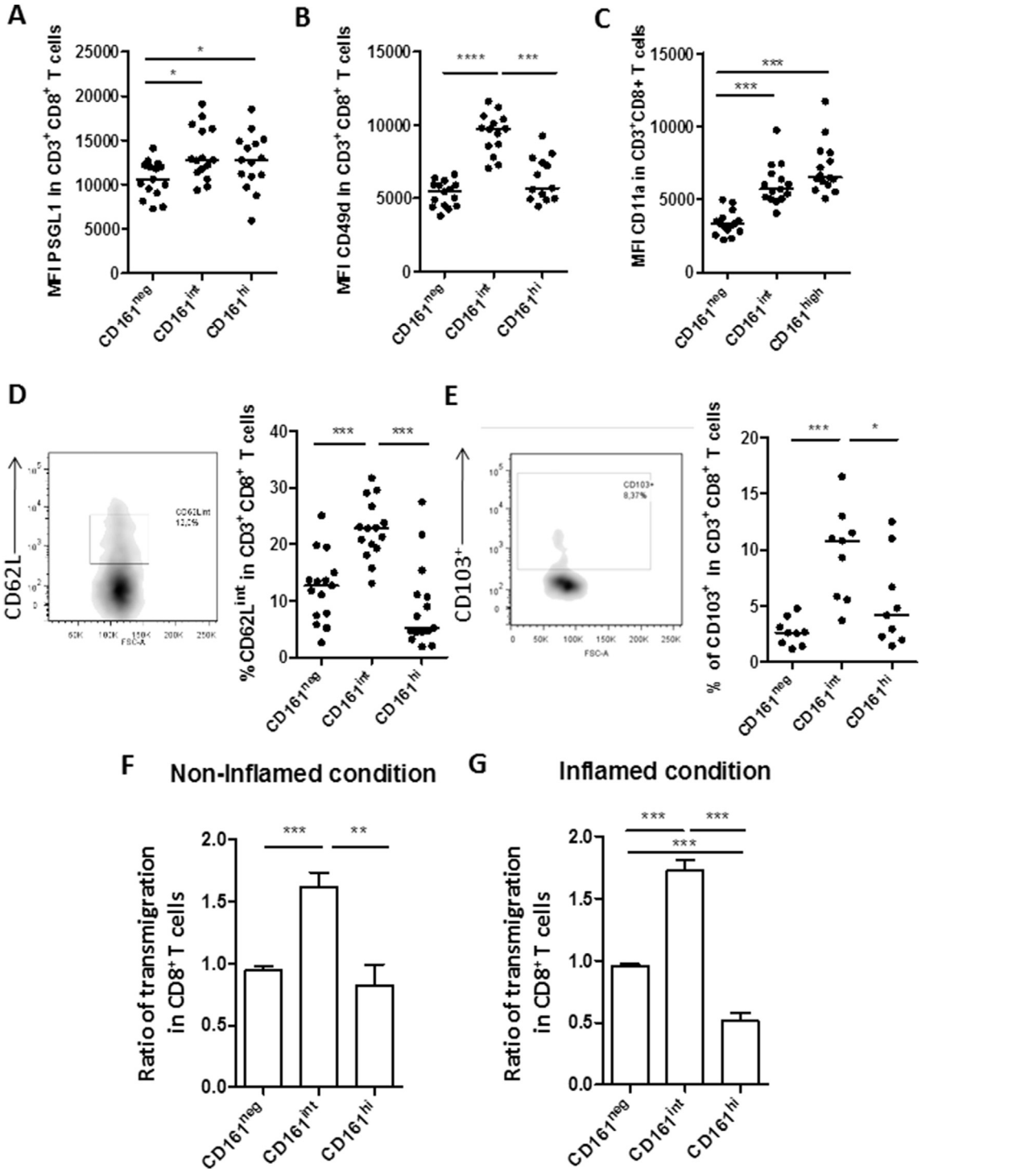

Fig. 3. CD8$^+$CD161^{int} T cells express homing markers with transmigratory abilities. (A) Frequency of CD3$^+$CD8$^+$ expressing CD62L^{int} and MFI of PSGL-1, CD49d, and CD11a in CD3$^+$CD8$^+$ CD161^{neg}, CD161^{int}, or CD161^{hi} cells in the blood of HV (n = 17, 15, 15, and 15, respectively). (B) Ratio of the frequency of CD3$^+$CD8$^+$CD161^{neg}, CD161^{int}, or CD161^{hi} in the transmigrated fractions as compared to the total non-transmigrated fraction under non-inflamed or inflamed conditions (n = 8 for the non-inflamed and n = 12 for the inflamed condition). (C) Frequency of CD3$^+$CD8$^+$ expressing GM-CSF, IL-22, or double producers in CD161^{int} and CD16^{neg} T cells in HV (n = 35).

studied the expression of several homing markers on these cells. We found an increased expression of PSGL-1 and CD11a in comparison to HV, two markers reported to be involved in CNS migration [2,23](the MFI of PSGL1 was 12,800 [9373–19,100] in HV vs. 16,600 [8214–26,700] in multiple sclerosis patients, and the MFI of CD11a was 5690 [4051–9734] in HV vs. 6923 [5105–15,400] in multiple sclerosis patients, $p < 0.05$ and $p < 0.01$, respectively) (Fig. 5D and E). MCAM has been reported to be a neuropathogenic marker of CD8$^+$ T cells in light of its association with IL-17 secretion in human and to neuro-inflammation in a murine model of multiple sclerosis [4]. After anti-CD3/CD28 polyclonal stimulation, CD8$^+$CD161^{int} T cells from the multiple sclerosis patients upregulated more MCAM as compared to those of the HV (13.5% [3.4%–31.5%] vs. 8.6% [3.6%–23.6%], respectively, $p < 0.05$) (Fig. 5F).

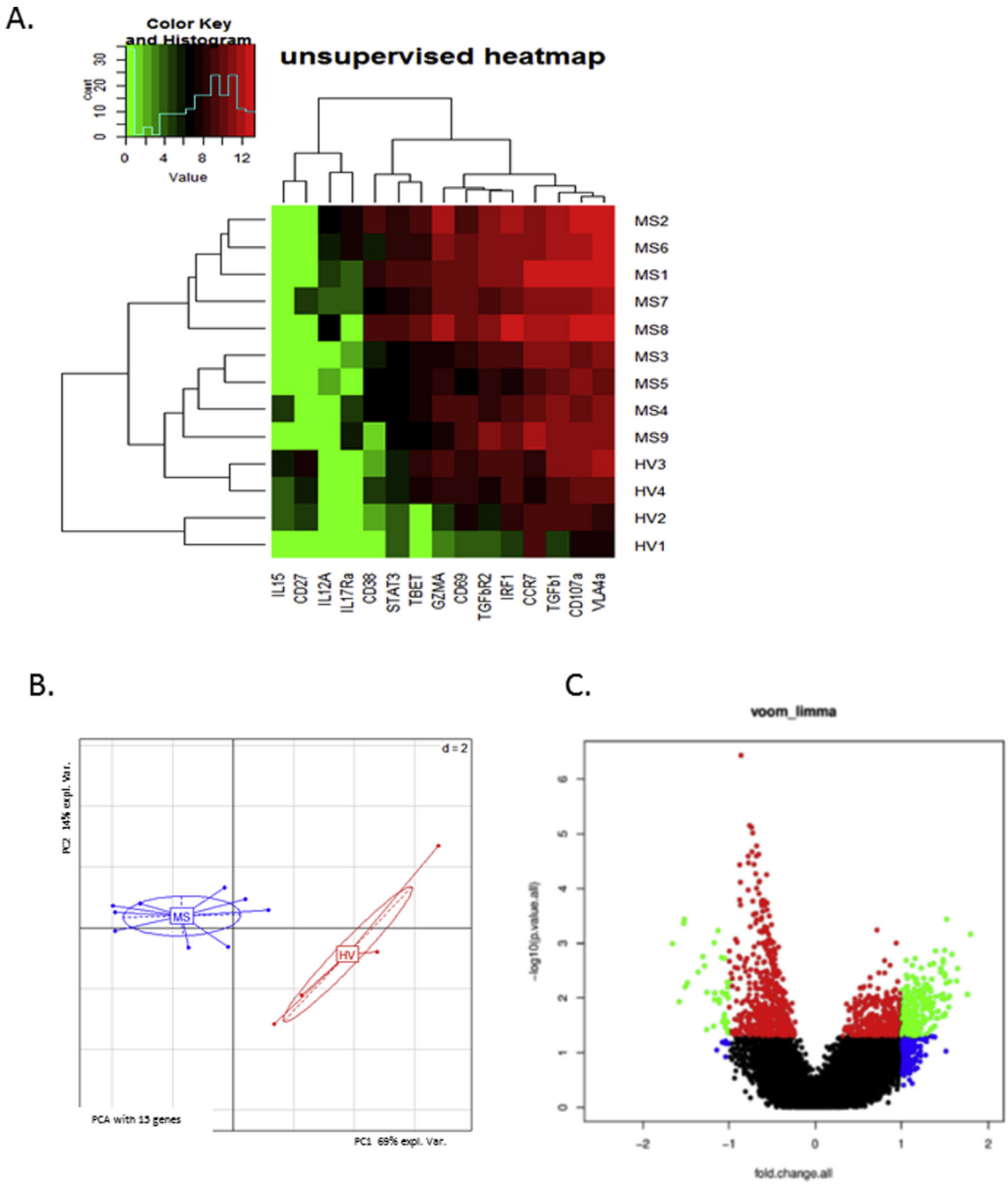

Fig. 4. RT-PCR and RNA sequencing analysis from untreated relapsing-remitting multiple sclerosis **patients and age- and gender-matched healthy volunteers.** (A) Unsupervised heatmap of the 15 genes differentially expressed between multiple sclerosis patients and HV. (B) Principal Component Analysis based on the 15 differentially expressed genes allowed for a clear distinction between multiple sclerosis patients and HV. (C) Volcano plot analysis revealed 170 genes differentially regulated in the $CD8^{+}CD161^{int}$ T cells from multiple sclerosis patients versus HV. The volcano plot represents all of the genes found in the multiple sclerosis patients minus the genes found in the HV. In red, the genes with an unadjusted p value < 0.05. In blue, the genes with an expression > log2 and in green, the genes fulfilling both conditions.

3.5. $CD8^{+}CD61^{int}$ T cells from multiple sclerosis patients secrete IL-17 specifically in CNS lesions

As the $CD8^{+}CD161^{int}$ T cells from multiple sclerosis patients had a phenotype suggestive of increased migration to the CNS, particularly after activation, as shown by increased MCAM expression, these cells were studied *in situ*, in CNS lesions. They were able to produce IL-17, which has been reported to be involved in the disease process. Indeed 11% [0.0%–47.5%] of the $CD8^{+}CD161^{+}$ T cells in the eight studied lesions were positive for IL-17, as shown in a typical example coming from one MS brain lesion (Fig. 6A). These $CD8^{+}CD161^{+}$ T cells are likely to belong to the $CD161^{int}$ subset, as $CD3^{+}CD161^{+}Va7.2^{+}$MAIT cells were absent from these lesions. More importantly, the *in situ* production of IL-17 appears to be specific to the CNS, as only a limited number of $CD8^{+}CD161^{+}$ T cells were labeled for IL-17 in the gut of the multiple sclerosis patients, as shown in a typical example of CD8, CD161 and IL-17 staining from a colon biopsy of one MS patient (Fig. 6B and C). Moreover, the frequency of $CD8^{+}CD161^{int}$ T cells producing IL-17 was less substantial *ex vivo* in the blood than in the CNS, but increased after CD3/CD28 stimulation (Fig. 6A and C, $p < 0.001$). This may suggest an *in situ* reactivation, likely by TCR engagement.

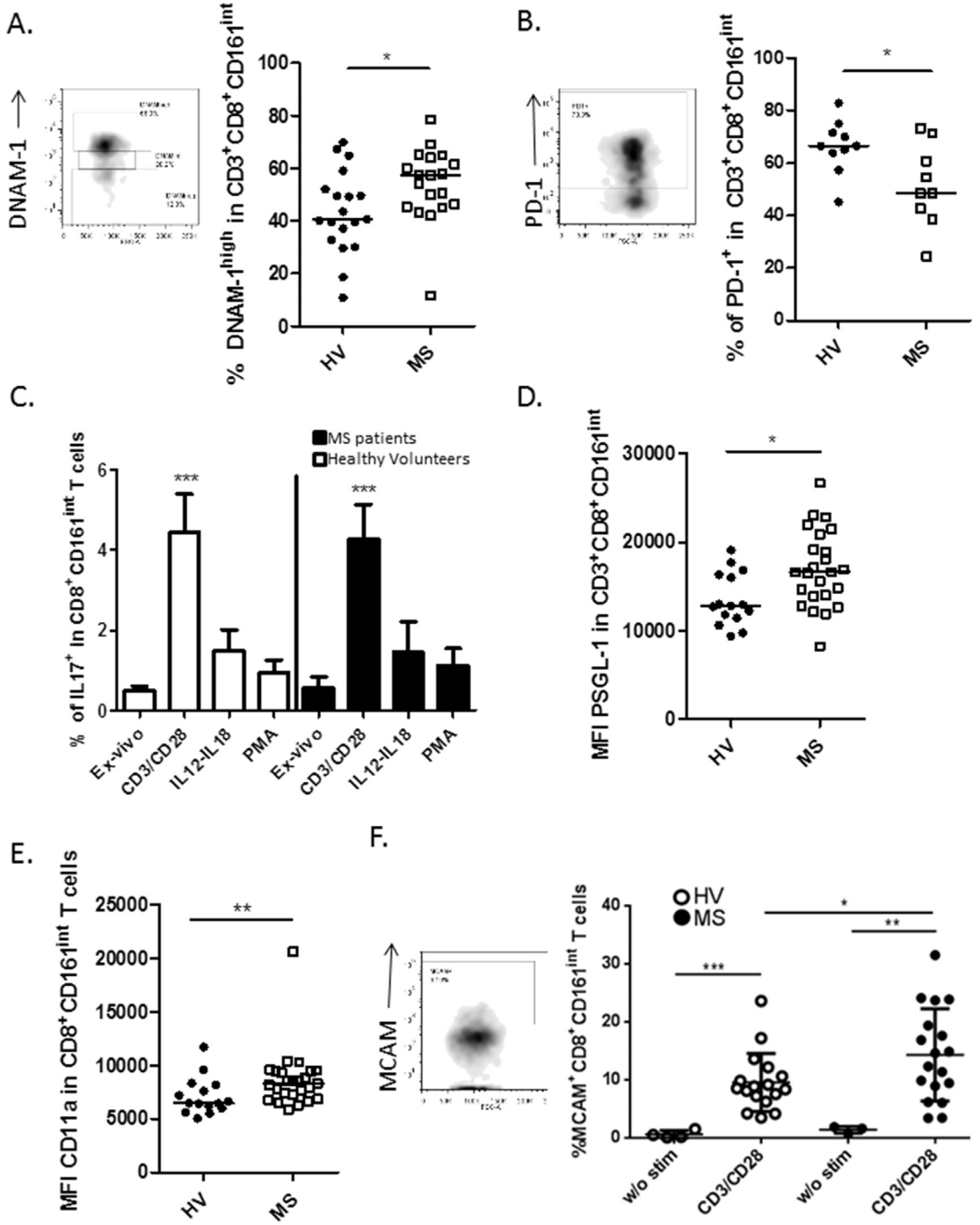

Fig. 5. CD8$^+$CD161^{int}T cells from patients with multiple sclerosis **display an activated phenotype and increased homing abilities compared to HV.** (A) Frequency of CD3$^+$CD8$^+$CD161^{int}T cells expressing high levels of DNAM-1, in the blood of HV (n = 19) and multiple sclerosis patients (n = 19) (B) Frequency of CD3$^+$CD8$^+$CD161^{int}T cells expressing PD-1 in the blood of HV (n = 10) and multiple sclerosis patients (n = 8), (C) Expression of IL-17 in the CD8$^+$CD161^{int} T cells from multiple sclerosis patients (black) and HV (white) after stimulation over four days with CD3/CD28 (MS, n = 18; HV, n = 17), four days with IL-12/IL-18 (MS, n = 15; HV, n = 14), 5 h with PMA and ionomycin (MS, n = 15; HV, n = 14) and without any stimulation (MS, n = 18; HV, n = 19). P < 0.001 for IL-17 secretion after CD3/CD28 stimulation compared to all other kinds of stimulation, (D) MFI of PSGL1 in the blood of HV (n = 15) and multiple sclerosis patients (n = 24), (E) MFI of CD11a in the blood of HV (n = 15) and multiple sclerosispatients (n = 25) and (F) Percentage of MCAM$^+$ cells among CD8$^+$CD161int T cells in the blood of HV and multiple sclerosis patients before (n = 4 and n = 3, respectively) and after stimulation with CD3/CD28 over four days (n = 17 and n = 18, respectively).

3.6. High-throughput sequencing of TCR repertoire analysis of circulating CD8$^+$CD161^{int} T cells reveals a more substantial skewing of TCR usage in multiple sclerosis patients

One challenging question that remained was whether these cells could recognize local cognate antigens in the multiple sclerosis lesions. While this question could not be directly addressed as the antigens recognized by the infiltrating CD8$^+$ T cells are not currently known in multiple sclerosis, an indirect assessment was carried out by studying the TCR repertoire. First, in order to explore a potential TCR skewing in the CD8$^+$CD161^{int} T cells from multiple sclerosis patients, we performed high-throughput

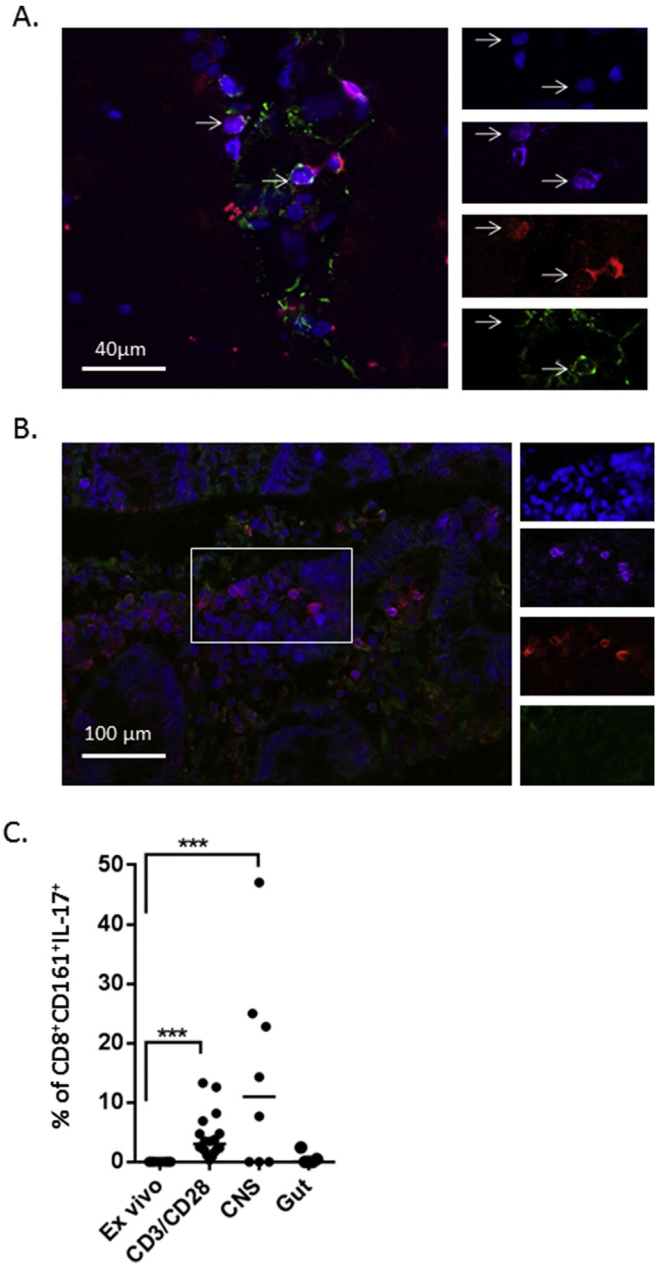

Fig. 6. CD8$^+$CD161^{int}T cells from patients with multiple sclerosis **secrete IL-17 specifically in the CNS lesions.** (A) Example of staining of CD8$^+$CD161^{int} T cells secreting IL-17 in a brain multiple sclerosis lesion. Blue: DAPI, purple: CD8, red: CD161, and green: IL-17. (B) Example of the same staining in a colon biopsy from a multiple sclerosis patient. (C) Frequency of IL-17 secreting CD8$^+$CD161^{int} T cells in different tissues in patients with multiple sclerosis. Blood (without stimulation, n = 18), blood after four days of stimulation with antiCD3/CD28 (n = 18), brain multiple sclerosis lesions (n = 7), and gut biopsies from the multiple sclerosis patients (n = 5).

sequencing of their TCR repertoire. We first compared the TCR repertoire of the Vβ chain observed in this subset of cells to the whole subset of CD45RA^{neg} memory CD8$^+$ T cells (after exclusion of MAIT cells). We were able to compare these two sets of experiments as the number of TCRB in-frame sequence reads was similar. We found an increased number of unique TRBV sequences in the CD8$^+$CD161^{int} subset ($p < 0.05$), thus indicating an increased number of unique clones in this subset (Fig. 7A) compared to the entire subset of memory clones. By examination of the repartition of these supplementary clones in the several frequencies, we observed a random distribution (Fig. 7B).

When comparing multiple sclerosis patients and HV for CD8$^+$CD161^{int} T cell TRBV repertoires, we also found a trend toward an increased diversity (corresponding to the number of unique clones per 100 cells) of CD8$^+$CD161^{int} clones in the multiple sclerosis patients (Fig. 7C, $p = 0.08$). Interestingly, these supplementary clones observed in the multiple sclerosis patients were not randomly distributed; rather, they were significantly increased in the frequency range above 0.01% (Fig. 7D).

Taken together these data suggest the presence of supernumerary clones in the CD8$^+$CD161^{int} T cell subset in multiple sclerosis patients compared to HV that may likely recognize cognate antigens associated to the pathology.

4. Discussion

This work represents the first investigation of the potential involvement of CD161^{int}CD8$^+$ T cells in multiple sclerosis. We first confirmed and extended the finding that these cells had specific homing and pro-inflammatory properties [18], with high expression of GZMB and perforin as well as CD49d, and to a lesser extent PSGL-1 and CD11a, thus making them suitable to invade and exert deleterious effects in the inflamed CNS. Furthermore, the overexpression of CD103 may retain the cells in the brain microenvironment, as has been previously demonstrated for brain tumors [23] or in mice after brain infection [24]. Indeed, we showed that these cells were more capable at transmigration through a BBB model than other CD8$^+$ T cells. In parallel, we showed a specific decreased frequency of CD8$^+$CD161^{int} T cells in the peripheral blood of the multiple sclerosis patients, but not in healthy or inflammatory controls, which is in line with an enrichment of these cells in the CSF and the CNS. This was confirmed by the fact that these cells normally accumulate in blood with age, which was not the case in multiple sclerosis. These cells overexpressed CCR5 in the CSF, which is also in line with our previous study showing an increased expression of this marker on clonally-expanded CD8$^+$ T cells from multiple sclerosis patients [2]. CCR5 has recently been highlighted as a key chemokine receptor in the trafficking of antigen-experienced T cells to the CNS [25], reinforcing our findings. All these characteristics indicate a subset of T cells with neuropathogenic properties. We then demonstrated that CD8$^+$CD161^{int} T cells from multiple sclerosis patients displayed peculiar properties compared with those from HV. We showed a phenotypic alteration at the RNA and protein levels in line with a proinflammatory and migratory profile. *In situ*, these cells are present in lesions at the same level as the CSF, and they were able to exert a proinflammatory role by secreting IL-17, in a similar way as in the blood after CD3/CD28 polyclonal stimulation, thus suggesting a local TCR engagement in CNS lesions. This is of particular interest as no IL-17 was secreted by these cells in non-specific conditions of stimulation either by IL-12/IL-18 or PMA-ionomycin, as already reported [8,9]. Moreover, whereas this phenotype was found in the CNS, no or few IL-17 secretion by these cells in the gut or in the blood of multiple sclerosis patients was observed, highlighting its specific feature in the inflammatory process of the CNS lesions. Interestingly CD8$^+$CD161^{int} T cells were also able to produce IL-22, which may directly act on astrocytes in multiple sclerosis lesions. Indeed, astrocytes have been recognized as being important players in the pathogenesis of multiple sclerosis [26], and they express both IL-22 receptor subunits, leading to IL-22-dependant pro-survival properties on primary human astrocyte cultures [27]. We also found that CD8$^+$CD161^{int} T cells are able to secrete GM-CSF, which is a key cytokine involved in the pathogenesis of multiple sclerosis by recruitment of myeloid cells and by promotion of tissue damage [28].

The high-throughput TRBV repertoire study revealed a more

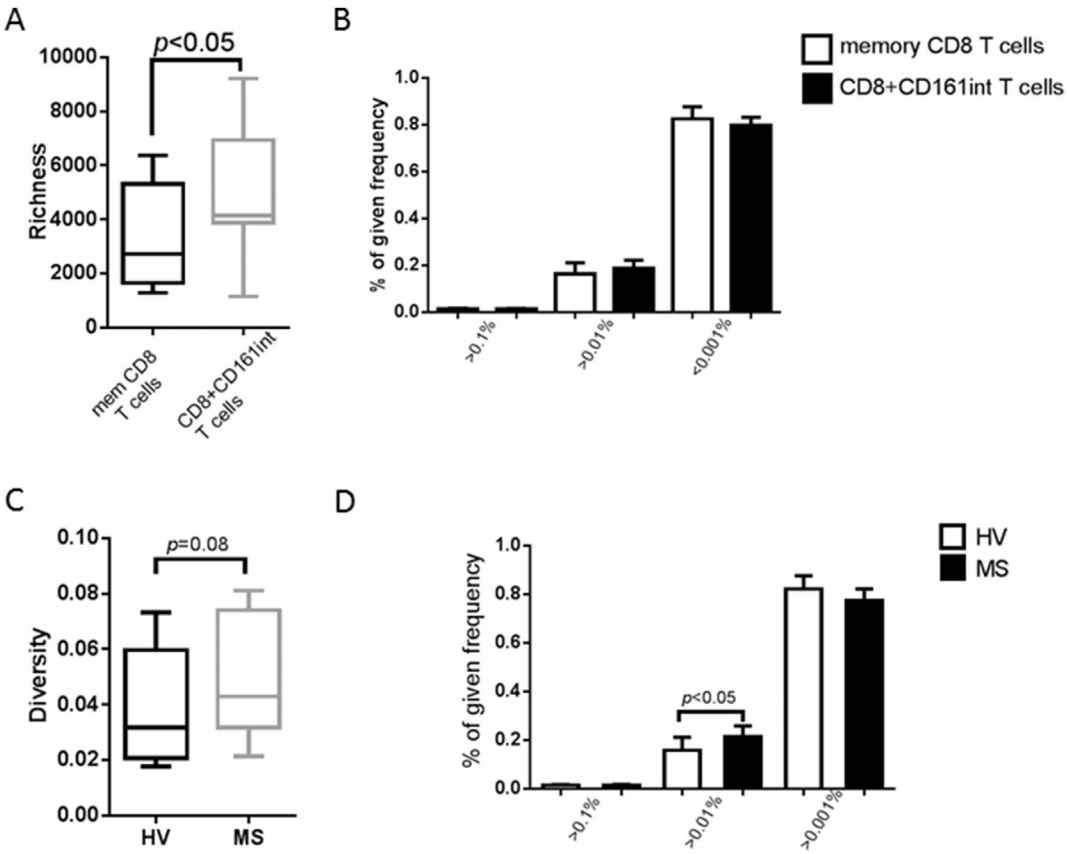

Fig. 7. High-throughput sequencing of the TCR Vβ repertoire from CD8$^+$CD161^{int} T cells and non-MAIT memory CD8$^+$ T cells from multiple sclerosis **patients and HV.** (A) Number of unique TCRBV sequences from non-MAIT memory CD8$^+$ T cells (black square, n = 10) and CD8$^+$CD161^{int} T cells (grey square, n = 10). (B) Percentage of a given frequency of clones >0.1%, >0.01%, and <0.01% in non-MAIT memory CD8$^+$ T cells (white, n = 10) and CD8$^+$CD161^{int} T cells (black, n = 10). (C) Diversity of TCRBV sequences in CD8$^+$CD161^{int} T cells (corresponding to the number of unique clones per 100 cells) from multiple sclerosis patients (grey, n = 10) and HV (black, n = 10). (D) Percentage of a given frequency in clones > 0.1%, >0.01%, and <0.01% in CD8$^+$CD161int T cells from multiple sclerosis patients (black, n = 10) and HV (white, n = 10).

diverse repertoire in CD8$^+$CD161^{int} T cells from the multiple sclerosis patients compared to the healthy controls. This suggests that peripheral supernumerary clones could be directed against CNS antigens. To date, the challenge has been determination of the actual antigen(s) recognized by multiple sclerosis lesion-infiltrating CD8$^+$ T cells. A previous study has reported that CD8$^+$CD161^{int} T cells display a diverse TCR repertoire, making them polyreactive under physiological conditions where they recognize various epitopes from viruses such as CMV, EBV, and Influenza [18]. These cells have also been reported to be enriched in the gut, with the expression of resident memory cell markers CD69 and CD103 [18]. This specific tropism may suggest a potential role in the modulation of the intestinal microbiome under pathological conditions such as multiple sclerosis and as has been demonstrated in an animal model [29], and more recently in the human disease itself [30].

In order to compare the phenotype of these cells in multiple sclerosis patients and in HV we also performed RNAseq experiments. These results point toward an overactivation of these cells, thus supporting the notion that they are involved in the disease process. Moreover, looking at the dysregulation of chemokine/chemokine-receptors, fractalkine-receptor CX3CR1 was found to be upregulated in the CD8$^+$CD161^{int} T cells from multiple sclerosis patients. Recent data have shown involvement of fractalkine as a key mediator for the presence of CD4$^+$ T cells in the CSF and CNS of patients with multiple sclerosis [31]. Fractalkine, which accumulates in the lesions and in the CSF of multiple sclerosis patients, is able to attract CX3CR1-expressing CD4$^+$ T cells *in vitro* [32]. Interestingly, we were also able to confirm that these CD8$^+$ T cells express several homing markers, such as CCR2, CCR5, CCR6, and CXCR6 which have been described in multiple sclerosis and other autoimmune diseases [33–36]. At the protein level, we were also able to show that CD8$^+$CD161^{int} T cells from multiple sclerosis patients overexpressed MCAM, which is a recently described adhesion molecule associated with direct pathogenic effector cells in multiple sclerosis; as MCAM$^+$ T cells secrete IL-17, have increased transmigratory abilities, and an increased capacity to kill target cells [4,37]. We also found overexpression of DNAM-1 on CD8$^+$CD161^{int} T cells from multiple sclerosis patients compared to HV. The DNAM-1 molecule is an activating receptor of CD8$^+$ T cells, and its blockade impairs costimulation, cytokine productions, and cytotoxicity [38]. CD8$^+$DNAM-1^{high} cells have also recently been shown to be involved in the pathophysiology of another autoimmune disease, namely systemic sclerosis, with an increased frequency of these cells in the blood of the patients [39].

5. Conclusions

In all, our findings demonstrate that CD8$^+$ T cells with an intermediate level of CD161 expression have effector functions and notably the ability to secrete pro-inflammatory cytokines such as IL-17, IL-22, IFNγ or GM-CSF. These cells are dysregulated at the transcriptomic and protein levels. They overexpress molecules involved in transmigration in multiple sclerosis patients, accounting for their enrichment in multiple sclerosis lesions. Moreover, these cells produce IL-17 *in situ*, thus supporting the notion that they are involved in the pathogenesis of the disease. Our study

highlights that CD161 is not only an interesting peripheral biomarker of T cell pathogenicity in multiple sclerosis but that it is also as a suitable target for therapeutic purposes in future studies.

Conflict of interest

The authors declare no commercial or financial conflict of interest in the context of this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2017.10.005.

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