doi:10.1093/brain/awq354 Brain 2011: 134; 542–554 | 542

BRAIN
A JOURNAL OF NEUROLOGY

$CD161^{high}CD8^+$ T cells bear pathogenetic potential in multiple sclerosis

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To identify differentially expressed genes in multiple sclerosis, microarrays were used in a stringent experimental setting—leukapheresis from disease-discordant monozygotic twins and gene expression profiling in $CD4^+$ and $CD8^+$ T-cell subsets. Disease-related differences emerged only in the $CD8^+$ T-cell subset. The five differentially expressed genes identified included *killer cell lectin-like receptor subfamily B, member 1*, also known as *natural killer receptor protein 1a/CD161*, presented by the International Multiple Sclerosis Genetics Consortium as one of the non-MHC candidate loci. Flow cytometric analysis on peripheral blood of healthy donors and patients with multiple sclerosis and rheumatoid arthritis confirmed an upregulation of CD161 at the protein level, showing also a significant excess of $CD161^{high}CD8^+$ T cells in multiple sclerosis. This subset prevalently included chemokine (C-C motif) receptor 6^+, cytokine-producing, effector-memory T cells with proinflammatory profiles. It also included all circulating interleukin-17^+CD8^+ T cells. In the $CD161^{high}CD8^+$ subset, interleukin-12 facilitated proliferation and interferon-γ production, with CD161 acting as a co-stimulatory receptor. $CD161^+CD8^+CD3^+$ T cells producing interferon-γ were part of intralesional immune infiltrates and ectopic B cell follicles in autopsy multiple sclerosis brains. Variations of CD161 expression on $CD8^+$ T cells identify a subset of lymphocytes with proinflammatory characteristics that have not been previously reported in multiple sclerosis and are likely to contribute to disease immunopathology.

Keywords: multiple sclerosis; gene expression; CD161; IL-17; CCR6

Abbreviations: CCR6 = chemokine (C-C motif) receptor 6; PBMC = peripheral blood mononuclear cell

Introduction

Multiple sclerosis is a CNS disease driven by inflammatory and neurodegenerative components (Compston and Coles, 2008). Its cause is unknown and the underlying pathogenic mechanisms are still poorly understood. As in other complex traits, technologies that allow non-hypothesis-driven investigations through high-throughput screening may increase the chances of detecting alterations with aetiopathogenetic relevance (Martin and Leppert, 2004). However, due to limitations in their reproducibility,

Received February 5, 2010. Revised August 19, 2010. Accepted October 25, 2010. Advance Access publication January 7, 2011
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complexities in data analysis and costs, these studies are—in some instances—descriptive and do not fully exploit their potential of being unbiased tools to pick up new clues.

In an attempt to optimize the reproducibility of data from microarrays, we reduced the number of variables by performing gene expression profiling in peripheral blood cells of disease-discordant monozygotic twins. The co-twin control design provides the best possible matching on a multitude of known and unknown potential confounding factors (MacGregor *et al.*, 2000), representing an ideal setting for investigating multifactorial diseases and performing high-throughput screenings (Baranzini *et al.*, 2010). Importantly, in our study the affected twin was free of disease-modifying therapies. To decrease further the number of variables, we avoided the admixture of different peripheral blood mononuclear cell (PBMC) types by separately investigating CD4^{+} and CD8^{+} T-cell subsets. The separate analysis of the two subpopulations allows a direct comparison of their involvement in disease pathogenesis and may disclose new interactions between subsets. In principle, given the increasing number of relevant subpopulations that are being described within the CD4^{+} and CD8^{+} T cells, even more discrete phenotypes should be investigated. Nonetheless, it is impractical to perform high-throughput screening on several cellular subsets in parallel. We therefore chose to put gene expression results into context while confirming microarray results with flow cytometry of a larger series of non-twin patients with multiple sclerosis, rheumatoid arthritis and of healthy controls. To this purpose, multicolour analyses were performed *ex vivo* and in functional *in vitro* assays. Finally, having found circulating CD8^{+} T cells with pathogenetic potential, we verified their presence in multiple sclerosis brain immune infiltrates.

In this study, microarrays in multiple sclerosis-discordant monozygotic twins and cytofluorimetric experiments in patients with multiple sclerosis, rheumatoid arthritis and in healthy controls all confirmed a multiple sclerosis-related overexpression of *killer cell lectin-like receptor subfamily B, member 1*, also known as *natural killer receptor protein 1a/CD161*, on CD8^{+} peripheral blood T cells. This gene was presented by the International Multiple Sclerosis Genetics Consortium as one of the non-MHC candidate loci (Hafler *et al.*, 2007) though it did not reach the suggestive evidence level in a subsequent meta-analysis (De Jager *et al.*, 2009). Virtually all the CD161^{high} were chemokine (C-C motif) receptor 6 (CCR6)$^{+}$, representing a major subset of the total circulating CD8^{+} T lymphocytes with proinflammatory characteristics; they were IFN-γ producers and harboured all of the relatively few IL-17 producing T cells within the CD8^{+} subpopulation. The simultaneous expression of CD161 and CCR6 indicates that these lymphocytes are also well equipped for entry into the CNS. In fact, CD161^{+}CD8^{+}CD3^{+} T cells producing IFN-γ were present in multiple sclerosis brain infiltrates in a percentage approaching 10% of all the infiltrating CD8^{+} T cells. These findings support a contribution of CD161^{+}CD8^{+} T lymphocytes to the pathogenesis of multiple sclerosis.

Materials and methods

Patients and healthy donors

Table 1 summarizes the characteristics of patients with multiple sclerosis. Gene expression analysis was performed on six pairs of monozygotic twins (mean age 38.33 ± 5.59; female : male ratio 4 : 2), who were discordant (four) and concordant (two) for multiple sclerosis. Cumulatively, 43 patients with relapsing–remitting multiple sclerosis (mean age 37.59 ± 9.98; female : male ratio 26:18), 10 patients with rheumatoid arthritis (mean age 57 ± 13.9; female : male ratio 10 : 0) and 47 healthy donors (mean age 47.4 ± 12; female : male ratio 34 : 13) were included for real-time polymerase chain reaction, flow cytometric analysis and enzyme-linked immunosorbent assay. All affected individuals were treatment naive, except for three who had stopped disease-modifying therapies two years before study enrolment. All the investigations were performed at least 3 months after the last steroid therapy. A contrast-enhanced MRI was obtained from the affected twins and the sporadic patients with multiple sclerosis within 24 h of sampling. The local ethics committee approved the protocol and all patients provided informed consent prior to entering the study.

Table 1 Demographic and clinical data of patients with multiple sclerosis

	Non-twin patients		Twin pairs					
	Group 1	Group 2	TW1	TW2	TW3	TW4	TW5[a]	TW6[a]
n	20	23	1	1	1	1	2	2
Female/male	12/7	14/11	F	F	F	M	F	M
Age (years)	40.95 ± 10.20	34.64 ± 8.88	34	38	41	37	46	34
Disease type	RR	RR	RR	RR	RR	SP	RR	RR
Disease duration (years)	0.4–20	0.4–19	1	3	4	10	8 1	6 4
EDSS	1–4	0–3.5	1	1	1	7	2 2	2 1
Disease-modifying therapies	17 Naïve[b]	All naïve	Naïve	Naïve	Naïve	Naïve	Naïve	Naïve

EDSS = Expanded Disability Status Scale; RR = relapse–remitting; SP = secondary progressive.
a Disease-concordant monozygotic twins.
b Three patients had interrupted disease-modifying therapies 2 years before study enrolment.

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Isolation of peripheral blood mononuclear cells and T-cell subsets

For gene expression analysis, PBMC were obtained by leukapheresis and were separated by density centrifugation over Ficoll–Hypaque according to standard procedures. Briefly, heparinized blood was diluted with one volume of Roswell Park Memorial Institute (RPMI) medium and gently layered over the Ficoll. Following centrifugation at 660 *g* for 30 min, cells at the interface of the gradient were collected and washed three times. Highly purified $CD4^+$ and $CD8^+$ (purity > 95%) cell subsets were then sorted by flow cytometry on MoFlo high-speed cell sorter (Beckman Coulter, Fullerton, CA, USA) and frozen in TRIzol® (Invitrogen, Carlsbad, CA, USA). For cytofluorimetric analysis and enzyme-linked immunosorbent assay, PBMC were isolated by density gradient, as described above, after venous puncture.

Microarray analysis

Total RNA was extracted using TRIzol® (Invitrogen), according to the manufacturer's instructions. RNA quality and purity was assessed with the use of the RNA 6000 Nano assay on Agilent 2100 Bioanalyser (Agilent). Copy RNA from each sample was prepared using GeneChip® One-Cycle Target Labelling and Control Reagents Kit (Affymetrix), according to the manufacturer's protocols. Briefly, 5 μg total RNA was first reverse transcribed using a T7-Oligo (dT) promoter primer in the first-strand complementary DNA synthesis reaction. Following RNase H-mediated second-strand complementary DNA synthesis, the double-stranded complementary DNA was purified and served as a template in the subsequent *in vitro* transcription reaction. This reaction is carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analogue/ribonucleotide mix for complementary RNA amplification and biotin labelling. The biotinylated copy RNA targets were then fragmented and hybridized to GeneChip® Human Genome U133 Plus 2.0 arrays (Affymetrix). After washing and staining, the images were acquired and analysed with Affymetrix's software. We compared the gene expression profiling between affected and healthy co-twins by the Rosetta Resolver® system (Rosetta Biosoftware). We selected the differentially expressed genes with the same trend in at least three twin pairs, as candidates to be validated by real-time polymerase chain reaction.

Real-time polymerase chain reaction

Total RNA (1 μg) was treated with DNase I (Invitrogen), according to the manufacturer's protocol, to remove genomic DNA contamination. Each sample was reverse-transcribed using SuperScript II RT (Invitrogen) according to the manufacturer's protocol. Multiplex real-time polymerase chain reaction was performed by LUX primer-detection (Invitrogen) using FAM-labelled primers for *IL-1B* (gaaaccggagcgaatgacagaggt[fam]tc and tctcctttcagggccaatcc), *IL-8* (cactgtgaaaggagaaaccaaggcacag[fam]g and tcaggaattgaatgggtttgct), *chemokine (C-C motif) ligand 3* (cactggtactttgagacgagcagccag[fam]g and catatttctggacccactcctca), *chemokine (C-C motif) ligand 4* (gacacattcctgagtatggaggagatgtg[fam]c and tgtgctgatcccagtgaatcct), *CD161* [gaacctttgctttgtcacgtatcagg(fam)tc and tggcagcaactccgagagaa] and JOE-labelled primers for the reference gene [Certified LUX. Primer Sets for *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenase*), Invitrogen]. Unlabelled and fluorophore-labelled primers were designed using the LUX designer software (Invitrogen, www.invitrogen.com/lux).

For each gene, a multiplex polymerase chain reaction was performed using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) in the presence of a reference gene. Cycling conditions were: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 60°C (or 61°C for *CD161)* and 30 s at 72°C. Each gene transcript level was normalized to *GAPDH* levels, using the two standard curves method. The fold-change was calculated comparing gene expression level between patients with multiple sclerosis and healthy donors. Statistical analysis was performed using Graph Pad Prism 5 for the non-twin patients and healthy donors. Statistical significance is expressed by *P*-values and was generated using a standard two-tailed unpaired *t*-test.

Flow cytometry

For polychromatic flow cytometry, aliquots of 0.5×10^6 fresh PBMCs were stained with monoclonal antibodies conjugated with the appropriate fluorochrome at previously defined optimal concentrations. The following antibodies were used: αβ TCR, CD4, CD8, CD45RA, CD161 and CD56 (Beckman Coulter); CD3, CCR4 and IFN-γ (BD Biosciences, San Jose, CA, USA); CD27, IL-13, IL-17, IL-18R, IL21 and IL-22 (eBioscience, San Diego, CA, USA); CCR7 (R&D Systems, MN, USA); Perforin and CCR6 (Pharmingen, San Diego, CA, USA); CX(3)CR1 (MBL, Woburn, MA, USA); IL-2 (BioLegend, San Diego, CA, USA). For intracellular staining, freshly isolated PBMCs were stimulated with plate bound anti-CD3 purified monoclonal antibody (5 μg/ml; Pharmingen, San Diego, CA, USA) alone or together with 10 or 20 μg/ml of purified antiCD161 (clone DX12, BD Biosciences) or Ig isotype control, in the presence of IL-2 (20 U/ml, Roche Diagnostic) and brefeldin A (10 μM; Sigma-Aldrich, St Louis, MO, USA). After 6 h of stimulation, cells were stained following standard procedures with anti-αβ TCR, anti-CD8 and anti-IFN-γ antibodies. For intracellular cytokine stainings, cells were stimulated with phorbol myristate acetate and ionomycin at 1 μg/ml (Sigma-Aldrich). For proliferation assay, freshly isolated PBMCs were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), (Invitrogen, Eugene, OR, USA) following standard protocols and stimulated with or without 20 ng/ml IL-12 (Pharmingen). After 48 h IL-2 was added (20 U/ml) to all samples and on Day 5 the cells were labelled and analysed by flow cytometry on a CyAn (Beckman Coulter) or on a fluorescence activated cell sorting Canto (Becton Dickinson). Data were compensated and analysed using FlowJo software (TreeStar, Ashland, OR, USA). Statistical analysis was performed using Graph Pad Prism 5. Statistical significance is expressed by *P*-values and was generated using a standard two-tailed unpaired *t*-test.

Enzyme-linked immunosorbent assay

$CD8^+$ T lymphocytes were sorted from freshly isolated PBMCs based on CD161 expression, using a MoFlo (Beckman Coulter). Sorted $CD161^{high}CD8^+$, $CD161^{low}CD8^+$ and $CD161^-CD8^+$ cell subsets were stimulated for 24 h at 37°C with antiCD3 and antiCD28-coated Dynabeads® (Invitrogen), with or without 20 ng/ml IL-12 (Pharmingen). Supernatants were harvested and the presence of IFN-γ was determined by a standard two-site sandwich enzyme-linked immunosorbent assay. Antibodies for IFN-γ were purchased from Endogen. Enhanced protein-binding enzyme-linked immunosorbent assay plates (Nunc Maxisorb; Nunc Maxi Corp., Roskilde, Denmark) were used.

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Immunofluorescence analysis of post-mortem multiple sclerosis brain tissue

Snap-frozen brain tissue blocks from four patients with secondary progressive multiple sclerosis and one control subject who died from cardiac failure were analysed. Tissues were provided by the UK Multiple Sclerosis Tissue Bank at Imperial College, London. A snap-frozen tonsil was used as positive control. Multiple sclerosis lesions were analysed and classified by histopathological methods, i.e. haematoxylin/eosin staining and Oil Red O, and the combined Luxol fast blue-periodic acid-Schiff reaction to estimate myelin breakdown and phagocytosis. For the multiple sclerosis cases, four cerebral tissue blocks containing areas of white matter demyelination (classified as chronic active lesions) and intact meninges were examined.

For triple immunofluorescence stainings, cryosections from tonsil and multiple sclerosis brain tissues were fixed in acetone for 10 min and stained using the following purified antibodies: anti-CD161 (natural killer receptor protein 1a) mAb (clone DX12, BD Biosciences), anti-CD8 rabbit polyclonal Ab (Affinity Bioreagent), and anti-CD3 (Santa Cruz Biotechnology) or anti-human IFN-γ (AbD Serotec, Oxford, UK) goat polyclonal antibodies diluted in phosphate buffered saline containing 2% bovine serum albumin. After an overnight incubation, sections were washed and treated with TRITC-conjugated donkey anti-rabbit IgG, fluorescein-conjugated donkey anti-mouse IgG and AMCA-conjugated donkey anti-goat IgG (Jackson Immunoresearch Laboratories, Cambridgeshire, UK) diluted in phosphate buffered saline containing 5% normal donkey serum (Jackson Lab.) for 1 h, washed again in phosphate buffered saline and sealed in the aqueous mounting medium Vectashield® (Vector Laboratories, Burlingame, CA, USA). For negative controls, the primary antibodies were replaced with pre-immune sera and IgG isotype control. Slides were viewed and analysed under epifluorescence with a Zeiss Axiophot microscope equipped with an Axiocam digital camera; images were acquired using the Axiovision 4 AC software.

Results

Gene expression analysis: microarray and real-time polymerase chain reaction

Microarray analysis was performed on four pairs of monozygotic twins discordant for multiple sclerosis. The gene expression profiles of peripheral blood CD4$^+$ and CD8$^+$ T cells were similar between each pair of twins and no transcript proved to be disease associated in all four twin pairs. We therefore chose to validate any trend that could be confirmed in at least three twin pairs. In spite of this relatively loose criterion, no gene was found to be differentially expressed between affected and healthy co-twins in CD4$^+$ T cells, whereas in CD8$^+$ T lymphocytes, *interleukin 1 beta (IL-1B)*, *interleukin 8 (IL-8)*, *chemokine (C-C motif) ligand 3* and *chemokine (C-C motif) ligand 4* genes were downregulated, and *CD161* upregulated, in the affected co-twins. All these differences were validated by real-time polymerase chain reaction (Tables 2 and 3). As evident from real-time polymerase chain reactions repeated on samples obtained from three twin pairs at least 48 months apart from the first time-point, the upregulation of CD161 persisted over time; in two concordant twin pairs, CD161 expression did not differ (Table 3).

Table 2 Real-time polymerase chain reaction results (fold-change) for downregulated genes in CD8$^+$ T cells from affected co-twins

Gene symbol	Gene name	Twin pairs			
		TW1	TW2	TW3	TW4
IL-1B	*Interleukin 1 beta*	2.0	1.1	4.1	2.0
IL-8	*Interleukin 8*	2.1	1.2	1.0	2.2
CCL3	*Chemokine (C-C motif) ligand 3*	1.6	8.0	1.1	1.6
CCL4	*Chemokine (C-C motif) ligand 4*	1.6	1.6	3.6	1.0

Table 3 Real-time polymerase chain reaction results (fold-change) for *CD161* in CD8$^+$ T cells from disease-discordant and -concordant monozygotic twins

Twin pairs	First validation	Second validation[a]
Discordant		
TW1	1.6	3.4
TW2	1.7	1.8
TW3	2.8	1.8
TW4	1.6	
Concordant		
TW5	1.2	
TW6	1.1	

a Performed on samples obtained at least 48 months apart from the first time-point.

Real-time polymerase chain reaction on CD161 transcript was also performed in 20 non-twin patients (mean age 39.45 $\pm$ 8.62; female : male ratio 17 : 3) and 18 healthy donors (mean age 40.59 $\pm$ 7.77; female : male ratio 14 : 4). The results confirmed the upregulation of the transcript in individuals with multiple sclerosis (2-fold change; $P < 0.05$; Supplementary Fig. 1).

CD161 expression on CD8$^+$ T cells in multiple sclerosis

To confirm the upregulation of CD161 expression at the protein level and in a larger series, flow cytometric analysis was performed on CD4$^+$ and CD8$^+$ T cells from the peripheral blood of 20 patients with relapse–remitting multiple sclerosis and 47 healthy donors. Ten patients with rheumatoid arthritis were also included in our analysis to evaluate the specificity of the results obtained from patients with multiple sclerosis against another autoimmune disease. The fraction of CD161$^+$ cells in the total CD8$^+$ T-cell population was higher in patients with multiple sclerosis (mean $\pm$ SD: 23.97 $\pm$ 9.8) compared to healthy donors (mean $\pm$ SD: 17.91 $\pm$ 8.8; $P = 0.03$). No significant difference was found between patients with multiple sclerosis and rheumatoid arthritis (mean $\pm$ SD: 21.58 $\pm$ 7.8), or between patients with rheumatoid arthritis and healthy donors (Fig. 1A). Furthermore, the expression of CD161 (quantified as median fluorescence intensity) on the surface of CD8$^+$ T cells was higher in patients with multiple sclerosis (mean $\pm$ SD: 385.7 $\pm$ 122.4) than in healthy

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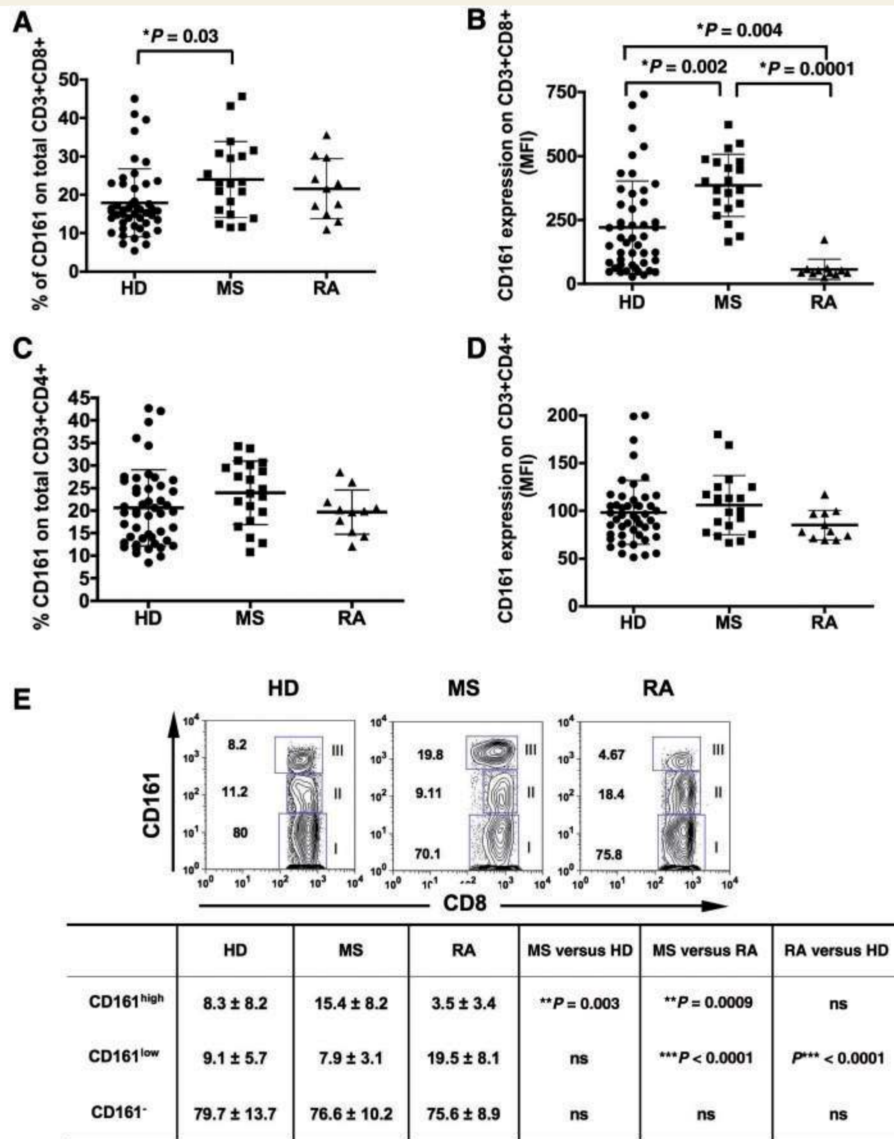

	HD	MS	RA	MS versus HD	MS versus RA	RA versus HD
CD161high	8.3 ± 8.2	15.4 ± 8.2	3.5 ± 3.4	**P = 0.003	**P = 0.0009	ns
CD161low	9.1 ± 5.7	7.9 ± 3.1	19.5 ± 8.1	ns	***P < 0.0001	P*** < 0.0001
CD161-	79.7 ± 13.7	76.6 ± 10.2	75.6 ± 8.9	ns	ns	ns

Figure 1 The percentages of $CD161^+CD8^+$ and expression of CD161 on $CD8^+$ T cells are significantly higher in the peripheral blood of patients with multiple sclerosis compared to controls. (**A** and **C**) Percentage of circulating $CD161^+$ cells within $CD8^+$ T cells and of $CD161^+$ T cells within $CD4^+$ T cells, in patients with multiple sclerosis (MS; n = 20), patients with rheumatoid arthritis (RA; n = 10) and healthy donors (HD; n = 47); (**B** and **D**) expression of CD161, presented as median fluorescence intensity (MFI), on the cell surface of $CD8^+$ T cells and $CD4^+$ T cells in patients with multiple sclerosis, patients with rheumatoid arthritis and healthy donors. (**E**) Flow data from three representative donors. Discrete populations of $CD8^+$ T cells are enclosed in gates I, II or III, corresponding to $CD161^-$, $CD161^{low}$ and $CD161^{high}$ subsets, respectively. The table shows the mean (±SD) percentages of $CD8^+$ T cells belonging to each subset. ns = not significant.

donors (mean ± SD: 221.8 ± 180.9; $P = 0.002$) and in patients with rheumatoid arthritis (mean ± SD: 57.14 ± 39.6; $P = 0.0001$) (Fig. 1B), as well as in healthy donors compared with patients with rheumatoid arthritis ($P = 0.004$). In agreement with the microarray results, the percentage of $CD161^+CD4^+$ T cells and the expression of CD161 on $CD4^+$ T cells were similar in the three groups (Fig. 1C and D). Based on levels of CD161 expression, $CD8^+$ cells can be divided into $CD161^{high}$ and $CD161^{low}$. Since it is known that CD161 expression levels define cell subsets with distinct functional properties (Takahashi *et al.*, 2006) we asked whether the increased staining intensity for CD161 was due to the selective expansion of the $CD161^{high}$ subset. Indeed, as shown in Fig. 1E, patients displayed a significantly higher percentage of $CD161^{high}CD8^+$ T cells, compared to healthy individuals and to patients with rheumatoid arthritis ($P = 0.003$ and 0.0009, respectively); $CD161^{low}$ $CD8^+$ T cells were higher in patients with rheumatoid arthritis than in healthy donors and patients with multiple sclerosis while no difference was observed between the three groups for $CD161^-CD8^+$ T cells.

In order to investigate possible correlations between the circulating $CD161^+CD8^+$ subset and demographic or clinical variables, we enrolled a second group of 23 patients with multiple

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sclerosis (Table 1). In this cumulative population of 43 patients with relapse-remitting multiple sclerosis, we found that the percentage of CD161^{high}CD8$^+$ T cells correlated with disease duration (Fig. 2). When we re-analysed the frequency of CD161$^+$CD8$^+$ T cells in this larger sample of patients with multiple sclerosis we were able to confirm a significant difference between patients with multiple sclerosis and healthy donors (Supplementary Fig. 2).

Functional analysis of CD161$^+$CD8$^+$ T lymphocytes in patients with multiple sclerosis and healthy donors

While the CD161^{high}CD8$^+$ T lymphocytes had an effector-memory, cytokine-producing phenotype (CD27$^{-/+}$CD45RA$^-$ perforin$^-$), the CD161^{low}CD8$^+$ subset displayed cytotoxic markers (CD27$^-$CD45RA$^+$perforin$^+$). This phenotypic distinction was confirmed by the expression profile of chemokine receptors: the CD161^{high}CD8$^+$ T cells were CX3CR1$^-$, while in the CD161^{low}CD8$^+$ T cells the expression of CX3CR1 was variable; both subsets expressed low, if any, amounts of CCR7 and CCR4. Virtually all the CD161^{high}CD8$^+$ T lymphocytes expressed CCR6 and the IL-18 receptor. The above functional characterization was comparable between patients and healthy donors (Fig. 3 and Supplementary Fig. 3).

We then studied the ability to produce Th1-, Th2- and Th17-like cytokines by CD8$^+$ T cells in patients with multiple sclerosis and healthy donors (Fig. 4). CD161$^+$CD8$^+$ T cells produced detectable levels of intracellular IFN-γ after stimulation with anti-CD3. Moreover, the combined stimulation of CD3 and CD161 induced higher IFN-γ levels compared to ligation of CD3 alone, suggesting a co-stimulatory role of CD161 for IFN-γ production; in contrast, ligation of CD161 alone did not trigger IFN-γ production (Fig. 4A). Given the known effect of IL-12 in promoting helper 1 profiles (and in particular IFN-γ production), as well as our previous studies on IL-12-mediated upregulation of CD161 (Poggi *et al.*, 1997, 1998, 1999), we stimulated sorted CD161^{high}CD8$^+$, CD161^{low}CD8$^+$ and CD161$^-$CD8$^+$ T cell subsets with a combination of anti-CD3 plus anti-CD28, with or without IL-12 (Fig. 4B): IL-12 induced an increase of IFN-γ release by all three subsets both in patients with multiple sclerosis and in healthy donors, without significant differences; however, the salient effect was a more than 10-fold increase on the CD161^{high}CD8$^+$ subset (multiple sclerosis, $P = 0,04$; healthy donors, $P = 0.03$). A small but measurable fraction of CD8$^+$ T cells produced IL-17. Notably, the CD161$^+$ subset contained virtually all IL-17$^+$CD8$^+$ T cells (Fig. 4C and Supplementary Fig. 4). IL-21 production was confined to the CD161^{low} and CD161$^-$, while IL-2 and IL-13 production could be detected mainly in CD161$^-$ lymphocytes. A small fraction of CD8$^+$ cells produced IL-22 regardless of CD161 expression levels. In general, cytokine production did not differ between patients with multiple sclerosis and healthy donors.

We also evaluated the effects of IL-12 on the proliferation of CD161$^+$CD8$^+$ T cells. The stimulation of PBMC with IL-2 in the presence or absence of IL-12 is shown in Fig. 5A. Proliferation was confined to the CD161^{high}CD8$^+$ T-cell subset in both patients with multiple sclerosis and healthy donors. As expected, culture with IL-12 determined a significant increase in the fraction of CD161$^+$ in the CD8$^+$ T-cell compartment (Fig. 5B).

Detection of CD161$^+$ CD8$^+$ T cells in the multiple sclerosis brain

We next investigated whether CD161$^+$CD8$^+$ T cells can migrate into the multiple sclerosis brain. To track CD161$^+$CD8$^+$ T cells in brain inflammatory infiltrates, we performed triple immunofluorescence stainings with anti-CD161, anti-CD8 and anti-CD3 antibodies in sections of post-mortem multiple sclerosis brain. We analysed brain samples from four cases with secondary progressive multiple sclerosis that contained chronic active white matter lesions and inflamed meninges. Ectopic B-cell follicles (Serafini *et al.*, 2004; Magliozzi *et al.*, 2007) were identified in the meninges of two cases. As previously reported (Friese and Fugger, 2009), a large proportion ($\geqslant$50%) of the CD3$^+$ T cells infiltrating the multiple sclerosis brain were CD8$^+$. Some triple-positive CD161$^+$CD8$^+$CD3$^+$ cells were found in the perivascular cuffs of active ($n = 2$) and chronic active ($n = 4$) white matter lesions (Fig. 6A), representing between ~8 and 12% of the CD3$^+$CD8$^+$ T-cell population. Some CD161$^+$CD8$^+$CD3$^+$ cells

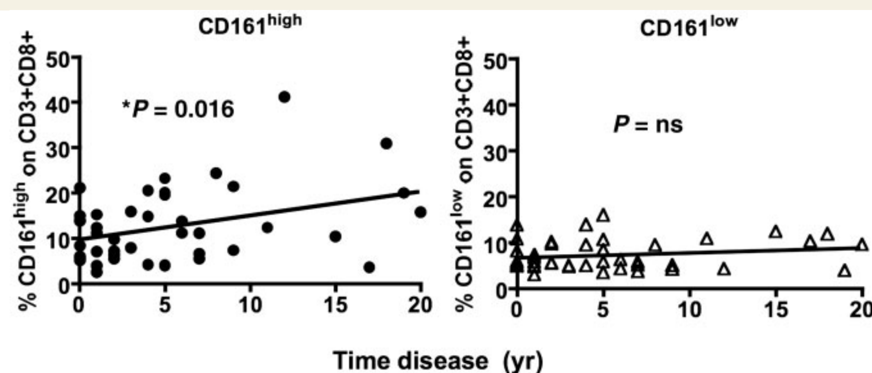

Figure 2 Correlation between CD161^{high}CD8$^+$ T cells and disease duration in a cohort of 43 patients with multiple sclerosis. ns = not significant.

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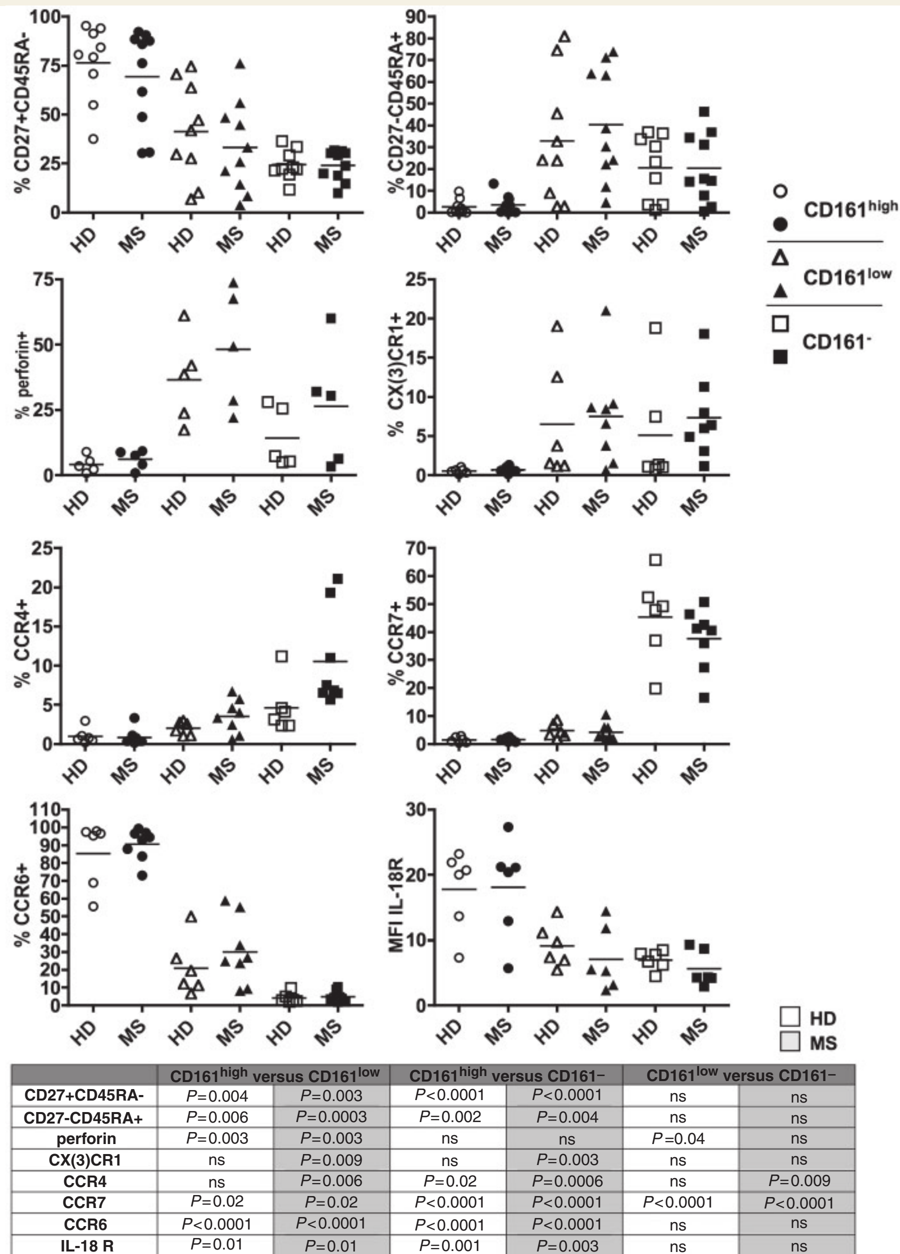

	CD161^{high} versus CD161^{low}		CD161^{high} versus CD161−		CD161^{low} versus CD161−	
CD27+CD45RA-	P=0.004	P=0.003	P<0.0001	P<0.0001	ns	ns
CD27-CD45RA+	P=0.006	P=0.0003	P=0.002	P=0.004	ns	ns
perforin	P=0.003	P=0.003	ns	ns	P=0.04	ns
CX(3)CR1	ns	P=0.009	ns	P=0.003	ns	ns
CCR4	ns	P=0.006	P=0.02	P=0.0006	ns	P=0.009
CCR7	P=0.02	P=0.02	P<0.0001	P<0.0001	P<0.0001	P<0.0001
CCR6	P<0.0001	P<0.0001	P<0.0001	P<0.0001	ns	ns
IL-18 R	P=0.01	P=0.01	P=0.001	P=0.003	ns	ns

Figure 3 CD161^{high}CD8^{+} T cells include effector-memory, non-cytotoxic, CCR6^{+} IL18R^{+} T cells. Expression of CD27, CD45RA, perforin and chemokine receptors in CD161^{high} CD8^{+}, CD161^{low} CD8^{+} and CD161^{-}CD8^{+} peripheral T lymphocytes. The table shows statistical comparisons between the CD161^{-}, CD161^{low}, and CD161^{high} subsets in healthy donors (HD) and patients with multiple sclerosis. ns = not significant.

were also identified in meningeal inflammatory infiltrates (data not shown) and inside ectopic B-cell follicles ($\leqslant$1% of the CD8^{+}CD3^{+} T-cell population) (Fig. 6B). No CD161, CD8 or CD3 immunoreactivity was detected in sections of non-pathological brain and no triple-positive CD161^{+}CD8^{+}CD3^{+} cells were detected in tonsils (data not shown). This finding is in agreement with the notion that the CD161^{+}CD8^{+}CD3^{+} T-cell subset mainly contains cells with an effector memory phenotype (Takahashi *et al.*, 2006) that would be expected to circulate in the peripheral blood and enter inflamed organs (such as the brain in multiple sclerosis), but not secondary lymphoid organs. Using triple immunofluorescence staining we observed that virtually all CD161^{+}CD8^{+} T cells in the three multiple sclerosis brains analysed were immunoreactive for IFN-γ, thereby confirming their ability to perform effector functions in the target tissue (Fig. 6C and D).

Discussion

By combining transcriptome analysis in multiple sclerosis-discordant monozygotic twins, flow cytometric studies in patients with multiple sclerosis and healthy donor controls, and

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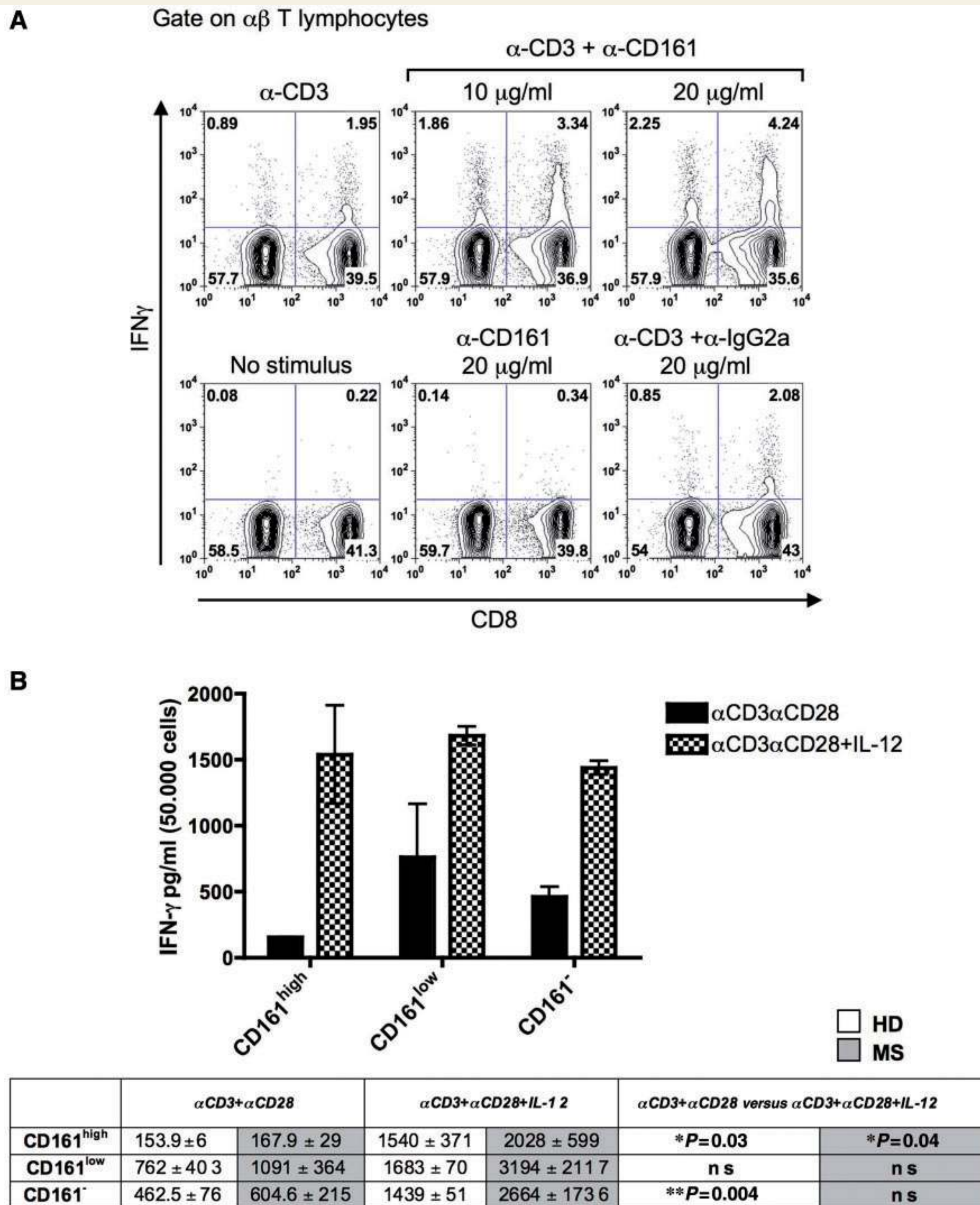

	αCD3+αCD28		αCD3+αCD28+IL-1 2		αCD3+αCD28 versus αCD3+αCD28+IL-12	
CD161^{high}	153.9 ± 6	167.9 ± 29	1540 ± 371	2028 ± 599	*P=0.03	*P=0.04
CD161^{low}	762 ± 40 3	1091 ± 364	1683 ± 70	3194 ± 211 7	n s	n s
CD161^{-}	462.5 ± 76	604.6 ± 215	1439 ± 51	2664 ± 173 6	**P=0.004	n s

Figure 4 CD161^{high}CD8^{+} T cells are cytokine-producing T cells, with a pro-inflammatory profile. (**A**) Intracellular staining for IFN-γ on PBMC after 6 h of stimulation with anti-CD3 and anti-CD161 in different combinations, in the presence of IL-2. Data are representative of five independent experiments performed on three patients with multiple sclerosis and three healthy donors (HDs); results from one patient with multiple sclerosis are shown. (**B**) Enzyme-linked immunosorbent assay for IFN-γ production after 24 h of stimulation with or without IL-12 of sorted CD161^{-} CD8^{+}, CD161^{low}CD8^{+} and CD161^{high}CD8^{+} T-cell subsets; representative data from three independent experiments performed in three patients with multiple sclerosis and three healthy donors are shown. The table shows statistical comparisons between the two stimuli (in the presence or the absence of IL-12) for IFN-γ production. (**C**) Cytokine production by the different CD8^{+} T-cell subsets. Data are expressed as percentage of cytokine-positive cells within CD8^{+} CD3^{+} lymphocytes. The table shows statistical comparisons between the CD161^{-}, CD161^{low} and CD161^{high} subsets in healthy donors and patients with multiple sclerosis. ns = not significant.

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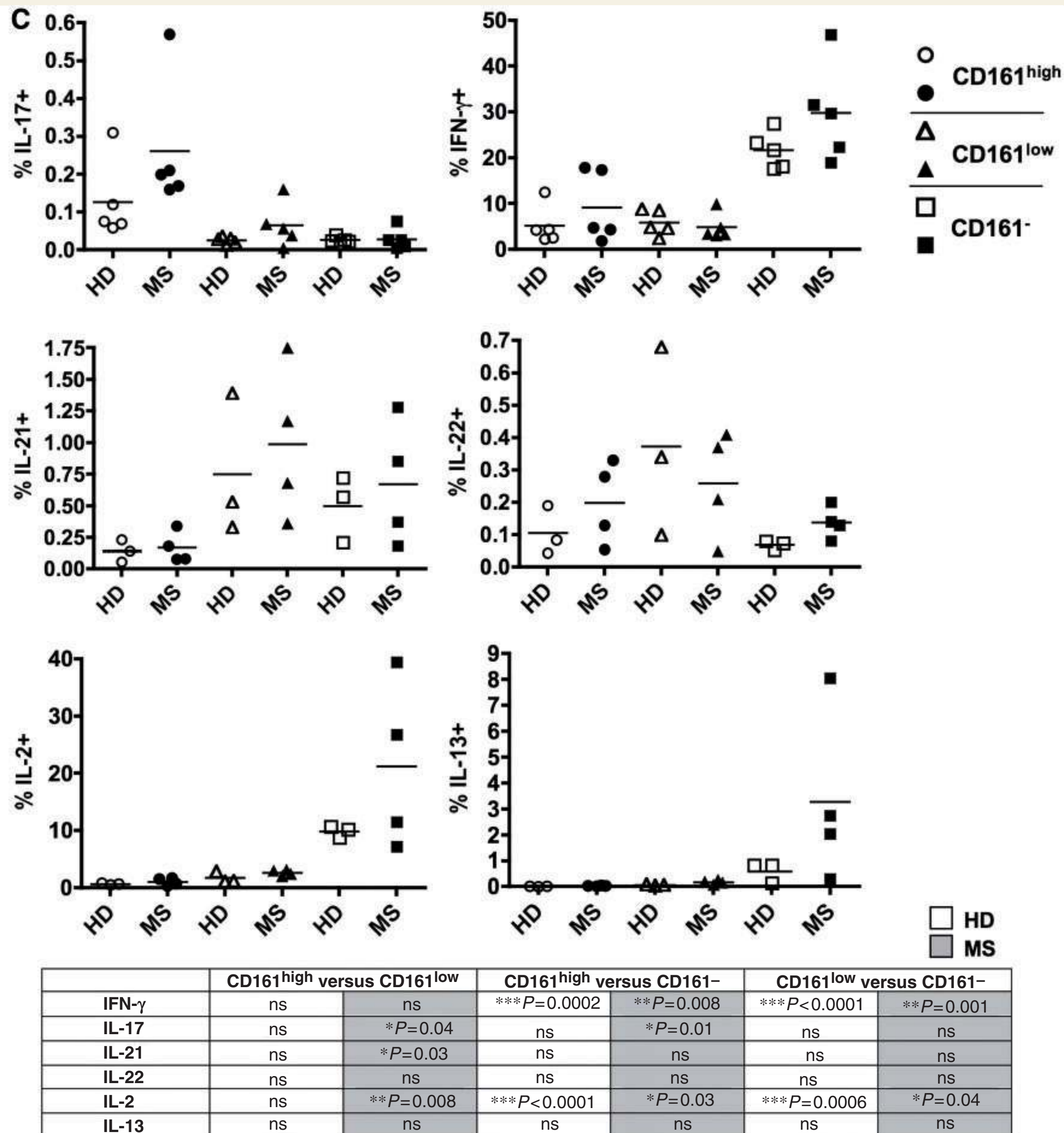

	CD161^high versus CD161^low		CD161^high versus CD161−		CD161^low versus CD161−	
IFN-γ	ns	ns	***P=0.0002	**P=0.008	***P<0.0001	**P=0.001
IL-17	ns	*P=0.04	ns	*P=0.01	ns	ns
IL-21	ns	*P=0.03	ns	ns	ns	ns
IL-22	ns	ns	ns	ns	ns	ns
IL-2	ns	**P=0.008	***P<0.0001	*P=0.03	***P=0.0006	*P=0.04
IL-13	ns	ns	ns	ns	ns	ns

Figure 4 Continued.

immunofluorescence on post-mortem brain sections, we show that patients with multiple sclerosis present an expansion of proinflammatory CD161^{high}CD8$^+$ T cells in the peripheral blood and that CD161$^+$CD8$^+$ T cells are detectable in brain immune infiltrates. In addition, this study contributes to the ongoing phenotypic characterization of CD161 expressing CD8$^+$ T cells.

Concerning the findings that pertain to multiple sclerosis, our data are in agreement with recent work on peripheral blood CD4$^+$ T cells in multiple sclerosis-discordant monozygotic twins, where no difference was found in the messenger RNA transcriptome between healthy and affected co-twins (Baranzini *et al.*, 2010). However, having included also the CD8$^+$ subset of T cells, the present study adds to the increasing appreciation of the role of CD8$^+$ T cells as effectors of the pathological immune response in multiple sclerosis (Friese and Fugger, 2009).

The International Multiple Sclerosis Genetics Consortium has reported an association between CD161 genetic variants and the diseases (Hafler *et al.*, 2007). The association of this locus with multiple sclerosis was not strong enough to reach the suggestive evidence level in a successive meta-analysis (De Jager *et al.*, 2009). Independently of the significance of the genetic data, it is intriguing that we could detect differences in CD161 expression also in monozygotic twins, suggesting that the alteration affects multiple sclerosis risk irrespective of its heritable or non-heritable origin. It will be of the utmost interest to verify whether and how genetic (if any) and environmental factors that affect CD161 expression interact and reciprocally amplify their impact on multiple sclerosis pathogenesis. A detailed analysis of CD161$^+$CD8$^+$ T cells revealed that this subset comprises CCR6$^+$, non-cytotoxic CD8$^+$ T lymphocytes with an effector-memory phenotype that may

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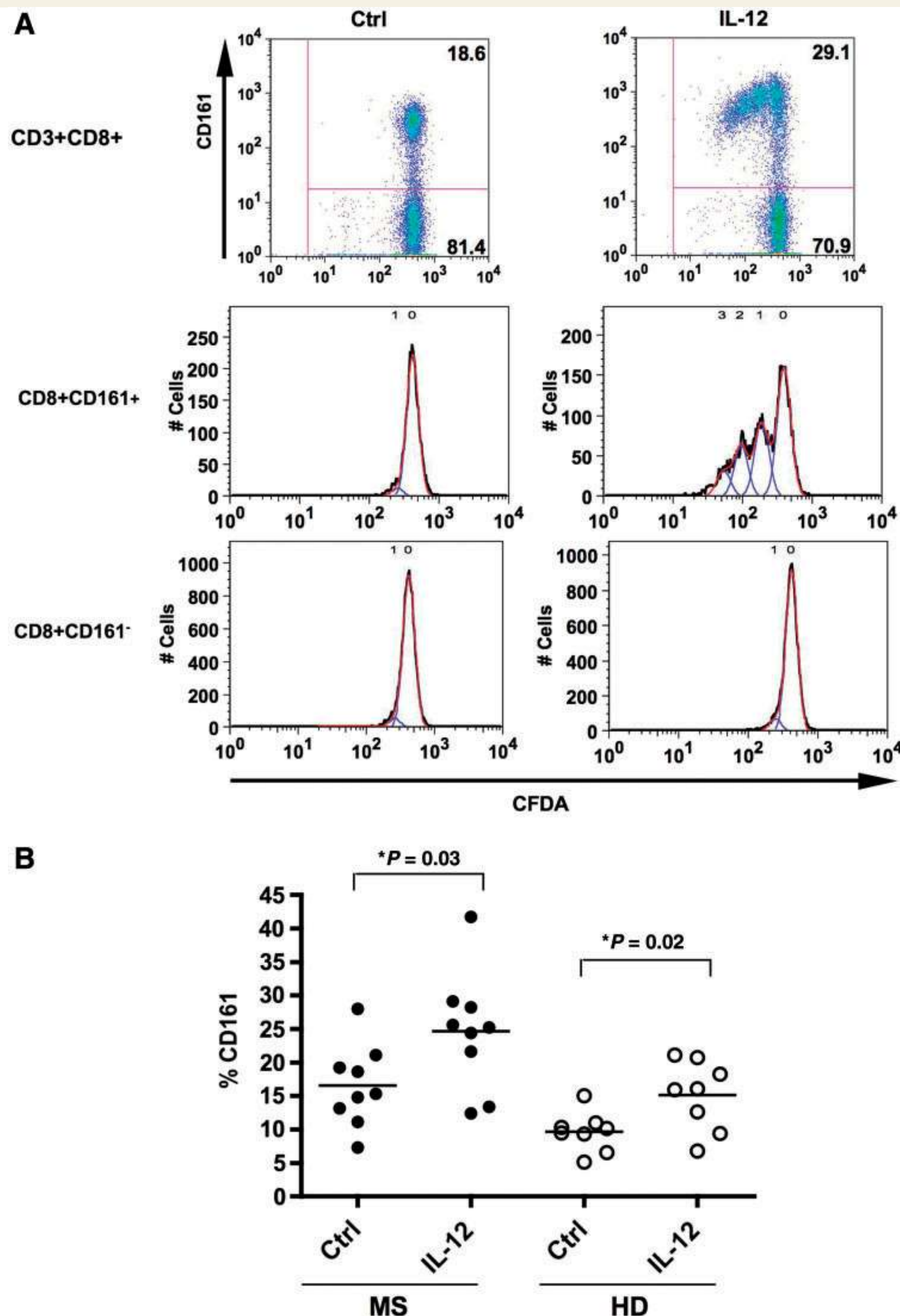

Figure 5 IL-12 induces preferential proliferation of CD161^{high}CD8$^+$ T cells. (**A**) Proliferation of CFDA-SE-stained PBMC cultured with (*right panels*) or without IL-12 (*left panels*). Gate on total CD8$^+$CD3$^+$ cells (*upper panels*). Proliferation analysis on CD161$^+$CD8$^+$CD3$^+$ and CD161$^-$CD8$^+$CD3$^+$ cells (*lower panels*). The figure is representative of data from nine patients with multiple sclerosis (MS) and eight healthy donors (HD). (**B**) Percentage of CD161$^+$ cells (gated on CD8$^+$ T cells) following culture with IL-12.

contribute to multiple sclerosis immunopathology through the production of proinflammatory cytokines (Takahashi *et al.*, 2006). Consistent with these and previous findings (Aldemir *et al.*, 2005), and supporting the relevance of this subset for multiple sclerosis pathogenesis, we show that CD161 co-stimulates IFN-γ production in CD8$^+$ T cells, with the CD161^{high}CD8$^+$ subset poised to secrete proinflammatory cytokines and to proliferate in the presence of IL-12. Further supporting its co-stimulatory role for T cells, CD161 has recently been reported to act as a ligand for proliferation-induced lymphocyte-associated receptor [PILAR

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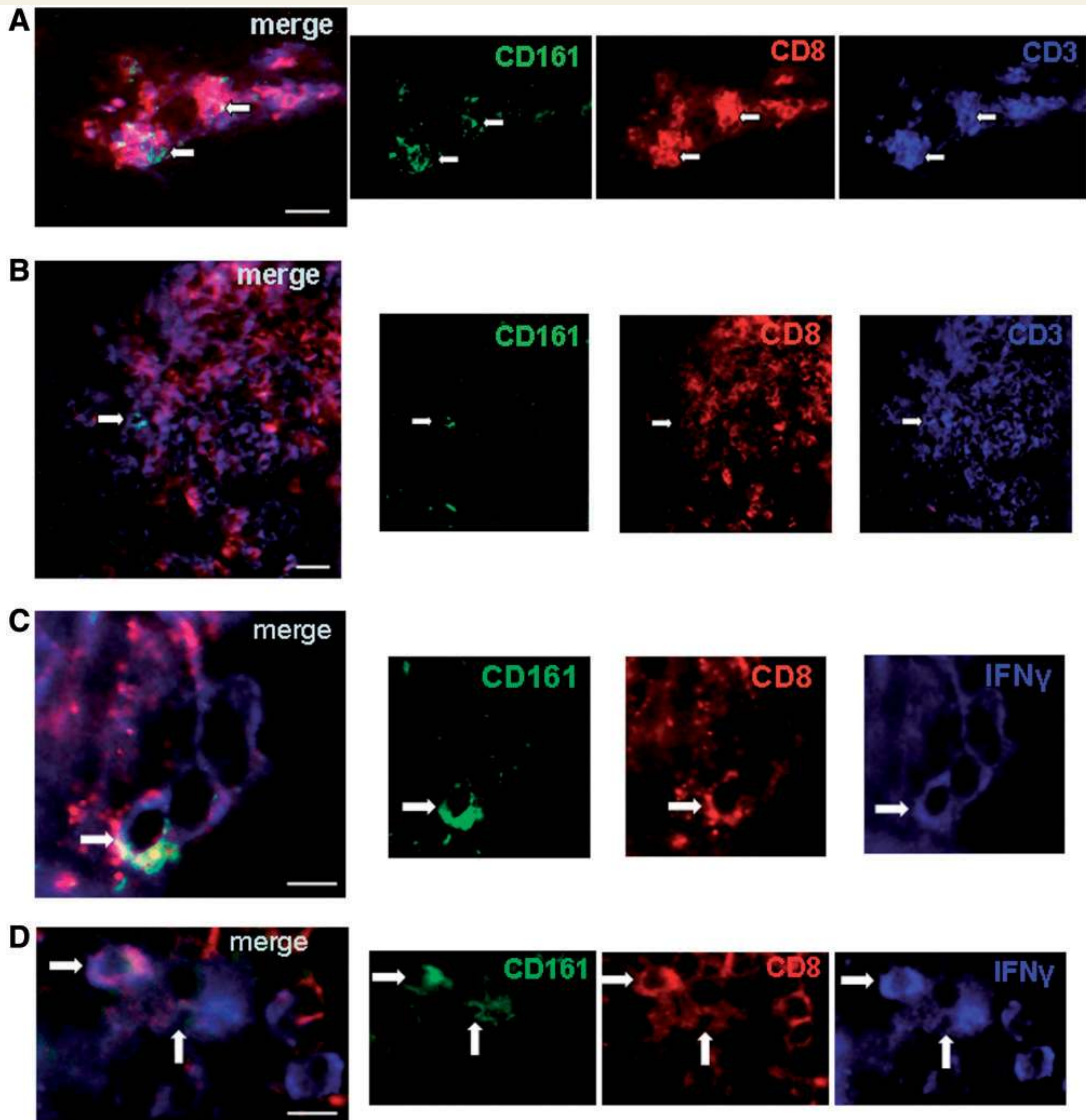

Figure 6 Detection of $CD161^+CD8^+$ T cells producing IFN-γ in the multiple sclerosis brain. Triple immunofluorescence stainings for CD161 (green), CD8 (red) and CD3 (blue) (**A** and **B**) and for CD161 (green), CD8 (red) and IFN-γ (blue) (**C** and **D**) were performed in snap frozen sections of post-mortem multiple sclerosis brain tissue. (**A**) Some $CD8^+CD3^+$ cells expressing CD161 (light blue in the merge) are present in a perivascular cuff of a chronic active white matter lesion. (**B**) One $CD161^+CD8^+CD3^+$ T cell is present in a meningeal B-cell follicle. (**C**) One triple stained $CD161^+CD8^+$IFN-γ^+ cell among several $CD161^-CD8^+$IFN-γ^+ in a sparse meningeal inflammatory cell infiltrate. (**D**) Two $CD161^+CD8^+$IFN-γ^+ cells among $CD161^-CD8^+$IFN-γ^+ and $CD161^-CD8^-$IFN-γ^+ cells in an ectopic B-cell follicle. Arrows indicate triple positive cells. Scale bars = 10 µm.

(Huarte *et al.*, 2008)], a novel modulator of T-cell expansion that shares homology with CLEC2D/LLT1 (Aldemir *et al.*, 2005; Rosen *et al.*, 2005). The presence of $CD161^+CD8^+$ T cells producing IFN-γ in multiple sclerosis lesions may be related to their migratory potential. Previous work showed that CD161 (Poggi *et al.*, 1997, 1999) and CCR6 (Reboldi *et al.*, 2009) alone, favour T-cell transmigration. The simultaneous expression of CD161 and CCR6 indicates that these lymphocytes may be particularly efficient at entering the CNS where they may form long-lasting contacts with local antigen presenting cells, as recently described for

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CD8$^+$ T cells (Serafini *et al.*, 2006). Potential target antigens include myelin autoantigens (Crawford *et al.*, 2004) and/or viral determinants (Tsunoda *et al.*, 2005). In view of the association between Epstein–Barr virus infection and multiple sclerosis (Ascherio and Munger, 2007), it is interesting that peripheral CD161$^+$CD8$^+$ T cells in the Epstein–Barr virus-specific repertoire increase during the latent/reactivating phase of infection (Poon *et al.*, 2005). Indeed, a dysregulated CD8$^+$ T-cell response to Epstein–Barr virus has been recently reported in multiple sclerosis (Jilek *et al.*, 2008; Pender *et al.*, 2009), though the presence of Epstein–Barr virus-infected B cells and plasma cells in multiple sclerosis brains remains controversial (Serafini *et al.*, 2007; Peferoen *et al.*, 2009; Pender, 2009; Willis *et al.*, 2009). In other chronic viral infections such as hepatitis C, CD161 expression is increased on virus-specific CD8$^+$ T cells and, further supporting the migratory capacity of these lymphocytes, is greatest on intrahepatic hepatitis C virus-specific T cells (Northfield *et al.*, 2008; Billerbeck *et al.*, 2010). A recent study by Turtle *et al.* (2009) shows that CD161^{high}CD8$^+$ T cells specific for Epstein–Barr virus, cytomegalovirus or influenza virus encompass IL-18Ra^{high}, IL-7 and IL-15 responsive memory cells, with high drug efflux capacity, higher levels of anti-apoptotic molecules and lower cell-cycle fraction. These properties may explain our finding of a relative increase of these cells during the course of the disease and suggest that the CD161^{high}CD8$^+$ phenotype may include cells that operate in hostile conditions such as those of the inflamed CNS. This view is supported by the high density of IL-18R that we and others (Billerbeck *et al.*, 2010) found on CD161^{high}CD8$^+$ T cells, as well as by the recently reported increasing relevance of IL-18R in multiple sclerosis pathogenesis (Gillett *et al.*, 2010).

At variance with the data in multiple sclerosis, we found that CD161^{low}CD8$^+$ T lymphocytes prevail in rheumatoid arthritis, a result that may be in agreement with observations on a decreased number of circulating CD161$^+$CD8$^+$ T cells in rheumatic diseases (Mitsuo *et al.*, 2006). The reasons for this difference between multiple sclerosis and rheumatoid arthritis are, at present, unclear and need further investigation, possibly in studies that also include patients with other autoimmune diseases.

IL-17-producing CD8$^+$ T cells are less frequent than the CD4$^+$Th17 and are confined to the CD27$^-$CD45RA$^-$CCR6$^+$ subset (Kondo *et al.*, 2009). In agreement with a very recent report (Billerbeck *et al.*, 2010), we now show a relationship between CD161 expression and production of IL-17 in the CD8$^+$ T cell subset, mirroring recent results obtained in CD4$^+$ T cells (Cosmi *et al.*, 2008; Kleinschek *et al.*, 2009). Th17 cells are key inducers of inflammation and autoimmunity (Bettelli *et al.*, 2008; Steinman, 2008). Looking again at the relevance of the CD161^{high}CCR6$^+$CD8$^+$ phenotype from a multiple sclerosis perspective, this finding appears to converge with the demonstration of IL-17$^+$CD8$^+$ T cells in multiple sclerosis brain lesions (Tzartos *et al.*, 2008; Kebir *et al.*, 2009), and with evidence that CCR6$^+$Th17 cells are required for the initiation of experimental autoimmune encephalomyelitis (Reboldi *et al.*, 2009). The relevance of CD161^{high}CCR6$^+$CD8$^+$ T cells with pro-inflammatory cytokine profiles for multiple sclerosis pathogenesis is also supported by a parallel study (Muraro P., personal communication) that shows near disappearance of the same T-cell subset after autologous haematopoietic stem cell transplantation and amelioration of clinical course in patients with multiple sclerosis.

In summary, our work shows that CD161 expression on CD8$^+$ T cells marks a subset of non-cytotoxic, proinflammatory, CCR6$^+$ T lymphocytes that may play a role in multiple sclerosis immunopathogenesis by acting as effectors and targeting the CNS. They also contribute to the ongoing characterization of CD161 expressing T cells.

Acknowledgements

We thank the Italian Study Group on Multiple Sclerosis in Twins supported by Associazione Italiana Sclerosi Multipla (AISM), Dr M. Simmaco for allowing us to use the facilities available at the DiMA Unit of the Sant'Andrea Hospital, and Dr B. Laganà (Department of Rheumatology-Sant'Andrea Hospital) for providing blood samples from 10 rheumatoid arthritis patients. Brain tissue samples were supplied by the UK Multiple Sclerosis Tissue Bank (www.ukmstissuebank.imperial.ac.uk).

Funding

Italian Ministry of Health (Finalizzato and Art. 56) to L.B.; FP6 Integrated Project NeuroproMiSe (LSHM-CT-2005-01863) to F.A.; and Italian Multiple Sclerosis Foundation grants to M.S., F.A. and G.B. Multiple sclerosis Society of Great Britain and Northern Ireland (registered charity 207495).

Supplementary material

Supplementary material is available at *Brain* online.

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