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Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis

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Autologous haematopoietic stem cell transplantation has been tried as one experimental strategy for the treatment of patients with aggressive multiple sclerosis refractory to other immunotherapies. The procedure is aimed at ablating and repopulating the immune repertoire by sequentially mobilizing and harvesting haematopoietic stem cells, administering an immunosuppressive conditioning regimen, and re-infusing the autologous haematopoietic cell product. 'Non-myeloablative' conditioning regimens to achieve lymphocytic ablation without marrow suppression have been proposed to improve safety and tolerability. One trial with non-myeloablative autologous haematopoietic stem cell transplantation reported clinical improvement and inflammatory stabilization in treated patients with highly active multiple sclerosis. The aim of the present study was to understand the changes in the reconstituted immune repertoire bearing potential relevance to its mode of action. Peripheral blood was obtained from 12 patients with multiple sclerosis participating in the aforementioned trial and longitudinally followed for 2 years. We examined the phenotype and function of peripheral blood lymphocytes by cell surface or intracellular staining and multi-colour fluorescence activated cell sorting alone or in combination with proliferation assays. During immune reconstitution post-transplantation we observed significant though transient increases in the proportion of $CD4^+FoxP3^+$ T cells and $CD56^{high}$ natural killer cell subsets, which are cell subsets associated with immunoregulatory function. $CD8^+CD57^+$ cytotoxic T cells were persistently increased after therapy and were able to suppress $CD4^+$ T cell proliferation with variable potency. In contrast, a $CD161^{high}$ proinflammatory $CD8^+$ T cell subset was depleted at all time-points post-transplantation. Phenotypic characterization

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revealed that the CD161^{high}CD8$^+$ T cells were mucosal-associated invariant T cells, a novel cell population originating in the gut mucosa but expressing the central nervous system-homing receptor CCR6. Detection of mucosal-associated invariant T cells in post-mortem multiple sclerosis brain white matter active lesions confirmed their involvement in the disease pathology. Intracellular cytokine staining demonstrated interferon γ and interleukin 17 production and lack of interleukin 10 production, a pro-inflammatory profile. Mucosal-associated invariant T cell frequency did not change in patients treated with interferon β; and was more depleted after autologous haematopoietic stem cell transplantation than in patients who had received high-dose cyclophosphamide ($n = 7$) or alemtuzumab ($n = 21$) treatment alone, suggesting an additive or synergistic effect of the conditioning regime components. We propose that a favourably modified balance of regulatory and pro-inflammatory lymphocytes underlies the suppression of central nervous system inflammation in patients with multiple sclerosis following non-myeloablative autologous haematopoietic stem cell transplantation with a conditioning regimen consisting of cyclophosphamide and alemtuzumab.

Keywords: stem cells; multiple sclerosis; T cells; proinflammatory cytokines; immune regulation

Abbreviations: AHSCT = autologous haematopoietic stem cell transplantation; IFN = interferon; IL = interleukin; MAIT = mucosal-associated invariant T cell; TNF = tumour necrosis factor

Introduction

Multiple sclerosis is an inflammatory and neurodegenerative disease of the CNS (Compston and Coles, 2008). Current immune-modifying treatments are incompletely effective in patients with aggressive multiple sclerosis phenotypes. Autologous bone marrow or haematopoietic stem cell transplantation (AHSCT) has been experimented in patients with aggressive forms of multiple sclerosis, aimed at suppressing CNS inflammatory activity and preventing further clinical deterioration (Saccardi *et al*., 2012). The mechanistic rationale for AHSCT is to first purge the mature immune system through intensive immune suppression and to then promote—with haematopoietic stem cell support—the reconstitution of a new immune system, free from aberrant responses that had previously developed within the individual's adaptive immune system.

Recent studies support a role for *de novo* regeneration of naïve T cells from the thymus (Hakim *et al*., 2005; Muraro *et al*., 2005), for enhanced immune regulation after AHSCT (de Kleer *et al*., 2006; Alexander *et al*., 2009; Zhang *et al*., 2009). In addition, a recent study by Darlington *et al*. (2013) showed abrogation of the T helper (Th)17 response following high-intensity AHSCT. However, the cellular and molecular mechanisms underlying improved clinical course post-AHSCT treatment are poorly understood and further complexity is added by the use of different immunosuppressive conditioning regimens. Non-myeloablative conditioning regimens have been proposed to improve tolerability and safety of AHSCT and allow treatment at earlier stages of disease than in the initial clinical trials (Burt *et al*., 2010). Current evidence for the immune modulatory mechanisms occurring after AHSCT remains scarce and no study to our knowledge has examined in detail the effects of a non-myeloablative conditioning transplantation regimen on the immune system. One recently published clinical trial of AHSCT using a non-myeloablative conditioning regimen in patients with highly active multiple sclerosis has demonstrated sustained clinical stabilization in all and even improvement of disability in some patients (Burt *et al*., 2009). To investigate the immunological mechanisms underlying the remission of CNS inflammation, we performed longitudinal analysis of immune reconstitution in a group of patients treated in that trial.

Our results show that following this non-myeloablative treatment protocol using cyclophosphamide and alemtuzumab for immunosuppressive conditioning, there were significant transient increases of CD4$^+$CD25^{high}FoxP3$^+$ T cells and of CD56^{high} natural killer cells, both phenotypes associated with immune regulatory function. We also observed robust and long-term increase of CD8$^+$CD57$^+$ T cells post-AHSCT. CD8$^+$CD57$^+$ T cells were, in some patients, able to suppress CD4$^+$ T cell proliferation in *ex vivo* cell co-cultures with superior efficiency. In contrast, we identified a population of CD161^{high}CD8$^+$ T cells that were readily detectable in the blood of all patients pre-transplant, but were maximally and permanently ablated during the 2-year post-AHSCT follow-up. Further characterization of the CD161^{high} CD8$^+$ T cell population found in multiple sclerosis patients' blood pre-AHSCT revealed that these cells are mucosal-associated invariant T (MAIT) cells, a T cell subset associated with the gut (Le Bourhis *et al*., 2010; Dusseaux *et al*., 2011). High CD161-expression defined a subset of pro-inflammatory T cells that includes the majority of interleukin (IL)-17 producing CD8$^+$ T cells and also produces IFN-γ and tumour necrosis factor (TNF)-α but not IL-10. We confirmed that MAITs are able to migrate to the brain as they were present in the white matter and perivascular infiltrate of post-mortem multiple sclerosis brain tissue. Comparison of MAIT frequency in patients that received other multiple sclerosis treatments, including the individual components of the conditioning regimen, high-dose cyclophosphamide and alemtuzumab monotherapy, revealed that autologous haematopoietic transplantation induced the most consistent depletion for up to 2 years post-therapy.

The data demonstrate that the adaptive immune system reconstituted in patients with multiple sclerosis following the non-myeloablative AHSCT regimen is characterized by a favourably modified balance of pro- and anti-inflammatory lymphocyte subsets in the circulation, characterized by the expansion of immunoregulatory cells and radical depletion of a gut-associated

CD161^{high}CD8^{+} MAIT population, which produces IFN-γ and IL-17, bears a pro-inflammatory profile and is able to infiltrate the CNS.

Materials and methods

Subjects, treatment and biological samples

Patients with aggressive multiple sclerosis who failed to respond to licensed immune-modifying treatments were recruited for an institutional review board-approved clinical trial of non-myeloablative immunosuppressive conditioning with cyclophosphamide and alemtuzumab and AHSCT at Northwestern University, Chicago, USA (Burt *et al*., 2009). Following informed consent, peripheral blood for the research study was donated by subjects with multiple sclerosis undergoing AHSCT in the trial ($n = 12$). The demographic and clinical characteristics of the non-myeloablative AHSCT trial patients who participated in this study are provided in Table 1. All these patients underwent non-myeloablative conditioning with 120 mg/kg cyclophosphamide and 20 mg alemtuzumab. Peripheral blood samples were obtained from the AHSCT patients at baseline within 1 month before haematopoietic stem cell mobilization, and at 6 months, 1 and 2 years post-haematopoietic stem cell reinfusion.

Peripheral blood mononuclear cell samples for comparative immunological analysis were obtained from non-AHSCT patients with multiple sclerosis ($n = 40$), and healthy individuals ($n = 7$). Of the non-AHSCT patients with sclerosis, five were untreated and seven received standard disease-modifying treatment with interferon-beta (IFN-β): Betaferon® ($n = 5$), Betaseron® ($n = 1$) or Avonex® ($n = 1$). The five patients receiving Betaferon® were studied longitudinally before and during the first 9 months of treatment. One additional group included patients who had participated in a study of high-dose cyclophosphamide ($n = 7$). Patients in this study received 50 mg/kg/day of cyclophosphamide intravenously for four consecutive days, followed by 5 μg/kg/day of granulocyte colony stimulating factor 6 days after completion of high-dose cyclophosphamide treatment, until the absolute neutrophil count exceeded 1.0×10^9 cells/l for two consecutive days. The protocol has been described in detail elsewhere (Krishnan *et al*., 2008). Of the seven patients, five had pretreatment baseline and post-treatment samples over a 2-year follow-up, one was available only at pretreatment baseline, and one only at 2 years post-therapy. The high-dose cyclophosphamide treated patients' clinical characteristics are given in Supplementary Table 1. The last group of patients received alemtuzumab monotherapy ($n = 21$) by participating in one of four studies: CAMMS-224 (an investigator-led study, REC 03/078); CAMMS-223 (a Phase 2 randomized controlled trial) or CARE-MS1 or CARE-MS2 (Phase 3 randomized controlled trials). Alemtuzumab was given for 5 days at baseline then for 3 days at Month 12 (12 or 24 mg/day); further cycles were given if there was clinical or radiological evidence of disease activity. All patients consented to long-term follow-up and venipuncture for research purposes (CAMSAFE REC-11/33/0007). Only post-treatment samples were available for analysis. Demographic and clinical information on alemtuzumab-treated patients is provided in Supplementary Table 2.

Peripheral blood mononuclear cells were freshly separated by density gradient centrifugation and cryopreserved according to rigorously standardized protocols for subsequent use in batched, parallel immune analysis. All laboratory studies received ethical approval from Imperial College Research Ethics Committee (Ref. ICREC62D).

Table 1 Demographic and clinical data of the AHSCT patients

Subject code	Sex	Age at AHSCT (years)	Multiple sclerosis course	Duration of disease (years from onset)	Previous treatments	Number of steroid-treated relapses in year preceding transplant	Post-treatment toxicity	Multiple sclerosis relapses post-AHSCT	EDSS score (pre-mobilization baseline)	EDSS score (6 months post-AHSCT)	EDSS score (12 months post-AHSCT)	EDSS score (24 months post-AHSCT)
CC 01	M	45	RR	8	MP, IFN-β, IVIG	3	None	0	2.0	1.0	0	0
CC 02	M	35	RR	9	IFN-β, GA, MP	3	None	0	6.0	5.0	5.0	3.0
CC 03	M	20	RR	3	IFN-β, MP	2	Dermatomal zoster at 20 months	0	3.0	3.0	1.5	1.5
CC 04	M	44	SP	12	MTX	0	none	0	5.5	6.0	6.0	6.0
CC 05	F	29	RR	5	IFN-β, GA, MP	2	None	1 at 12 months	3.5	2.0	3.5	2.5*
CC 07	M	34	RR	8	IFN-β, MP	3	None	0	3.5	3.5	3.5	2.5
CC 08	M	22	RR	3.5	IFN-β, PE, CY, MP	2	None	1 at 12 months	5.5	5.5	6.0	2.0
CC 09	M	36	RR	4	GA, IFN-β, DCZ, MP	2	None	1 at 16 months	2.5	1.5	1.5	1.0
CC 10	F	38	RR	6	IFN-β, GA, IVIG, MP	1	None	0	3.5	2.0	2.0	2.0
CC 11	M	38	RR	5	IFN-β, MP	2	None	0	2.5	2.5	1.5	2.5
CC 12	M	25	RR	4	IFN-β, AZA, MP	2	None	1 at 6 months	3.0	3.5	3.0	3.0
CC 13	F	53	RR	2	IFN-β, MP	2	ITP at 14 months	0	3.5	2.0	NA[ll]	0

AZA = azathioprine; CY = cyclophosphamide; DCZ = daclizumab; EDSS = Expanded Disability Status Scale; GA = glatiramer acetate; ITP = idiopathic thrombocytopenic purpura; IVIG = intravenous immunoglobulin; MP = methylprednisolone; MTX = mitoxantrone; NA = not available; PE = plasma exchange; RR = relapsing-remitting; SP = secondary-progressive.
*Assessment at 36 months.

Flow cytometry

Peripheral blood mononuclear cells were thawed in Dulbecco's PBS and washed in fluorescence activated cell sorting staining buffer (Dulbecco's PBS, 1% foetal bovine serum and 0.01% sodium azide). Surface staining was performed on ice for 20 min and the cells were then analysed on a two laser, four colour FACSCalibur flow cytometer, or for multicolour analysis on a five laser, 18 colour LSRFortessa (Becton Dickinson). Data were analysed using CellQuest (Becton Dickinson) and FlowJo software (TreeStar).

Immune reconstitution and phenotyping studies

Multiplexed dilutions of monoclonal antibodies (mAbs) were used to characterize lymphocyte populations. The following antibodies from BD Biosciences were used: CD3-Cy-Chrome, CD3-RPE-Cy5, CD4-RPE-Cy5, CD4-APC, CD5-APC, CD8-FITC, CD8-RPE-Cy5, CD8-PerCP, CD8-APC, CD11a-PE, CD14-APC, CD19-FITC, CD20-PE, CD25-FITC, CD25-PE, CD27-FITC, CD28-PE, CD31-PE, CD45RA-FITC, CD45RA-PE, CD45RO-PE, CD45RO-APC, CD54-PE (ICAM-1), CD56-PE, CD57-FITC, CD58-FITC (LFA-3), CD62L-PE, CD69-FITC, CD95-PE, CD161-FITC (clone DX12), and T-cell receptor (TCR)$\alpha\beta$-FITC. Other monoclonal antibodies included CD49d-FITC and ILT2-PE (clone HP-F1) from Beckman Coulter.

Regulatory CD4$^+$ cell quantification

Staining for CD3-PE-Cy7 (BD Biosciences), CD4-BV711, CD8-BV785, CD25-BV421, CD45RA-BV510 (BioLegend), and CD127-FITC (eBioscience) was performed before fixation and permeabilization of the cells. Intranuclear cell staining was performed according to the manufacturer's instructions using FoxP3 Alexa Fluor® 647 (clone PCH101) and Ki-67 PerCP-eFluor710 (eBioscience). Blue Live/Dead Stain (Life Technologies) was added to the samples before FoxP3 staining to for live versus dead cells discrimination.

Characterization of mucosal-associated invariant T cells

Multicolour staining was performed with the following antibodies: TCRVα7.2-APC, TCRVα7.2-PE (clone 3C10), CD4-BV711, CD4-BV785, CD8-BV711, CD8-BV785, CD45RA-BV510, CD161-BV421 or CD161-BV605, CCR6 (CD196) PerCP-Cy5.5, from BioLegend; CD161-PE and CD161-APC (clone 191B8) from Miltenyi Biotec; CD3-PE, ILT2 (CD85j)-APC, ILT2-PE, CD150 PE and CD218 (IL-18R) FITC from eBioscience; CCR6-PE from R&D Systems; CD3-APC-H7 and CD57 PE-CF594, CCR7 (CD197) PE-Cy7 (BD Biosciences) and CD45RO-ECD (Beckman Coulter). Blue Live/Dead Stain (Life Technologies) was included for exclusion of dead cells.

Characterization of T cell receptor variable region expression

TCRVα7.2-FITC and TCRVα7.2-PE (clone 3C10) were obtained from BioLegend. For TCRBV usage CD8$^+$ T cells, peripheral blood mononuclear cells were stained with anti-CD8-PerCP (clone SK3, BD Bioscience) and anti-CD161-APC (clone 191B8, Miltenyi Biotec), in combination with pairs of FITC and PE-conjugated antibodies to the following TCRVβ chains and assessed as previously described (Muraro *et al*., 2000): BV1-PE (clone BL37.2), BV2-PE (clone MPB2D5), BV3-FITC (clone CH92), BV5S1-FITC (clone Immu157), BV5S2-FITC (clone 36213), BV5S3-PE (clone 3D11), BV6S7-FITC (clone OT145), BV7-PE (clone ZOE), BV8-FITC (clone 56C5.2), BV9-PE (clone FIN9), BV11-FITC (clone C21), BV12-FITC (clone VER2.32.1), BV13S1-PE (clone Immu222), BV13S2-PE (clone H132), BV14-PE (clone CAS1.13), BV16-FITC (clone TAMAYA1.2), BV17-FITC (clone E17.5F3), BV18-PE (clone BA62.6), BV20-PE (clone ELL1.4), BV21S3-FITC (clone IG125), BV22-FITC (clone Immu546) and BV23-PE (clone AF23). TCRBV6S7 was obtained from Endogen (Thermo Fisher Scientific), TCRBV13S2 from Santa Cruz Biotechnology, and all other TCRBV monoclonal antibodies were purchased from Immunotech.

Intracellular staining for cytotoxic enzymes

Cytotoxic potential was assessed by intracellular staining after fixation and permeabilization of the cells. Antibodies to perforin (PE) and granzyme B (Alexa Fluor® 647) were purchased from BD Biosciences.

Cytokine production assay

Peripheral blood mononuclear cells were stimulated for 5 h with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml, all from Sigma-Aldrich), *ex vivo* after overnight recovery in cell incubator with RPMI-1640 with 10% foetal bovine serum. The cells were harvested and stained for relevant surface markers before fixation in 1% paraformaldehyde and permeabilization in 0.2% saponin. Intracellular cytokine production was assessed by IFN-γ Horizon V450, TNF-α PE-Cy7, IL-10 PE (BD Biosciences) and IL-17A Alexa Fluor® 647 (eBioscience).

Suppression assays

Peripheral blood mononuclear cells were thawed and left to recover overnight in RPMI-1640 with 10% foetal bovine serum and 20 U/ml of IL-2. The following day, CD8$^+$CD57$^+$ and CD8$^+$CD57$^-$ cells were obtained from peripheral blood mononuclear cell using a magnetic microbead kit from Miltenyi Biotec. The percentage of natural killer cells found in all cases was $\leqslant$5%. The CD8-depleted fraction was stained with carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) and used as 'effector' (E) cells, while CD8$^+$CD57$^+$ and CD8$^+$CD57$^-$ cells were used as 'regulatory' (R) cells. Co-cultures were conducted at different R:E ratios in the presence of soluble anti-CD3 antibody (OKT3, 0.5 μg/ml, eBioscience). On Day 4, the cells were washed and stained. Propidium iodide (1 μg/ml) was used for dead cell exclusion. The percentage of cell proliferation was quantified on live (propidium iodide-negative) CD4-gated cells. To normalize the data providing from different donors, the proliferation in the absence of CD8$^+$ cells (0:1 ratio) was considered 100% and the normalized proliferation was defined as the percentage of normalized proliferation at test ratio = (% dividing cells at test ratio / % dividing cells at 0:1 ratio) $\times$ 100, and % suppression was defined as (100 $-$ % proliferation).

Immunostaining of multiple sclerosis brain tissue

Brain tissue blocks were provided by the UK Multiple Sclerosis Tissue Bank at Imperial College, London, UK. Post-mortem tissues were collected with fully informed consent through a prospective donor scheme with ethical approval by the National Research Ethics Committee (08/MRE09/31). Tissue blocks were screened and chronic active white matter lesions were identified as described previously

(Magliozzi *et al*., 2007). Snap frozen brain white matter tissue blocks containing active lesions were selected from a subset of nine cases with progressive multiple sclerosis with high levels of CNS inflammation that formed part of a larger, well-described case series (Howell *et al*., 2011). Tissue blocks (four per case) cut at 10 µm, fixed for 10 min in ice-cold methanol and stained using a double sequential immunofluorescence technique, first using anti-CD161 antibody (clone: B199.2, AbD Serotec) followed by anti-Vα7.2 antibody (clone: 3C10, BioLegend). Sections were incubated overnight at 4°C with the first primary antibody. Binding of biotinylated secondary antibody was visualized with the avidin-biotin horseradish peroxidase complex (Vector Laboratories) followed by 30-min incubation with tyramine (Sigma) in PBS containing 0.03% H_2O_2 according to a modified version of a method described by Adams (1992). The negative control consisted in the same protocol without incubation with tyramine. After 1 h incubation with Alexa Fluor® 546-streptavidin (Invitrogen) sections were washed, blocked with normal serum and incubated overnight at 4°C with the second primary antibody that was then visualized with an Alexa Fluor® 488 secondary antibody (Invitrogen). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for the localization of the nuclei and coverslipped with aqueous mounting medium Vectashield (Vector Laboratories).

Statistical analysis

Statistical significance was calculated using unpaired *t*-test, or by signed rank test for paired data that was not normally distributed. For multiple group comparisons, statistical significance was evaluated by parametric repeated measures ANOVA and Holm-Sidak's *post hoc* test, or if the normality test failed, by non-parametric ANOVA on ranks and Dunn's *post hoc* test. An overall P-value < 0.05 was considered significant. Values are given as mean ± standard deviation (SD) for parametric comparisons, or median and interquartile range (IQR) for non-parametric comparisons. Graphical presentations were created with GraphPad Prism 5 and the statistical analyses were performed using SigmaStat v3.1 software (Systat Software).

Results

Moderate contribution of thymic reactivation to immune reconstitution

We first evaluated the basic lymphocyte reconstitution after non-myeloablative AHSCT. Total absolute lymphocyte counts measured in whole blood were decreased up to the first year after treatment. The proportion of $CD4^+$ cells within the total T cell population remained reduced for the entire 2-year follow-up whereas no significant differences were detected in the percentages of $CD8^+$ T cells, hence CD4/CD8 ratios were persistently decreased (Supplementary Fig. 1 and Supplementary Table 3).

Previous work from our group and others has shown that AHSCT following myeloablative conditioning promoted increased output of *de novo* generated naïve T cells (Hakim *et al*., 2005; Muraro *et al*., 2005). We now examined immune reconstitution after non-myeloablative AHSCT through quantification of functional differentiation stages of T cells, including naïve ($T_{naïve}$), central-memory (T_{CM}), and effector memory (T_{EM}) cells (Fig. 1 A and Supplementary Table 3). Post-AHSCT the proportion of $T_{naïve}$ cells ($CD45RO^-CD27^+$) was unaltered in the $CD4^+$ subset and decreased in the $CD8^+$ subset (from mean 61 ± 14% at baseline to 33 ± 12% at 2 years, $P = 0.001$). Conversely, T_{EM} ($CD45RO^+CD27^-$) cells constituted a larger proportion of the total $CD8^+$ pool than at baseline (from mean 4.2 ± 3.0% to 26 ± 14% at 2 years post-therapy, $P < 0.001$). There was a trend to an increase of $CD4^+$ T_{EM} cells. These results suggested that T cell reconstitution during the first 2 years post-transplantation was predominantly driven by peripheral expansion.

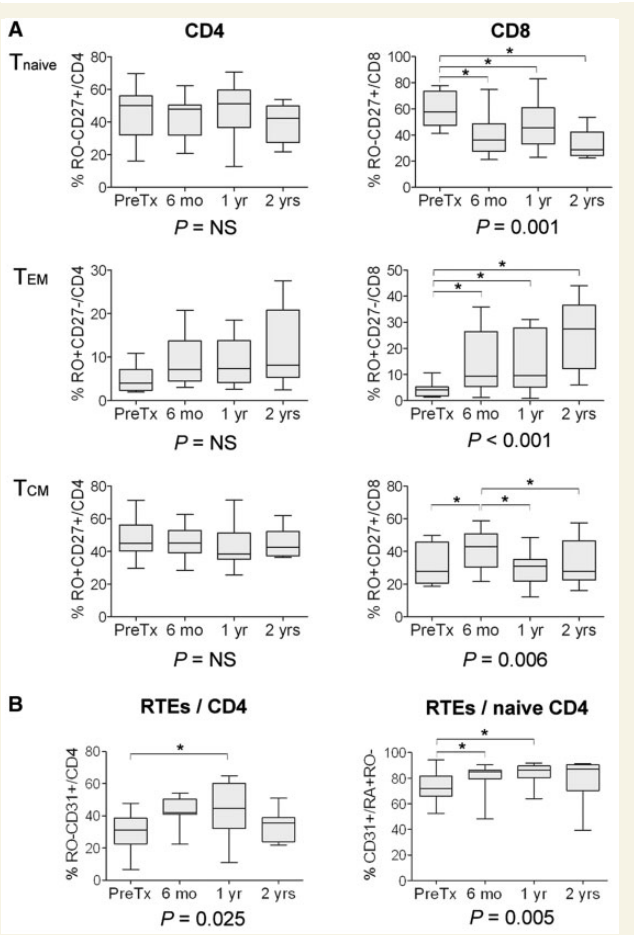

Figure 1 Moderate thymic reactivation during T cell reconstitution. The proportions of peripheral blood T cells in functional differentiation stages are shown at pretreatment baseline and at indicated time points post-AHSCT. (**A**) Reconstitution of naïve, effector memory (T_{EM}), and central-memory (T_{CM}) cell populations are expressed as the percentage of cells $CD4^+$ versus $CD8^+$ cells Statistical tests performed were repeated measures ANOVA (naïve $CD4^+$ cells and all $CD8^+$) and non-parametric ANOVA (CD4 T_{EM} and T_{CM}). (**B**) Reconstitution of recent thymic emigrant (RTEs) $CD4^+$ cells. The proportions of recent thymic emigrants ($CD31^+CD45RO^-$) in the total and in the naïve ($CD45RA^+CD45RO^-$) $CD4^+$ cell populations are increased after treatment. Statistical test performed was repeated measures-ANOVA. Pretreatment (PreTx) $n = 11$, 6 months (6 mo) $n = 10$, 1 year (1 yr) $n = 10$, 2 years (2 yrs) $n = 7$.

To ensure that effective thymic output was not masked by peripheral expansion (either antigen-driven or homeostatic) of naïve

cells resulting in conversion into effector/memory phenotypes, we enumerated naïve $CD4^+$ T cells expressing CD31, a marker of recent thymic origin (Kimmig *et al*., 2002). Such recent thymic emigrant naïve cells constituted a higher proportion of all $CD4^+$ cells at 1 year after therapy (from mean $30 \pm 12\%$ to $45 \pm 18\%$, a 50% increase, $P = 0.025$, Fig. 1B). Also the proportion of naïve $CD45RA^+CD45RO^-$ cells expressing CD31 was increased for up to the first year post-AHSCT (from mean $73 \pm 13\%$ to $84 \pm 9\%$, $P = 0.005$). These results show that during the first 2 years after non-myeloablative AHSCT the reconstitution of the circulating T cell pool is dominated by the expansion in the periphery of differentiated T cells acquiring effector cell phenotypes, with a moderate but significant activation of thymic output.

Immunoregulatory cell surge early post-therapy

To investigate the evolution of immunoregulatory cell populations post-therapy, we used in our analysis a number of markers specifically defining regulatory lymphocytes. At 6 months post-AHSCT there was a significant increase in the frequency of $CD25^{high}$ CD127-$FoxP3^+$ cells in the $CD4^+$ T cell subset [from median 0.11% (IQR 0.03–0.20) to median 1.5% (IQR 0.9–1.9), $P = 0.002$, Fig. 2A], as well as an expansion of $CD56^{high}$ natural killer cells [from median 0.5% (IQR 0.3–1.3] to 3.7% (IQR 2.8–5.1), $P = 0.001$, Fig. 2B). The frequency of both cell subsets subsequently returned to near baseline levels at 1 year after treatment. The changes in the absolute counts were not significant (Supplementary Table 3). These data show that the proportions of regulatory T and natural killer cells are increased post-AHSCT. Their transient increase may help modulate activated effector cells during the early stages of antigen re-experiencing (O'Gorman *et al*., 2009).

To assess whether the increased proportions of $CD4^+$ regulatory T cells were a result of increased homeostatic proliferation at 6 months, we assessed the expression of Ki-67, a cellular marker for proliferation. Whereas the percentage of Ki-67-expressing cells in the total $CD4^+$ T cell population increased at 6 months (from $2.2 \pm 1.1\%$ to $5.6 \pm 3.0\%$, $P = 0.035$; Supplementary Fig. 2A), the percentage of actively proliferating regulatory T cells, which was nearly four times that of the total $CD4^+$ T cell population at pretreatment baseline, did not change post-therapy (from $26 \pm 12\%$ to $20 \pm 9\%$, $P =$ not significant; Supplementary Fig. 2B). These results suggested that the increased relative frequency post-transplantation of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells was not due to an increase in their proliferation rate.

We were also interested in the potential contribution to treatment effect of $CD8^+CD57^+$ cells, a suggested immunoregulatory

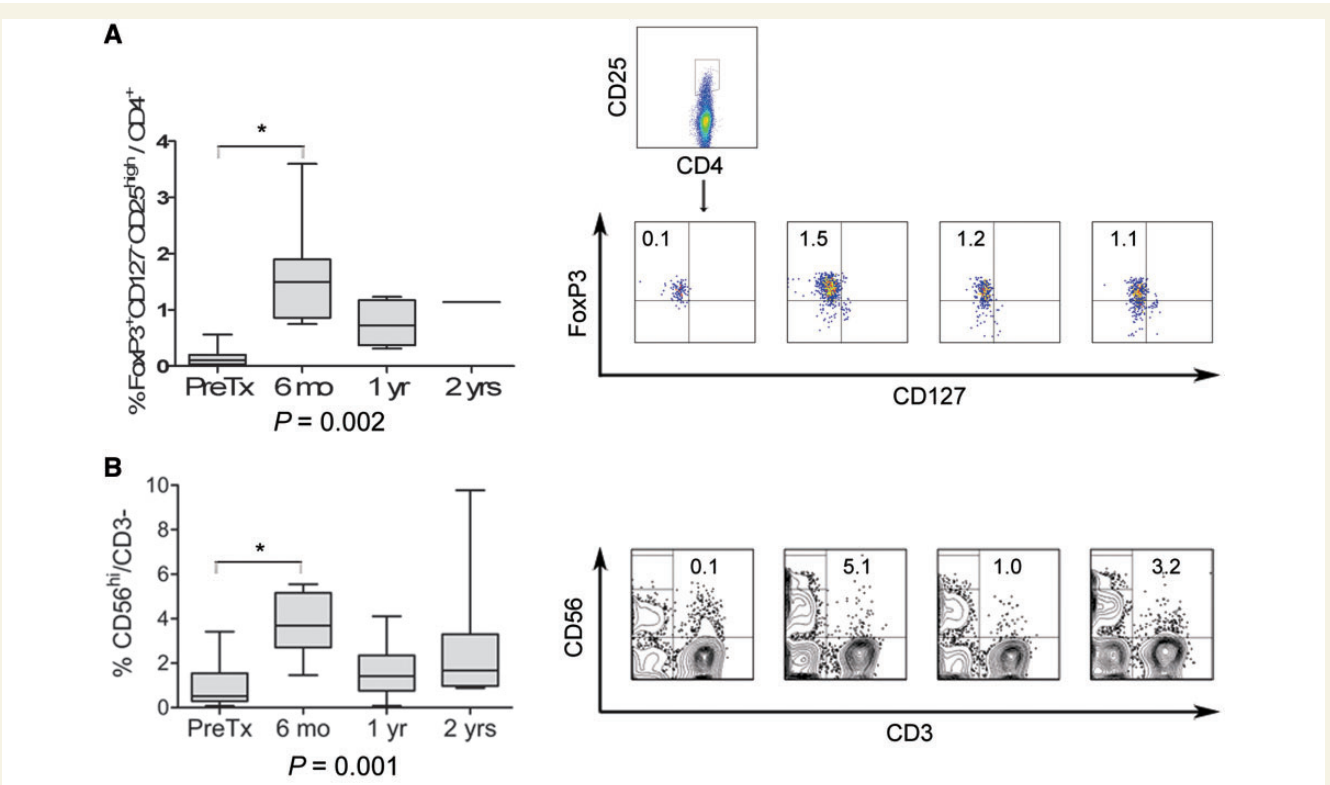

Figure 2 Post-therapy surge of lymphocytes with regulatory phenotype. A significant relative increase of cells with well-described immunoregulatory phenotypes in major lymphocyte populations ($CD4^+$ cells and natural killer cells) was detected at 6 months post-treatment follow-up. (**A**) The figure shows the gating strategy and the percentages of $FoxP3^+CD127^-CD25^{high}$ in the $CD4^+$ T cell population. The cells were first gated on live $CD25^{high}CD4^+CD3^+$ cells and then on the $FoxP3^+CD127^-$ populations. The numbers in the dot plots indicate the percentages of $FoxP3^+CD127^-CD25^{high}$ cells in the $CD4^+CD3^+$ population T cells. Pretreatment (PreTx) $n = 6$, 6 months (6 mo) $n = 6$, 1 year (1 yr) $n = 5$, 2 years (2 yrs) $n = 1$. (**B**) Percentage of $CD56^{high}$ natural killer cells. Pretreatment (PreTx) $n = 11$, 6 months (6 mo) $n = 10$, 1 year (1 yr) $n = 10$, 2 years (2 yrs) $n = 5$. Statistical test performed was non-parametric ANOVA.

population (Autran *et al*., 1991; Mollet *et al*., 1998) that is significantly increased in the periphery of patients with multiple sclerosis after treatment with myeloablative AHSCT (Muraro *et al*., 2005) as well as during treatment with glatiramer acetate (Ratts *et al*., 2006). $CD57^+$ cells occupied a significantly greater proportion of the $CD8^+$ T cell pool at all post-AHSCT time points when compared with the baseline before haematopoietic cell mobilization, reaching almost four times the baseline levels at 2 years post-transplantation (from mean $16 \pm 9\%$ to $59 \pm 13\%$ of all $CD8^+$T cells, $P < 0.001$; Supplementary Fig. 3A). $CD8^+CD57^+$ T cells after treatment were mainly effector memory cells (not shown) that produced high levels of IFN-γ, granzyme B (Supplementary Fig. 3B and C) and perforin (not shown). Their phenotype was therefore consistent with that of classic cytotoxic $CD8^+$ T cells (Chattopadhyay *et al*., 2009).

To investigate the potential immunoregulatory ability of $CD8^+CD57^+$ T cells, we carried out suppression assays in which CD8-depleted peripheral blood mononuclear cells (effector cells) were stained with the vital dye carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with increasing numbers of $CD8^+CD57^+$ and $CD8^+CD57^-$ ('regulatory cells') in the presence of a polyclonal (anti-CD3 antibody) stimulus. We then assessed the inhibition of $CD4^+$ T cell proliferation by measuring CFSE dilution in effector cells after 4 days in culture in the presence of both $CD8^+CD57^+$ and $CD8^+CD57^-$ cells (Supplementary Fig 3D). The degree of inhibition by $CD8^+CD57^+$ cells varied in different subjects and was either greater or equal to that of their $CD57^-$ counterpart at all 'regulatory' to 'effector' (R:E) ratios (Supplementary Fig. 3E). These experiments demonstrated that cytotoxic $CD8^+CD57^+$ T cells are massively increased in number after AHSCT and, although CD57 was not a marker for inhibition *per se*, the subset comprised in some patients cells with strong suppressive activity.

$CD161^{high}CD8^+$ are radically depleted after autologous haematopoietic transplantation and are invariant T cells associated with the gut mucosa

Extensive multi-colour fluorescence activated cell sorter phenotypic characterization of pretreatment $CD3^+CD8^+$ cells (data not shown) revealed a distinct $CD161^{high}CD8\alpha^{high/dim}$ population (Fig. 3A). $CD161^{high}CD8^+$ T cell populations were present in the

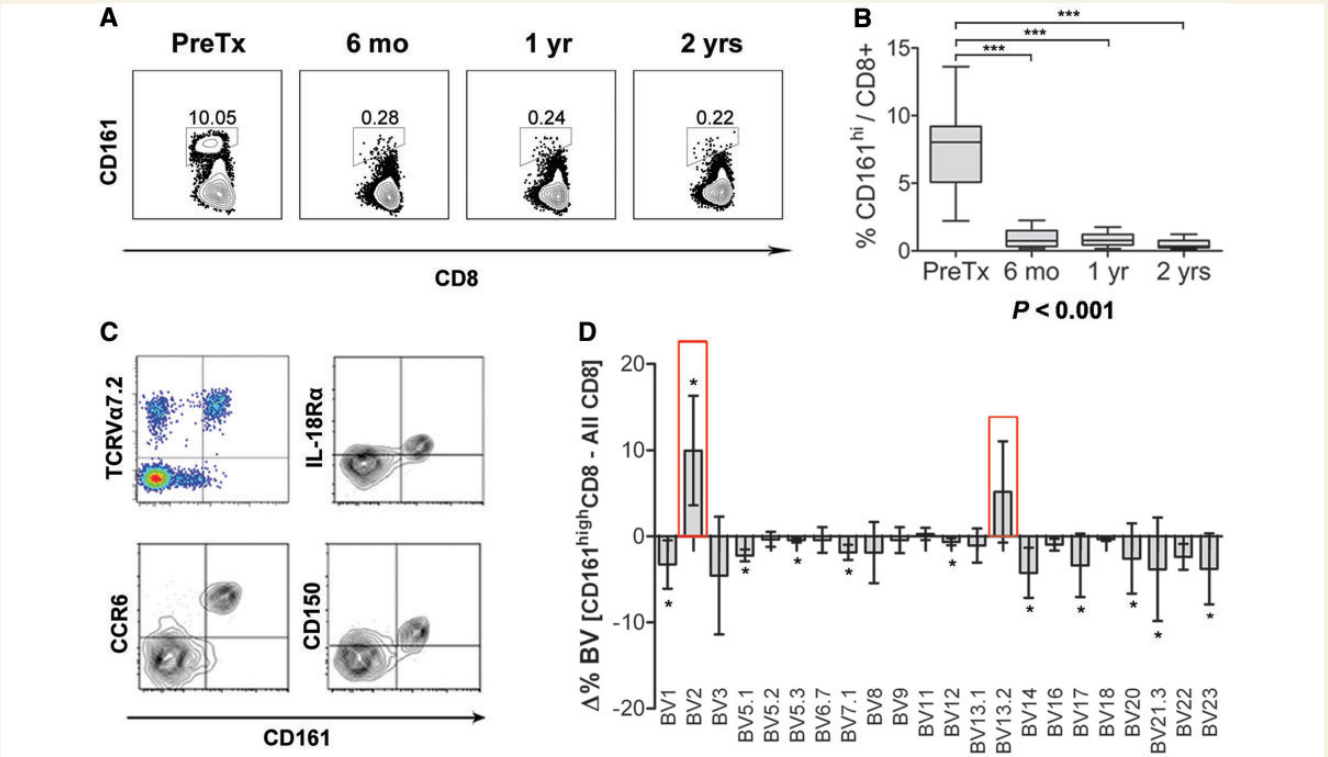

Figure 3 $CD161^{high}CD8$ are depleted following AHSCT and are MAITs. The $CD161^{high}$ cells represented on average ~8% of the $CD8^+CD3^+$ cell population and were almost undetectable after the therapy with AHSCT. (**A**) Representative example of CD161 expression by $CD8^+CD3^+$ cells in one patient at pre-therapy and at indicated time-points after AHSCT. (**B**) The box plots represent the proportion of $CD161^{high}$ $CD8^+CD3^+$ cells at pretreatment and at indicated time points after AHSCT. Pretreatment (PreTx) $n = 11$, 6 months (6 mo) $n = 9$, 1 year (1 yr) $n = 8$, 2 years (2 yrs) $n = 6$. (**C**) Representative example of MAIT markers expressed by $CD161^{high}CD8^+$ cells show an almost exclusive usage of TCR Vα7.2 and expression of IL-18Rα, CCR6, and CD150. (**D**) Difference in T cell receptor beta variable (BV) gene expression of $CD161^{high}CD8^+$ cells with reference to the total $CD8^+$ population [% BV ($CD161^{high}CD8$ – all CD8 cells)] shows a preferential usage of Vβ2 and Vβ13.2 by $CD161^{high}CD8^+$ cells (red boxes, $n = 7$). The bars indicate the mean and standard deviation. Asterisks indicate significant *P*-values (< 0.05, unpaired *t*-test). BV = T cell receptor beta variable (BV) gene.

blood of all the patients before treatment (mean 7.8 ± 3.2% of total $CD8^+CD3^+$ cells). Of note, this population was radically depleted after AHSCT, and remained nearly undetectable for the whole follow-up period of 2 years ($P < 0.001$; Fig. 3B).

High expression of CD161 in $CD8^{high/dim}$ T cells defines the MAIT subset (Dusseaux *et al.*, 2011), a $CD4^-$ T cell subset defined by the expression of innate immune receptors and usage of the semi-invariant TCR Vα7.2-Jα33 (Treiner *et al.*, 2003), with a preferential Vβ2 and Vβ13 gene expression (Tilloy *et al.*, 1999; Treiner *et al.*, 2005). We confirmed that >90% of the $CD161^{high}CD8^+$ T cell population in patients with multiple sclerosis expressed TCR Vα7.2 and that high expression of CD161 was associated with expression of IL-18Rα, CCR6 and the SLAM molecule CD150 (Fig. 3C), consistent with a recent defining description of MAITs (Dusseaux *et al.*, 2011). In contrast, a high expression of CD161 in the less frequent $CD4^-CD8^-$ T cell subset did not identify MAITs *per se,* as a large proportion of these $CD4^-CD8^-CD161^{high}$ cells did not express Vα7.2 (Supplementary Fig. 4A). The $CD161^{high}CD8^+$ subset was also enriched in cells expressing TCR Vβ2 (mean 15.4 ± 6.4% of $CD161^{high}CD8^+$) and Vβ13.2 (mean 7.3 ± 3.3%), consistent with MAITs, and readily appreciated when expressed as the difference of TCR expression in the $CD161^{high}CD8^+$ T cells compared with all $CD8^+$ T cells (Fig. 3D). The $CD161^{high}CD8^+$ T cell population showed a pattern of markers typical of antigen primed ($CD45RA^-CD45RO^+CD27^{+/-}CD28^+CD62L^-$) effector memory cells (Supplementary Fig. 4B).

$CD161^{high}CD8^+$ mucosal-associated invariant T cells are proinflammatory

To assess the cytokine profile of $CD161^{high}$, $CD161^{dim}$, and $CD161^-$ $CD8^+$ T cells, we carried out intracellular cytokine staining. $CD161^{high}CD8^+$ T cells from patients with multiple sclerosis before AHSCT produced the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 (Fig. 4 A), but not the immunoregulatory IL-10 (results not shown). Of all $CD8^+$ subsets, the $CD161^{high}CD8^+$ subset contained the highest frequency of IFN-γ (77 ± 4%, $P < 0.001$, Fig. 4B) and TNF-α producing cells (56 ± 12%, $P < 0.001$, Fig. 4C). The frequencies of $CD8^+$ T cells producing IL-17 tended to be higher in the $CD161^{high}$ (mean 1.76 ± 1.57% and 1.15 ± 1.4%, $P = 0.040$, Fig. 4D), but the analysis was not powered to detect the differences between the groups. However, the $CD161^{high}CD8^+$ subset had the highest frequencies of cells producing both $IL\text{-}17^+$ and IFN-γ (mean 0.82 ± 0.69%, $P = 0.002$, Fig. 4E).

When comparing the frequencies of $CD161^{high,}$ $CD161^{dim}$, and $CD161^-$ cells within the cytokine-producing $CD8^+$ T cells, the majority of the cells producing the widely expressed IFN-γ and TNF-α were $CD161^-CD8^+$ cells (that constitute 80–90% of the $CD8^+$ T cell pool). Despite their considerably smaller numbers, $CD161^{high}$ cells constituted half of the total $IL\text{-}17^+$ (mean 49 ± 28%) and $IL\text{-}17^+IFN\text{-}\gamma^+$ double positive cells (mean 51 ± 26%, Fig. 4F).

Together these data confirmed that the $CD161^{high}CD8^+$ T cells present in patients with multiple sclerosis before treatment and ablated post-therapy were proinflammatory effector MAITs, which contained a major fraction of IL-17 producing and IL-17/IFN-γ co-producing $CD8^+$ T cells.

Mucosal-associated invariant T cells are present in multiple sclerosis lesions

The almost exclusive expression of CCR6 on $CD8^+$ MAITs suggested their ability to enter the CNS (Reboldi *et al.*, 2009). We then investigated whether CD161-expressing MAITs were also present in multiple sclerosis lesions in post-mortem brain tissue from nine cases with high levels of inflammatory CNS infiltration. Staining with antibodies against CD8 and CD161 revealed the presence of double positive $CD161^+CD8^+$ cells within the inflammatory infiltrates of chronic active white matter lesions (Fig. 5A). Since distinction of the $CD161^{high}$ versus $CD161^{dim+}$ cells is not possible in tissue, we also stained for TCRVα7.2, which together with CD161 defines the MAIT population. We confirmed by dual immunofluorescent staining that CD161 and TCRVα7.2 double positive MAITs were indeed present in white matter lesion inflammatory infiltrates in all nine multiple sclerosis cases (Fig. 5B and C). The presence of MAITs in active white matter lesions in the multiple sclerosis brain suggested their involvement in multiple sclerosis pathogenesis, based on their cytokine profile, possibly as proinflammatory effectors.

Circulating mucosal-associated invariant T cell numbers are differentially affected by diverse immunotherapies

We next asked whether depletion of MAITs in the periphery was specific for the autologous haematopoietic transplantation protocol and to which extent other treatments for multiple sclerosis affected MAIT frequencies. We first confirmed the depletion of $CD161^{high}V\alpha7.2^+$ $CD8^+$ MAITs from the periphery of AHSCT patients (Fig. 6A). After AHSCT, the frequencies of $CD8^+$ MAITs decreased (from a mean 12.1 ± 7.4% of $CD8^+$ cells at baseline, to mean 0.6 ± 0.2% at post-therapy follow-up 2 years, $P < 0.001$, Fig. 6B). In contrast, the frequency of $CD161^{high}CD8^+$ cells, a good surrogate of $CD8^+$ MAITs (Supplementary Fig. 4A) in the blood of patients treated with IFN-β was unchanged 6 months after treatment (Supplementary Fig. 5).

We then set out to dissect the effects on MAITs *in vivo* from each of the two components of the immunosuppressing conditioning regime, cyclophosphamide and alemtuzumab, by studying peripheral blood mononuclear cell samples from patients who had been treated with either high-dose cyclophosphamide or alemtuzumab alone. We measured the frequencies of $CD8^+$ MAITs at pre-therapy baseline and at 2 years post-therapy in five patients who underwent immunosuppression with high-dose cyclophosphamide. An additional two patients had only pre- or post-therapy samples available. The $CD8^+$ MAITs were profoundly reduced in four of six patients, but persisted at high levels post-therapy in two subjects (Fig. 6C). We also measured MAIT frequency in patients who received treatment with alemtuzumab monotherapy ($n = 21$). Patient samples were taken at different time points

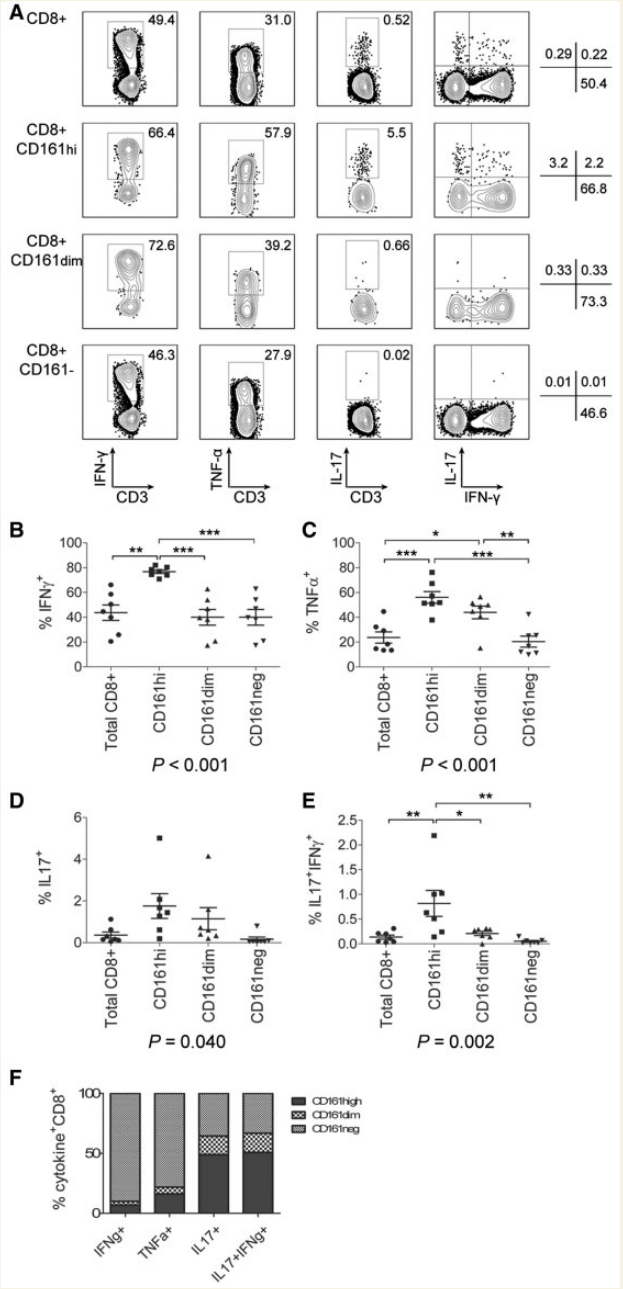

Figure 4 CD161^{high}CD8$^+$ cells are proinflammatory MAITs. Characterization of CD161^{high}CD8$^+$ cells in multiple sclerosis patients. (**A**) Representative example of cytokine production by CD8$^+$CD3$^+$ cells in function of CD161-expression. Peripheral blood mononuclear cells from patients before treatment were stimulated in culture with phorbol-12-myristate-13-acetate (PMA) and ionomycin after overnight recovery in cell incubator. The contour blots show the production of IFN-γ, TNF-α, and IL-17 by the total CD8$^+$CD3$^+$ populations, and by CD161^{high}, CD161^{dim} and CD161$^-$ CD8$^+$ T cells. (**B–E**) The scatter plots show the percentage of cytokine producing cells in the total CD8$^+$CD3$^+$ population, and in CD161^{high}, CD161^{dim} and CD161$^-$ CD8$^+$ T cells: (**B**) IFN-γ, (**C**) TNF-α, (**D**) IL-17, and (**E**) IL17$^+$IFN-γ^+ cells. (**F**) The distribution of CD161^{high}, CD161^{dim} and CD161^{neg} in the total cytokine-producing population is shown, and expressed as a percentage of cytokine$^+$CD8$^+$ T cells ($n = 7$). Statistical tests performed were non-parametric ANOVA.

after the last alemtuzumab infusion (range 2–38 months); however, the time seemed not to affect the CD8$^+$ MAIT frequencies within the sampled period as they did not correlate with the time after treatment (Fig. 6D).

When comparing AHSCT, high-dose cyclophosphamide and alemtuzumab monotherapy post-treatment samples, MAIT frequencies in both the CD8$^+$ (Fig. 6E) and the CD4$^-$CD8$^+$ (Fig. 6F) cell subsets, as well as in the total CD3$^+$ population (Fig. 6G) were low in all three groups, being lowest in the AHSCT-treated patients. MAITs in all subsets (CD8$^+$, CD4$^-$ and CD3$^+$) were significantly higher in the high-dose cyclophosphamide patient samples compared with both the AHSCT and alemtuzumab (monotherapy) treated patients (CD8$^+$ MAITs: $P = 0.011$; CD4$^-$ MAITs: $P = 0.004$; CD3$^+$ MAITs: $P = 0.002$). These results were driven by MAIT numbers remaining high in two of six high-dose cyclophosphamide patients. Whereas the mean frequencies were not statistically different between AHSCT and alemtuzumab treated patients (*t*-test, CD8$^+$ MAITs: $P = 0.09$; CD4$^-$ MAITs: $P = 0.12$: CD3$^+$ MAITs: $P = 0.07$), the variance between the two patient groups was different (*F*-test, CD8$^+$ MAITs: $F < 0.001$: CD4$^-$ MAITs: $F = 0.006$: MAIT/total CD3$^+$: $F = 0.012$), indicating that the frequencies of all MAIT populations were differently distributed in the two treatment groups, with a greater variability of MAIT numbers in the alemtuzumab monotherapy treated group.

Relationship of circulating mucosal-associated invariant T cell frequency with clinical response to treatment

Our study was not designed to ascertain if a correlation might exist between the degree of activity after the treatments. We attempted, however, to detect a potential association by stratifying patients according to post-therapy circulating CD8$^+$ MAIT frequency and examining their clinical course. As the putatively therapeutic range of depletion of MAITs is unknown, we applied statistical cut-off values to stratify patients in groups having low, intermediate and high post-therapy MAIT frequencies (Supplementary Fig. 6). The patients who underwent AHSCT had predominantly low (<0.8%) and none had high (>2.38) frequencies of circulating CD8$^+$ MAITs at any time point post-therapy. There were only two post-therapy samples that had intermediate (0.8–2.38%) CD8$^+$ MAIT frequency in the AHSCT cohort ($n = 12$) and they were the 12-month sample from Patient CC09 who relapsed at 16 months and the 6-month sample from Patient CC12 who relapsed at 6 months (CD8 MAIT frequencies 1.1% in both). Interestingly, CD4$^+$ T cells in the sample associated with relapse at 6-month follow-up showed a greatly enhanced proliferation to myelin basic protein (Supplementary Fig. 7). The remaining two patients who had a relapse after AHSCT (both at 12 months), however, had low MAIT frequencies at all the post-transplantation time points assessed. In the high-dose cyclophosphamide treated cohort ($n = 6$ with post-therapy samples) there were two patients (Patients HiCy1 and HiCy2) who had high MAIT frequency post-therapy (10.4% and 15.7%, respectively). Notably, they were also the two patients with highest MAIT frequency at the pretreatment baseline (13.8% and 17.0%,

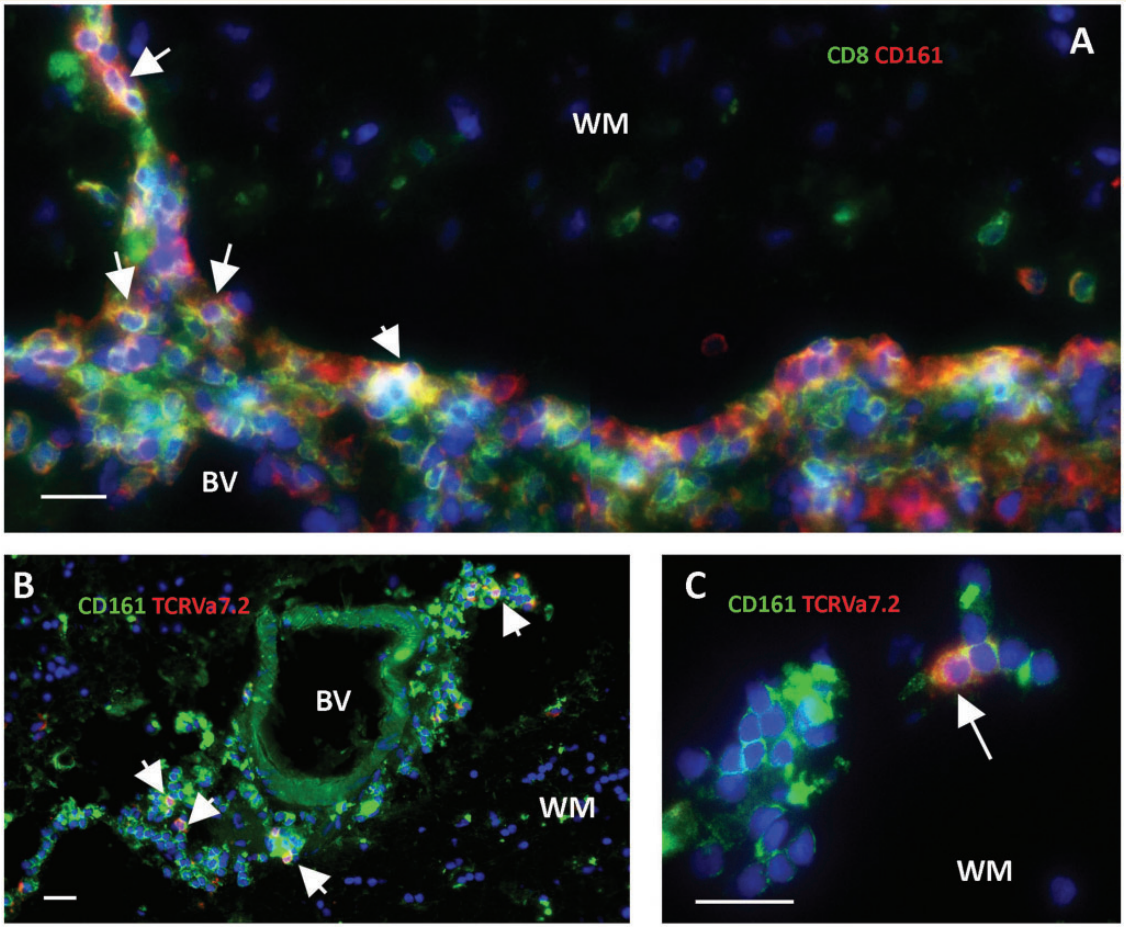

Figure 5 CD8$^+$CD161$^+$ and MAITs are present in perivascular infiltrates within chronic active lesions in the brain of patients with multiple sclerosis. (**A**) An example of CD8$^+$ (green) and CD161$^+$ (red) single and double positive cells (indicated by white arrows) within the inflammatory cellular infiltrates of chronic active white matter (WM) lesions in the brain of a patient with multiple sclerosis. (**B** and **C**) CD161$^+$ (green) and TCRVα7.2$^+$ (red) double positive MAITs are also found in white matter lesions inflammatory infiltrates. Blue (DAPI) stains all nuclei. All images were acquired at $\times$20 magnification except **C** at $\times$40. All scale bars = 20 μm. BV = blood vessel.

respectively), and the highest disability both pretreatment (Expanded Disability Status Scale 7) and post-treatment (Expanded Disability Status Scale 6.5 and 7, respectively) in the cohort (Supplementary Table 1). Of these two patients, one (Patient HiCy1) had a stable post-treatment course; the other (Patient HiCy2) had a high inflammatory activity in the CNS, with 20 gadolinium-enhancing lesions at Month 27 post-therapy. The clinical course post-therapy in high-dose cyclophosphamide treated patients with intermediate (Patients HiCy3 and HiCy4) or low (Patients HiCy6 and HiCy9) MAIT frequencies was variable (Supplementary Table 1). In the larger cohort of alemtuzumab-only treated patients (n = 18 after excluding three patients who were being treated with IFN-β at the alemtuzumab baseline, in order to avoid potential confounding effects on the clinical measures) a fair range of MAIT levels and of disease activity following therapy were documented (Supplementary Fig. 6A). Of note, the two patients with high frequency of CD8 MAITs after alemtuzumab treatment (Patients Alem10 and Alem20, 3.56% and 7.43% CD8 MAIT at 24 months and 12 months since last infusion of three courses of alemtuzumab, 80 months and 66 months since first infusion, respectively) had highly active multiple sclerosis before treatment (both had three relapses in the preceding 12 months), were at the high end of the range of disabilities at baseline within the cohort (Expanded Disability Status Scale 6 and 6.5, respectively) and relapsed during a long-term post-treatment follow-up (four relapses and one relapse; annualized relapse rate 0.6 and 0.18, respectively). Their disability, however, markedly improved after alemtuzumab treatment; Patient Alem10 had Expanded Disability Status Scale 2.5 and Patient Alem20 had Expanded Disability Status Scale 3.5 at their last assessments (at 80 months and at 74 months, respectively, since the first course of alemtuzumab). In the low- and intermediate CD8 MAIT frequency group there were four patients who had no relapses post-treatment (two and two, respectively); one or more relapses post-therapy over a variable duration of follow-up were documented in five of seven and eight of ten patients in these groups, respectively (Supplementary Fig. 6B–D).

Discussion

In this study we investigated the immune reconstitution in patients who underwent non-myeloablative conditioning AHSCT for

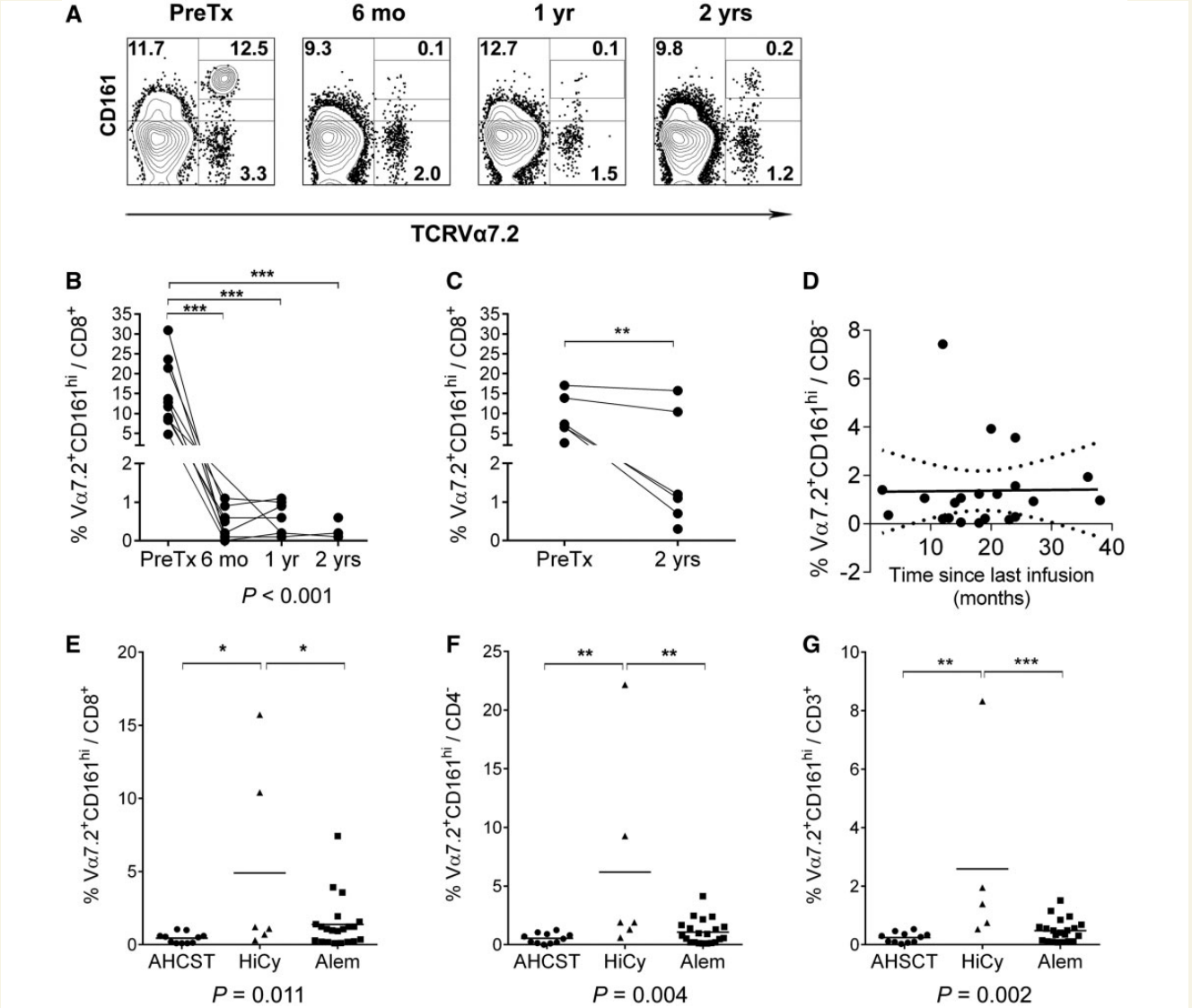

Figure 6 Effects of high-dose cyclophosphamide monotherapy and alemtuzumab monotherapy on MAIT frequency. MAITs defined by the expression of Vα7.2 and CD161 are reduced following AHSCT and reduced after monotherapy with high-dose cyclophosphamide (HiCy), and after alemtuzumab alone (Alem). (**A**) Representative dot plots gated on live $CD3^+CD8^+$ cells, showing expression of TCRVα7.2 on the *x*-axis and CD161 on the *y*-axis. (**B**) Frequencies of MAITs ($V\alpha7.2^+CD161^{high}$) in patients with multiple sclerosis before and after AHSCT. Note the segmented *y*-axis. Pretreatment (PreTx) $n = 10$, 6 months (6 mo) $n = 9$, 1 year (1 yr) $n = 7$, 2 years (2 yrs) $n = 3$. Statistical test was repeated measures-ANOVA. (**C**) Frequencies of MAITs in patients with multiple sclerosis before and after receiving high-dose cyclophosphamide. Patients with samples available at both time points are connected with a line. Note the segmented y-axis. Pretreatment (PreTx) $n = 6$, 2 years (2 yrs) $n = 6$. Statistical test performed was paired *t*-test. (**D**) Frequency of $CD8^+$ MAITs in peripheral blood mononuclear cells of patients ($n = 21$) collected at different time points after their last infusion of alemtuzumab. The middle line represents the mean and the sideline the confidence interval (95% onfidsence interval). (**E–G**) Comparison of MAIT frequencies in patients receiving AHSCT ($n = 11$, range 6–24 months post-therapy) monotherapy with either high-dose cyclophosphamide (HiCy, $n = 6$, 24 months post-therapy) or alemtuzumab (Alem, $n = 21$, range 2–38 after last infusion).

treatment of their highly active, conventional treatment-resistant forms of multiple sclerosis. We observed immunological changes that were consistent with a favourably redistributed balance of regulatory versus proinflammatory lymphocytes, resulting from the relative increase of cells with regulatory profile and the radical, virtually complete depletion of a $CD8^+$ T cell subset expressing high levels of CD161 and producing IFN-γ, TNF-α, and IL-17. Based on their chemokine and cytokine receptor profile as well as its semi-invariant T cell receptor rearrangement, we characterized $CD8^+CD161^{high}$ cells as MAITs and demonstrated their presence in brain white matter lymphomononuclear cell infiltrates in post-mortem multiple sclerosis white matter tissue, supporting its potential pathogenic relevance.

Different AHSCT conditioning regimens have been explored for treatment of aggressive forms of immune-mediated disorders. Non-myeloablative regimens have been proposed for two main

reasons: (i) the treatment should primarily target the lymphoid, not the myeloid compartment; and (ii) improved safety and tolerability facilitate treating patients during the appropriate window of therapeutic opportunity at earlier stages of disease (Burt *et al*., 2008). A recent clinical trial showed that non-myeloablative AHSCT can arrest or reverse neurological deterioration in patients with highly active, treatment-resistant relapsing-remitting multiple sclerosis with acceptable safety (Burt *et al*., 2009). Here we investigated key aspects of immune reconstitution in 12 patients, who had all received the same (cyclophosphamide/alemtuzumab) non-myeloablative conditioning regimen in that trial.

Analysis of the naïve and memory T cell repertoire after non-myeloablative conditioning AHSCT revealed a markedly different quality of immune reconstitution when compared with our previous study of a myeloablative conditioning regimen using cyclophosphamide and total body irradiation (Muraro *et al*., 2005). Whereas naïve T cells following cyclophosphamide and total body irradiation had a biphasic reconstitution with initial depletion followed by recovery and increase to twice their baseline frequency (Muraro *et al*., 2005), in the present study the frequencies of naïve $CD4^+$ T cells and of naïve $CD8^+$ T cells were unchanged and reduced throughout the post-therapy follow-up. We detected, however, a moderate but statistically significant degree of $CD4^+$ recent thymic emigrant expansion during the first year of follow-up, demonstrating a degree of reactivation of the thymus. Together, these results suggest that the non-myeloablative conditioning regimen that we investigated in our study induced a less extensive replacement of the mature T cell repertoire than the previously reported myeloablative regimen. We have used CD31 as an established marker to enumerate T cells recently differentiated from haematopoietic stem cells; however, these methods cannot distinguish whether the haematopoietic stem cells were survivors from the bone marrow or from the infused autologous graft. Since a non-myeloablative conditioning protocol by definition causes minimal bone marrow suppression, it is likely that haematopoietic stem cells from both the patient's bone marrow and the graft contribute to immune reconstitution.

These results prompted us to hypothesize that the therapeutic effect of AHSCT on CNS inflammation in multiple sclerosis may not require a complete renewal of the T cell repertoire but could be mediated by the normalization of a balance between pro-inflammatory and immunoregulatory cells. Abnormalities in the number or function of $CD4^+$ regulatory cells have been previously reported in multiple sclerosis (Viglietta *et al*., 2004; Venken *et al*., 2008). Our longitudinal analysis of regulatory T cell frequency showed a significant surge in $CD4^+CD25^+CD127^-FoxP3^+$ cell proportions relatively early (6 months) post-transplantation. These results are consistent with previous work in juvenile idiopathic arthritis and in systemic lupus erythematosus, suggesting that recovery of $CD4^+CD25^{high}$ T cells might play a role in the mode of action of AHSCT (de Kleer *et al*., 2006; Alexander *et al*., 2009). Expansion of $CD4^+$ regulatory T cells has also been shown after alemtuzumab monotherapy (Cox *et al*., 2005) leaving open the possibility that increased regulatory T cell numbers are part of a common response following intensive lymphodepletion.

We considered the possibility that the transiently increased numbers of regulatory T cells after AHSCT were the result of a boosted homeostatic proliferation at 6 months. Regulatory T cells had a higher proliferation than the whole $CD4^+$ population at baseline and there was no change at 6 months post-therapy, despite that the total $CD4^+$ patient population was proliferating much more actively than at baseline. These results suggest that increased active proliferation did not account for the higher numbers of regulatory T cells post-transplantation; rather, it appears that regulatory T cells either are more resistant to the immunosuppressive regime or they recommence to be exported from the thymus and steadily proliferate in the periphery, with a gain of their relative proportion in the lymphopenic $CD4^+$ compartment, which is likely to give them a regulatory advantage over re-emerging effector T cells.

We also describe a transient increase of $CD56^{high}$ natural killer cells, a natural killer cell subset with immunoregulatory potential (Jiang *et al*., 2011). $CD56^{high}$ natural killer cells have been reported to expand and mediate therapeutic effects in patients with multiple sclerosis treated with daclizumab (Bielekova *et al*., 2006) and IFN-β (Vandenbark *et al*., 2009). We speculate that the early expansion of $CD4^+$ regulatory T cells and regulatory $CD56^{high}$ natural killer cells may be important for the control of the immune system during the early antigen priming of re-emerging lymphocytes, as previously suggested (O'Gorman *et al*., 2009).

Next, we focused our analyses on the functional significance of different types of phenotypic $CD8^+$ T cell effector memory cell subpopulations, which demonstrated significant changes in their frequencies in pre- to post-AHSCT blood samples. We have previously reported a prominent increase of $CD57^+CD28^-CD8^+$ T cells, a subset of $CD8^+$ T cells with proposed suppressor function (Mollet *et al*., 1998), after myeloablative AHSCT (Muraro *et al*., 2005). We have reproduced and extended this observation in the present study of non-myeloablative AHSCT. $CD8^+CD57^+$ T cells were significantly and persistently increased throughout the post-transplantation follow-up. Functional characterization of $CD57^-$ and $CD57^+$ CD8 T cells through co-culture suppression assays showed that although both populations contained cells with suppressive activity, the suppressive effect of $CD57^+CD8^+$ cells was variable and either much greater or equal to their $CD57^-$ counterpart. This variability suggested that CD57 may not be *per se* a marker defining immunoregulatory $CD8^+$ T cells. Interestingly, we observed that ILT2, a marker associated with regulatory function, is expressed by a majority of $CD57^+CD8^+$ cells and is differentially regulated during the post-AHSCT follow-up (data not shown). Further work is required to define the key determinants of regulatory activity of cell populations within the $CD8^+CD57^+$ subset.

Our detailed characterization of $CD8^+$ T cell reconstitution included several natural killer markers known to be expressed on T, natural killer T and natural killer-like T cells (data not shown) and revealed that a $CD161^{high}CD8^+$ T subset, readily detectable in all patients before treatment, was radically depleted after AHSCT. We also demonstrate that >90% of the $CD161^{high}CD8^+$ cell population in multiple sclerosis patients express TCRVα7.2 and IL-18Rα, and therefore represent MAITs, a subset recently described for their antimicrobial activity (Le Bourhis *et al*., 2010; Kjer-Nielsen *et al*., 2012). MAITs have been described as non-cycling, tissue-targeted cells that secrete IL-17 and express high

levels of the multiple drug transporter protein ABCB1, which confers them resistance to ABCB1-effluxed chemotherapy (Dusseaux *et al*., 2011). We confirmed that high expression of CD161 defines a proinflammatory $CD8^+$ memory MAIT population that produces IFN-γ and contains the IL-17 producing $CD8^+$ T cells in patients with multiple sclerosis, in agreement with recent studies describing $CD161^{high}$ cells as a chemotherapy-resistant and tissue-homing proinflammatory $CD8^+$ subpopulation (Turtle *et al*., 2009; Billerbeck *et al*., 2010). These MAITs likely possess some plasticity depending on the inflammatory environment; it was recently shown that their IL-17 production was enhanced by co-stimulation with IL-1β, whereas presence of IL-12 induced a Tc1-like function (Turtle *et al*., 2011). Our description of a post-treatment depletion of $CD8^+$ T cells that produce IL-17 (Tc17) is novel and complementary to recent data from a study of AHSCT employing a high-intensity immunosuppressive protocol. Darlington *et al*. (2013) demonstrate that the capacity to mount Th17 responses is diminished post-therapy, although the Th1 responses remain unaltered. Together, their study and ours suggest that alterations of the Th17 and of the Tc17 pathways might be critically involved in the therapeutic mechanism of AHSCT in multiple sclerosis.

The demonstration that MAITs are pro-inflammatory contrasts with the interpretation of Miyazaki *et al*. (2011) who despite describing MAITs as a population that secrete high levels of proinflammatory cytokines IL-17 and IFN-γ, interpret them as immunoregulatory cells able to suppress Th1 responses in multiple sclerosis. We believe that without supportive data from intracellular cytokine staining assays it is difficult to ascertain whether MAITs truly inhibit IFN-γ production from T cells. The interpretation of results from their co-culture system is made problematic by the fact that 'MAIT-depletion' in their co-culture assays was performed by depletion of CD161 expressing cells, and thus would have depleted not only $CD161^{high}$ MAITs, but also the widely present $CD161^{dim}$ effector CD4 (IFN-γ and IL-17 producing cells, and even some regulatory populations), cytotoxic CD8 T cells including $CD57^+CD8^+$ cells, and natural killer cells. After the depletion of several functionally important cell subsets, it is difficult to define the effect of any one specific cell population. In our study, by intracellular cytokine staining and selective gating on CD161high, -dim and -negative cells we demonstrate production of IL-17, IFN-γ and TNF-α and no production of IL-10 by $CD161^{high}$ cells, consistent with a pro-inflammatory function and in agreement with a majority of other studies (Billerbeck *et al*., 2010; Annibali *et al*., 2011; Dusseaux *et al*., 2011; Walker *et al*., 2012).

The involvement of IL-17 producing $CD161^{high}CD8^+$ T cells in multiple sclerosis pathology is plausible when considering that the majority (>70%) of CD8 T cells in acute and chronic active multiple sclerosis lesions were reported to express IL-17 (Tzartos *et al*., 2008). The potential relevance of $CD161^{high}CD8^+$ cells in multiple sclerosis was recently underpinned by the study by Annibali *et al*. (2011) that showed that the expression of *KLRB1*, the gene coding for CD161 and one of the non-major histocompatibility complex risk alleles with the highest statistical association to multiple sclerosis (Hafler *et al*., 2007), was increased in affected monozygotic twins as compared to their healthy co-twins. In that study, the frequency of $CD161^+$ cells in the $CD8^+$ T cell subset was significantly increased in the blood of patients with multiple sclerosis when compared with healthy controls. Furthermore, $CD161^+CD8^+$ T cells were detected amongst tissue-infiltrating cells in post-mortem multiple sclerosis brain tissue (Annibali *et al*., 2011).

Here, by examining immune cell infiltrates in post-mortem multiple sclerosis brain white matter tissue from cases with high levels of inflammation we confirm that MAITs are present in white matter lesions, as predicted from their tissue-homing receptor profile. In a separate study extensively characterizing $CD161^+$ lymphocytes in multiple sclerosis tissue we have quantified the number of MAITs in the white matter as well as in the meningeal inflammatory infiltrates, where they represented 17% and 8% of total $CD161^+$ cells, respectively (Carassiti *et al*., manuscript submitted). These data extend the previous demonstration of the TCRVα7.2Jα33-transcript in white matter lesions (Illes *et al*., 2004), and their frequency in highly active cases' lesions suggest that MAITs are probably implicated in the development of CNS immune-mediated injury in multiple sclerosis.

$CD161^{high}CD8^+$ MAITs express CCR6, a receptor that is involved in transmigration of T cells into the CNS and in the initiation of experimental autoimmune encephalomyelitis (Reboldi *et al*., 2009). CD161 itself also plays a role in trans-endothelial migration of T cells (Poggi *et al*., 1997). MAITs produce high levels of inflammatory cytokines IFN-γ and TNF-α, and are the highest $CD8^+$ producers of IL-17; IL-17R is expressed on blood–brain barrier epithelial cells in multiple sclerosis lesions and IL-17 increases the permeability of the barrier (Kebir *et al*., 2007). Furthermore, the commensal gut flora has been shown to enhance the IL-17 response and to be required for the development of myelin-specific autoimmunity in an experimental model of demyelination (Berer *et al*., 2011), which corroborates the implication of gut immunity in autoimmune disease. Taken together, these studies and our own data strongly suggest an important role for $CD161^{high}CD8^+$ MAITs in multiple sclerosis pathogenesis.

It was, therefore, of great interest to consider to which extent the two principal components of the immunoablative chemo–biological therapy conditioning regimen, high-dose cyclophosphamide and alemtuzumab contributed to the observed depletion of MAITs. We hence obtained peripheral blood mononuclear cell from patients treated in a protocol using high-dose cyclophosphamide at Johns Hopkins University, USA; and in a protocol of alemtuzumab at the University of Cambridge, UK. There was no post-therapy depletion of MAITs in two of six analysed high-dose cyclophosphamide treated patients. The frequency of MAITs peripheral blood mononuclear cells obtained from patients treated with alemtuzumab was not statistically different from AHSCT treated patients although the significantly greater variance suggested that the frequency was higher in some alemtuzumab monotherapy-treated individual. This variance was independent of the time since the last treatment infusion. Our data suggest that both high-dose cyclophosphamide and alemtuzumab alone have the potential to deplete circulating MAITs but larger numbers of cells may escape depletion from either treatment alone than from autologous haematopoietic stem cell transplantation with a conditioning regimen that includes both cyclophosphamide and alemtuzumab. Based on our data we speculate that alemtuzumab may account

for the majority of the MAIT-depleting effect, but that the combination of alemtuzumab with cyclophosphamide in the transplantation conditioning regime (which was also preceded by a few weeks by the use of cyclophosphamide for haematopoietic cell mobilization, which may contribute to the ablative effect) may have additive or synergistic effects, resulting in a more complete depletion.

Differentiating responders and non-responders to a given treatment and measuring potentially relevant biological variables might allow us to gain valuable insight into its mechanism of action. Dichotomizing clinical responses to therapy, however, is not always straightforward. Clinical and MRI follow-up of the AHSCT patients showed complete disease remission post-therapy in 8 of 12 patients. Four patients had a single relapse during the first 2 years post-transplantation (versus two relapses each in the 12 months before transplantation) yet at the end of the $\geqslant$24 month follow-up post-AHSCT the Expanded Disability Status Scale scores of all relapsing patients were either improved ($n = 3$) or unchanged ($n = 1$) compared to pretreatment baseline, suggesting that their multiple sclerosis course had been stabilized or at least attenuated. For the interpretation of our immunological studies, therefore, we felt more appropriate to regard the clinical outcomes in these four patients as partial/incomplete responses rather than treatment failure, and we show here the immunological results from all patients conjointly. We applied the same consideration to the other treatments. Indeed, stratification analysis showed no statistically significant differences between the complete and partial responders groups in any of the immune parameters being investigated (data not shown), including recovery of $CD4^+$ T cell numbers and CD4/8 ratios that were recently described as surrogate marker of treatment response to alemtuzumab (Cossburn *et al*., 2013), although we cannot rule out that some differences might have been detected if larger numbers of patients had been available.

Based on the pro-inflammatory profile of MAITs, their radical post-therapy depletion of MAITs observed after AHSCT and the more variable depletion observed after other treatments we hypothesized that their frequency might align most closely with therapeutic efficacy. Our study had not been designed to examine a correlation of MAIT numbers and clinical or imaging response to treatment and was statistically underpowered to detect such potential correlations. Only a descriptive analysis of a potential association, therefore, was performed. In the AHSCT cohort, it was of note that the only two post-therapy samples in which $CD8^+$ MAITs frequency exceeded 1% (1.1% in both) were from two time points of two patients who subsequently had a relapse. Interestingly, we detected enhanced T cell reactivity to myelin basic protein at one of these time points. In the high-dose cyclophosphamide cohort, one of two patients with high (>10%) $CD8^+$ MAIT frequency post-therapy had an inflammatory flare with high number of enhancing lesions; the other patient remained stable. In the alemtuzumab cohort, the two patients with high $CD8^+$ MAIT frequency post-therapy (3.6% and 7.4%) both had relapses post-therapy, although their annualized relapse rate post-treatment was much lower than in the 12 months preceding treatment with alemtuzumab. Interestingly, all four patients with high MAIT frequency post-therapy in the high-dose cyclophosphamide, and in the alemtuzumab cohorts, had high disability levels pretreatment, although disability improved (even if relapses occurred) in the two patients who received alemtuzumab, which has been suggested to exert neuroprotective effects *in vivo* based on the demonstration of induction of neurotrophic mediators *in vitro* (Jones *et al*., 2010).

Although these observations are intriguing and support the implication that MAITs are active perpetrators of inflammatory disease activity in multiple sclerosis, it should be noted that in all three cohorts (AHSCT, high-dose cyclophosphamide and alemtuzumab), there were patients with low circulating MAITs who had relapses or MRI activity post-therapy; and there was one case (Patient HiCy1) who had high circulating MAIT frequency yet remained clinically stable. These observations suggest that there is no exclusive association of low frequency of circulating MAITs with disease remission; or exclusive association of high frequency of circulating MAITs with disease relapse. The results, therefore, should be interpreted with caution. Future studies in larger numbers of patients, with prospective enumeration of MAITs and clinical monitoring before and during treatment are warranted to establish if a correlation of MAIT number and clinical course exists. These studies could address the hypotheses generated from the present study that MAITs are implicated as detrimental inflammatory mediators of disease and may represent a biomarker of treatment response in multiple sclerosis.

In conclusion, our data show significant qualitative and functional changes in the reconstituted immune response following the non-myeloablative AHSCT regimen. Data on differentiation factors describing a reciprocal relationship between regulatory T cells and Th17 cells (Bettelli *et al*., 2007; Mucida *et al*., 2007) suggest that the balance between regulatory and pro-inflammatory cells is reset by tightly controlled processes that coordinate functional differentiation of lymphocytes during immune reconstitution. Indeed, following AHSCT we demonstrate increased numbers of circulating cells with regulatory potential as well as the effective depletion of a pro-inflammatory IL-17, TNF-α and IFN-γ-producing $CD8^+$ cell subset, which corresponds phenotypically and functionally to the recently described gut-derived MAIT population. We further show that $CD8^+$ MAITs, which express CCR6, infiltrate multiple sclerosis lesion tissue. Taken together, our results suggest that $CD8^+$ MAITs might be involved in multiple sclerosis as inflammatory mediators and could represent a disease and a treatment biomarker as well as, potentially, a therapeutic target.

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Supplementary material

Supplementary material is available at *Brain* online.

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