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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ABSTRACT

Multiple sclerosis is an inflammatory disease of the central nervous system. 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 utilizing an attenuated cytotoxic chemotherapy to achieve lymphocytic ablation without marrow suppression have been proposed to improve safety and tolerability. A recent clinical trial with non-myeloablative autologous haematopoietic stem cell transplantation reported clinical improvement and inflammatory stabilization in treated patients with highly active multiple sclerosis. 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 two 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} NK cell subsets, which are cell subsets associated with immunoregulatory function. Proportions of CD8^{+}CD57^{+} cytotoxic T cells were persistently increased after therapy and were able to suppress CD4^{+} T cell proliferation \textit{in vitro} with variable potency. In contrast, a CD161^{high} proinflammatory CD8^{+} T cell subset was virtually ablated at all time-points post-transplantation. The expression of T cell receptor Va7.2 and IL-18RA 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 central nervous system-infiltrating mucosal-associated invariant T cells in post-
mortem multiple sclerosis brain white matter active lesions confirmed their involvement in the disease pathology. Characterization of this T cell subset by intracellular cytokine staining demonstrated IFN-γ and IL-17 production and lack of IL-10 production, demonstrating a pro-inflammatory cytokine profile. Mucosal-associated invariant T cell frequency did not change after interferon-β treatment; and was more profoundly depleted after autologous haematopoietic stem cell transplantation than in patients who had received high-dose cyclophosphamide or alemtuzumab 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.
INTRODUCTION

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease of the central nervous system (CNS) (Compston and Coles 2008). Current immune-modifying treatments are incompletely effective in patients with aggressive MS phenotypes. Autologous bone marrow- or haematopoietic stem cell transplantation (AHSCT) has been experimented in patients with aggressive forms of MS, 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 (Alexander et al. 2009; de Kleer et al. 2006; Zhang et al. 2009). In addition, a very recently published study by Darlington showed abrogation of the Th17 response following high-intensity AHSCT (Darlington et al. 2012). 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 recently proposed in order 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 utilizing a non-myeloablative conditioning regimen in highly active MS patients, 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 utilizing
cyclophosphamide and alemtuzumab for immunosuppressive conditioning, there were significant transient increases of CD4+CD25^{high}FoxP3^{+} T cells and of CD56^{high} NK 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 \textit{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 whole 2-year post-AHSCT follow-up. Further characterization of the CD161^{high}CD8^{+} T cell population found in MS patients’ blood pre-AHSCT revealed that these cells are mucosal-associated invariant T (MAIT) cells, a T cell subset associated with the gut (Dusseaux \textit{et al.} 2011; Le Bourhis \textit{et al.} 2010). High CD161-expression defined a subset of pro-inflammatory T cells that includes the majority of IL-17 producing CD8^{+} T cells and also produces IFN-\gamma and TNF-\alpha but not IL-10. We confirmed that MAIT cells are able to migrate to the brain as they were present in the white matter and perivascular infiltrate of post-mortem MS brain tissue. Comparison of MAIT frequency in patients that received other MS 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 two years post-therapy.

The data demonstrate that the adaptive immune system reconstituted in patients with MS 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 cell population, which produces IFN-\gamma and IL-17, bears a pro-inflammatory profile and is able to infiltrate the central nervous system.
SUBJECTS, MATERIALS AND METHODS

Subjects, treatment and biological samples

Patients with aggressive MS who failed to respond to licensed immune-modifying treatments were recruited for an IRB-approved clinical trial of non-myeloablative immunosuppressive conditioning with cyclophosphamide and alemtuzumab and AHSCT at Northwestern University, Chicago, IL (Burt et al. 2009). Following informed consent, peripheral blood for the research study was donated by subjects with MS 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 cyclophosphamide 120 mg/kg and alemtuzumab 20 mg. Peripheral blood samples were obtained from the AHSCT patients at baseline within one month before haematopoietic stem cell (HSC) mobilization, and at 6 months, 1 and 2 years post-HSC reinfusion.

PBMC samples for comparative immunological analysis were obtained from non-AHSCT MS patients (n = 40), and healthy individuals (n = 7). Of the non-AHSCT MS patients, 5 were untreated and 7 received standard disease-modifying treatment with interferon-beta (IFN-β): Betaferon® (n = 5), Betaseron® (n = 1), or Avonex® (n = 1). The 5 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 (HiCy, n = 7). Patients in this study received 50 mg/kg/d of cyclophosphamide intravenously for 4 consecutive days, followed by 5 µg/kg/day of granulocyte colony–stimulating factor 6 days after completion of HiCy treatment, until the absolute neutrophil count exceeded $1.0 \times 10^9$ cells/L for 2 consecutive days. The protocol has been described in detail elsewhere (Krishnan et al. 2008). Of the 7 patients, 5 had pretreatment baseline and post-treatment samples over a two 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 randomised controlled trial) or CARE-MS1 or CARE-MS2 (Phase 3 randomised controlled trials). Alemtuzumab was given for 5 days at baseline then for 3 days at month 12 (12 or 24mg/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 (PBMCs) 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).

Flow cytometry

PBMCs were thawed in Dulbecco’s PBS and washed in FACS staining buffer (D-PBS, 1% FBS and 0.01% sodium azide). Surface staining was performed on ice for 20 minutes and the cells were then analyzed on a 2-laser, 4 colour FACSCalibur flow cytometer, or for multicolour analysis on a 5-laser, 18 colour LSRFortessa (Becton Dickinson, Franklin Lakes, NJ). Data were analysed using CellQuest (Becton Dickinson) and FlowJo software (TreeStar, Ashland, OR).

Immune reconstitution and phenotyping studies: Multiplexed dilutions of monoclonal antibodies (mAbs) were used to characterize lymphocyte populations. The following antibodies from BD Biosciences (Oxford, UK) 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, CD62L-PE, CD69-FITC, CD95-PE, CD161-FITC (clone DX12), and TCRαβ-FITC.
Other mAbs included CD49d-FITC and ILT2-PE (clone HP-F1) from Beckman Coulter (High Wycombe, UK).

**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 staining was performed according to the manufacturer’s instructions using Foxp3 AlexaFluor647 (clone PCH101) and Ki-67 PerCP-eFluor710 (eBioscience). Blue Live/Dead Stain (Life Technologies, Grand Island, NY) was added to the samples before Foxp3 staining to for live versus dead cells discrimination.

**Characterization of MAIT 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, San Diego, CA); CD161-PE and CD161-APC (clone 191B8) from Miltenyi Biotec (Gladbach, Germany); CD3-PE, ILT2 (CD85j)-APC, ILT2-PE, CD150 PE, and CD218 (IL-18R) FITC, from eBioscience (San Diego, CA); CCR6-PE from R&D Systems (Abingdon, UK), 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 TCRV expression:** TCRVα7.2-FITC and TCRVα7.2-PE (clone 3C10) were obtained from Biolegend. For TCRBV usage CD8⁺ T cells, PBMCs 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 Abs 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
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 (AlexaFluor647) were purchased from BD Biosciences.

Cytokine Production Assay

PBMCs were stimulated for 5 hours with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml) and ionomycin (1µg/ml) in the presence of brefeldin A (10 µg/ml, all from Sigma-Aldrich, Dorset, UK), ex vivo after overnight recovery in cell incubator with RPMI-1640 with 10% FBS. 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 AlexaFluor647 (eBioscience).

Suppression assays

PBMCs were thawed and left to recover overnight in RPMI-1640 with 10% FBS and 20 U/ml of IL-2. The following day, CD8+CD57+ and CD8+CD57- cells were obtained from PBMC using a magnetic microbead kit from Miltenyi Biotec. The percentage of NK cells found in all cases was ≤5%. The CD8-depleted fraction was stained with 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 Ab (OKT3, 0.5 µg/ml, eBioscience). On day 4, the cells were washed and stained. Propidium iodide (PI, 1 µg/ml) was used
for dead cell exclusion. The percentage of cell proliferation was quantified on live (PI-) 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) * 100, and % suppression was defined as (100 - % proliferation).

**Immunostaining of MS 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 via 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 9 progressive MS cases with high levels of CNS inflammation that formed part of a larger, well described case series (Howell et al. 2011). Tissue blocks (4 per case) cut at 10µm, 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, Oxford, UK) 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, Burligame, CA) followed by 30 min incubation with tyramine (Sigma) in PBS containing 0.03% H2O2 according to a modified version of a method described by Adams (Adams 1992). The negative control consisted in the same protocol without incubation with tyramine. After 1h incubation with AlexaFluor546-Streptavidin (Invitrogen, Eugene, OR) sections were washed, blocked with normal serum and incubated overnight at 4°C with the second primary antibody that was then visualized with an AlexaFluor488 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 analysis of variance (RM-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 ± 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, Chicago, IL).
RESULTS

Moderate contribution of thymic reactivation to immune reconstitution

We first evaluated 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 two-year follow-up whereas no significant differences were detected in the percentages of CD8\(^+\) T cells, hence CD4/CD8 ratios were persistently decreased (Supplementary figure 1, 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\(_{\text{naïve}}\)), central-memory (T\(_{\text{CM}}\)), and effector-memory (T\(_{\text{EM}}\)) cells (Figure 1 A, Supplementary table 3). Post-AHSCT the proportion of T\(_{\text{naïve}}\) 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\(_{\text{EM}}\) (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\(_{\text{EM}}\) cells. These results suggested that T cell reconstitution during the first two years post-transplantation was predominantly driven by peripheral mechanisms of expansion.

To ensure that effective thymic output was not masked by peripherally expanded naïve cells that had survived the preconditioning regimen, we enumerated naïve CD4\(^+\) T cells expressing CD31, a marker of recent thymic origin (Kimmig et al. 2002). Such recent thymic emigrant naïve cells, RTEs, constituted a higher proportion of all CD4\(^+\) cells at 1 year after therapy (from mean 30 ± 12% to 45 ± 18%, a 50% increase, \(P = 0.025\), Figure 1 B). Also the proportion of naïve CD45RA\(^-\)CD45RO\(^-\) cells expressing CD31 was increased for up to the first year post AHSCT (from mean 73 ± 13% to 84 ± 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 contribution of recent thymic output.

**Immunoregulatory cell surge early post-therapy**

To investigate the evolution of immunoregulatory cell populations post-therapy, we utilized 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]%, \( P = 0.002 \), Figure 2 A), as well as an expansion of CD56^{high} NK cells (from median 0.5 [IQR 0.3 - 1.3]% to 3.7 [IQR 2.8 – 5.1]%, \( P = 0.001 \), Figure 2 B). The frequency of both cell subsets subsequently had 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 NK 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^{+} Tregs 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 ± 1.1% to 5.6 ± 3.0%, \( P = 0.035 \); Supplementary figure 2 A), the percentage of actively proliferating Tregs, which was nearly 4 times that of the total CD4^{+} T cell population at pre-treatment baseline, did not change post-therapy (from 26 ± 12% to 20 ± 9%, \( P = \text{NS} \); Supplementary figure 2 B). These results suggested that the increased relative frequency post-transplantation of CD4^{+}CD25^{high}FoxP3^{+} Tregs was not due to a further increase in their proliferation rate.

We were also interested in the potential contribution to treatment effect of CD8^{+}CD57^{+} cells, a suggested immunoregulatory population (Autran *et al.* 1991; Mollet *et al.* 1998) that is significantly increased in the periphery of MS patients after treatment with myeloablative AHSCT (Muraro *et al.*
and 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 to before HSC mobilization, reaching almost four times the baseline levels at 2 years post-transplantation (from mean 16 ± 9% to 59 ± 13% of all CD8+ T cells, \( P < 0.001 \); Supplementary figure 3 A). CD8+CD57+ T cells after treatment were mainly effector-memory cells (not shown) that produced high levels of IFN-γ, granzyme B (Supplementary figure 3 B-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 PBMCs (‘effector cells’) were stained with CFSE and co-cultured with increasing numbers of CD8+CD57+ and CD8+CD57- (‘regulatory cells’) cells in the presence of a polyclonal (anti-CD3 Ab) stimulus. We then assessed the inhibition of CD4+ T cell proliferation after 4 days in culture in the presence of both CD8+CD57+ and CD8+CD57- cells (Supplementary figure 3 D). The degree of inhibition by CD8+CD57+ cells varied in different subject and was either superior or equal to that of their CD57- counterpart at all ‘regulatory’ to ‘effector’ (R:E) ratios (Supplementary figure 3 E). 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.

**CD161highCD8+** are radically depleted after autologous haematopoietic transplantation and are invariant T cells associated with the gut mucosa

Extensive multi-colour FACS phenotypic characterization of pre-treatment CD3+CD8+ cells (data not shown) revealed a distinct CD161highCD8αhigh/dim population (Figure 3 A). CD161highCD8+ T cell populations were present in the 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 (\( P < 0.001 \); Figure 3 B).
High expression of CD161 in CD8^{high/dim} T cells defines the mucosal-associated invariant T (MAIT) cell 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 more than 90% of the CD161^{high}CD8^+ T cell population in MS patients expressed TCR Vα7.2 and that high expression of CD161 was associated with expression of IL-18Rα, CCR6 and the SLAM molecule CD150 (Figure 3 C), consistent with a recent defining description of MAIT cells (Dusseaux et al. 2011). In contrast, a high expression of CD161 in the less frequent CD4^-CD8^- T cell subset did not define MAIT cells per se, as a large proportion of these CD4^-CD8^-CD161^{high} cells did not express Vα7.2 (Supplementary figure 4 A). 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 MAIT cells, and readily appreciated when expressed as the difference of TCR expression in the CD161^{high}CD8^+ T cells compared to all CD8^+ T cells (Figure 3 D). 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 figure 4 B).

CD161^{high}CD8^+ MAIT 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 MS patients before AHSCT produced the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 (Figure 4 A), but not the immuno-regulatory 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, Figure 4 B) and TNF-α producing cells (56 ± 12%, P < 0.001, Figure 4 C). The frequencies of CD8^+ T cells producing IL-17 tended to be higher in the CD161^{high} (median 1.4 [IQR 0.6-2.1]%) and CD161^{dim} (median 0.6 [IQR 0.3-1.6]%), P = 0.040, Figure 4 D), but the analysis was not powered to detect the differences between the groups. However,
the CD161\textsuperscript{high}CD8\textsuperscript{+} subset had the highest frequencies of cells producing both IL-17\textsuperscript{+} and IFN-γ (mean 0.14 ± 0.11%, \(P = 0.002\), Figure 4 E).

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

Together these data confirmed that the CD161\textsuperscript{high}CD8\textsuperscript{+} T cells present in MS patients before treatment and ablated post-therapy were proinflammatory effector MAIT cells, which contained the majority of IL-17 producing and IL-17/IFN-γ co-producing CD8\textsuperscript{+} T cells.

**MAIT cells are present in MS lesions**

The almost exclusive expression of CCR6 on CD8\textsuperscript{+} MAIT cells suggested their ability to enter the central nervous system (Reboldi et al. 2009). We next investigated whether CD161-expressing MAIT cells were also present in MS lesions in post-mortem brain tissue from 9 cases with high levels of inflammatory CNS infiltration. Staining with antibodies against CD8 and CD161 revealed the presence of double positive CD161\textsuperscript{+}CD8\textsuperscript{+} cells within the inflammatory infiltrates of chronic active WM lesions (Figure 5 A). Since distinction of the CD161\textsuperscript{high} versus CD161\textsuperscript{+} cells is not possible in tissue, we also stained for TCRVα7.2, which together with CD161 defines the MAIT cell population. We confirmed by dual immunofluorescent staining that CD161 and TCRVα7.2 double positive MAIT cells were indeed present in WM lesions inflammatory infiltrates in all 9 MS cases (Figure 5 B-C). The presence of MAIT cells in active white matter lesions in the MS brain suggested their involvement in MS pathogenesis, based on their cytokine profile, possibly as proinflammatory effectors.
Circulating MAIT cell numbers are differentially affected by diverse immunotherapies

We next asked whether depletion of MAIT cells in the periphery was specific for the autologous haematopoietic transplantation protocol and to which extent other treatments for MS affected MAIT cell frequencies. We first confirmed the depletion of CD161\textsuperscript{high}\alpha 7.2\textsuperscript{+} CD8\textsuperscript{+} MAIT cells from the periphery of AHSCT patients (Figure 6 A). After AHSCT, the frequencies of CD8\textsuperscript{+} MAIT cells decreased (from a mean 12.1 ± 7.4% of CD8\textsuperscript{+} cells at baseline, to mean 0.6 ± 0.2% at post-therapy follow-up 2 years, \(P < 0.001\), Figure 6 B). In contrast, the frequency of CD161\textsuperscript{high}CD8\textsuperscript{+} cells, a good surrogate of CD8\textsuperscript{+} MAIT’s (Supplementary figure 4 A) in the blood of patients treated with IFN-β was unchanged 6 months after treatment (Supplementary figure 5).

We then set out to dissect the effects on MAIT cells in vivo from each of the two components of the immunosuppressing conditioning regime, cyclophosphamide and alemtuzumab, by studying PBMC samples from patients who had been treated with either high-dose cyclophosphamide (HiCy) or alemtuzumab alone. We measured the frequencies of CD8\textsuperscript{+} MAIT cells at pre-therapy baseline and at 2 years post-therapy in 5 patients who underwent immunosuppression with high-dose cyclophosphamide. An additional 2 patients had only pre or post-therapy samples available. The CD8\textsuperscript{+} MAIT cells were profoundly reduced in 4 out of 6 patients, but persisted at high levels post-therapy in two subjects (Figure 6 C). We also measured MAIT frequency in patients who received treatment with alemtuzumab monotherapy (n = 21). Patient samples were taken at different time points after the last alemtuzumab infusion (range 2-38 months), however the time seemed not to affect the CD8\textsuperscript{+} MAIT cell frequencies within the sampled period as they did not correlate with the time after treatment (Figure 6 D).

When comparing AHSCT, HiCy and alemtuzumab monotherapy post-treatment samples (Table 1), MAIT cell frequencies in both the CD8\textsuperscript{+} (Figure 6E) and the CD4 CD8\textsuperscript{+} (Figure 6 F) cell subsets, as well as in the total CD3\textsuperscript{+} population (Figure 6 G) were low in all three groups, being lowest in the AHSCT-treated patients. MAIT cells in all subsets (CD8\textsuperscript{+}, CD4\textsuperscript{+}, and CD3\textsuperscript{+}) were significantly higher in the HiCy samples as compared to both the AHSCT and alemtuzumab
(monotherapy) treated patients (CD8⁺ MAIT cells: \( P = 0.013 \); CD4⁺ MAIT cells: \( P = 0.006 \); CD3⁺ MAIT cells: \( P = 0.002 \)). These results were driven by MAIT cell numbers remaining high in 2 out of the 6 high-dose cyclophosphamide patients. Whereas the mean frequencies were not statistically different between AHSCT and alemtuzumab treated patients (CD8⁺ MAIT cells: \( P = 0.08 \); CD4⁺ MAIT cells: \( P = 0.08 \); CD3⁺ MAIT cells: \( P = 0.09 \)), the variance between the two patient groups was different (CD8⁺ MAIT cells: \( F < 0.001 \); CD4⁺ MAIT cells: \( F = 0.004 \); MAIT / total CD3⁺: \( F = 0.011 \)) indicating that the frequencies of all MAIT cell populations were differently distributed in the two treatment groups, with a greater variability of MAIT cell numbers in the alemtuzumab monotherapy treated group.

Our study was not designed to ascertain if a correlation might exist between the degree of ablation of circulating MAIT cells and the persistence or recurrence of MS disease activity after the treatments. We attempted, however, to detect a potential association by stratifying patients according to post-therapy circulating CD8⁺ MAIT cell frequency and examining their clinical course. The patients who underwent AHSCT had predominantly low frequencies (<1%) of circulating CD8⁺MAIT cells post-therapy. There were only two post-therapy samples that had CD8⁺ MAIT frequency >1% 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). The remaining two patients who had a relapse after AHSCT (both at 12 months), however, had low (<1%) MAIT frequencies at all the post-transplantation time points assessed. In the high-dose cyclophosphamide treated cohort (n = 7) there were two patients (HiCy1 and HiCy2) who had high MAIT cell frequency post-therapy (10.4% and 15.7%, respectively). Interestingly, they were also the two patients with highest MAIT frequency at the pre-treatment baseline (13.8% and 17.0%, respectively), and the highest disability both pre-treatment (EDSS 7) and post-treatment (EDSS 6.5 and EDSS 7, respectively) in the cohort (Supplementary Table 1). Of these two patients, one (HiCy1) had 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. In the larger cohort of alemtuzumab-only treated patients (n=19 after excluding two
patients who were being treated with IFN-B at the alemtuzumab baseline, in order to avoid potential confounding effects on the clinical measures), where a fair range of MAIT cell levels and range of disease activity following therapy were documented, we were able to stratify patients in 3 groups according to CD8+ MAIT cell frequency: low (<1%), intermediate (between 1% and 90th percentile value in the cohort) and high (90th percentile and higher; Supplementary Fig. 6A); and examined the clinical data in the three groups (Supplementary Fig. 6B-D). Of note, the two patients with high frequency of CD8 MAIT cells after alemtuzumab treatment (Patients Alem10 and Alem20) had highly active MS before treatment (both had 3 relapses in the preceding 12 months), were at the high end of the range of disabilities at baseline within the cohort (EDSS 6 and 6.5, respectively) and relapsed post-treatment (1 and 4 relapses respectively; the latter being the highest number observed within the cohort). Their disability, however, markedly improved after alemtuzumab treatment (decreasing to EDSS 2 in both patients). In the low- and intermediate CD8 MAIT frequency group there were 4 patients who had no relapses post-treatment (2 and 2, respectively); one or more relapses post-therapy were documented in 5/7 and 8/10 patients in these groups, respectively.
DISCUSSION

In this study we investigated the immune reconstitution in patients who underwent non-myeloablative conditioning AHSCT for treatment of their highly active, conventional treatment-resistant forms of MS. We observed immunological changes which were consistent with a favourably redistributed balance of regulatory vs. 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 TCR rearrangement we characterized CD8+CD161high cells as MAIT cells and demonstrated their presence in brain white matter lymphomononuclear cell infiltrates in postmortem MS 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: (1) the treatment should primarily target the lymphoid, not the myeloid compartment; and (2) 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 MS 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 utilizing cyclophosphamide and total body irradiation (CY/TBI) (Muraro et al. 2005). While naïve T cells following CY/TBI 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, respectively, 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 (HSCs), however these methods cannot distinguish whether the HSCs 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 HSCs 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 MS 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. Several studies have described defects in the number or function of CD4+ regulatory cells in MS (Venken et al. 2008; Viglietta et al. 2004). Our longitudinal analysis of regulatory T-cell frequency showed a significant surge in CD4+CD25+CD127-Foxp3+ cells proportion 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<sup>high</sup> T cells might play a role in the mode of action of AHSCT (Alexander et al. 2009; de Kleer et al. 2006). Expansion of CD4+ Tregs has also been shown after alemtuzumab monotherapy (Cox et al. 2005) leaving open the possibility that increased Treg numbers are part of a common response following intensive lymphodepletion.

We considered the possibility that the transiently increased numbers of Tregs after AHSCT were the result of a boosted homeostatic proliferation at 6 months. Tregs 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 Tregs 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 NK cells, a NK cell subset with immunoregulatory potential (Jiang et al. 2011). CD56⁺ high NK cells have been reported to expand and mediate therapeutic effects in MS patients receiving daclizumab (Bielekova et al. 2006) and IFN-β (Vandenbark et al. 2009). We speculate that the early expansion of CD4⁺ Tregs and regulatory CD56⁺ high NK 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. 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. These observations suggested that CD57 may not be per se a marker defining immunoregulatory CD8⁺ T cells, as previously suggested. Interestingly, 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 CD8⁺CD57⁺ regulatory activity.

Our detailed characterization of CD8⁺ T cell reconstitution included several NK markers known to be expressed on T, NKT and NK-like T cells (data not shown) and revealed that a
CD161<sup>high</sup>CD8<sup>+</sup> T subset, readily detectable in all patients before treatment, was radically depleted after AHSCT. We also demonstrate that more than 90% of the CD161<sup>high</sup>CD8<sup>+</sup> cell population in MS patients express TCRVα7.2 and IL-18Rα, and therefore represent mucosal-associated invariant T (MAIT) cells, a subset recently described for their antimicrobial activity (Kjer-Nielsen et al. 2012; Le Bourhis et al. 2010). MAIT cells have been described as noncycling, 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<sup>+</sup> memory MAIT population that produces IFN-γ and contains the IL-17 producing CD8<sup>+</sup> T cells in MS patients, in agreement with recent studies describing CD161<sup>high</sup> cells as a chemotherapy-resistant and tissue-homing proinflammatory CD8<sup>+</sup> subpopulation (Billerbeck et al. 2010; Turtle et al. 2009). These MAIT cells likely possess some plasticity depending on the inflammatory environment – It was recently shown that their IL-17 production was enhanced by costimulation with IL-1β, while presence of IL-12 induced a Tc1-like function (Turtle et al. 2011). Our description of a post-treatment depletion of CD8<sup>+</sup> 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. The study by Darlington et al shows that the capacity to mount Th17 responses is diminished post-therapy, although the Th1 responses remain unaltered (Darlington et al. 2012). Together, their study and ours suggest that alterations of the Th17/Tc17 pathways are crucial mechanisms of immune reconstitution post-AHSCT.

The demonstration that MAIT cells are pro-inflammatory contrasts with the interpretation of Miyazaki et al., who despite describing MAIT cells 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 MS (Miyazaki et al. 2011). We believe that without intracellular cytokine staining assays it is difficult to ascertain whether MAIT cells 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<sup>high</sup> MAIT cells, but also the widely present CD161<sup>dim</sup>
effector CD4 (IFN-\(\gamma\) and IL-17 producing cells, and even some regulatory populations), CD8 T cells, including CD57\(^+\)CD8\(^+\) cells), and NK 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 CD161\(^{\text{high}}\), -\(^{\text{dim}}\) and negative cells we demonstrate production of IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) and no production of IL-10 by CD161\(^{\text{high}}\) cells, consistent with a pro-inflammatory function and in agreement with a majority of other studies (Annibali et al. 2011; Billerbeck et al. 2010; Dusseaux et al. 2011; Walker et al. 2012).

The involvement of IL-17 producing CD161\(^{\text{high}}\)CD8\(^+\) T cells in MS pathology is plausible when considering that the majority (> 70%) of CD8 T cells in acute and chronic active MS lesions were reported to express IL-17 (Tzartos et al. 2008). The potential relevance of CD161\(^{\text{high}}\)CD8\(^+\) cells in MS was recently underpinned by the study by Annibali et al. (Annibali et al. 2011) that showed that the expression of KLRB1, the gene coding for CD161 and one of the non-MHC risk alleles with the highest statistical association to MS (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 MS patients when compared to healthy controls. Furthermore, CD161\(^+\)CD8\(^+\) T cells were detected amongst tissue-infiltrating cells in post-mortem MS brain tissue (Annibali et al. 2011).

Furthermore, by examining immune cell infiltrates in postmortem MS brain white matter tissue from cases with high levels of inflammation we confirm that MAIT cells are present in white matter lesions, confirming their ability to migrate into the central nervous system as suggested by their tissue-homing receptor profile. In a separate study extensively characterizing CD161\(^+\) lymphocytes in MS tissue the presence of MAIT cells was quantified 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 in preparation). These data extend the previous demonstration of the TCR\(\alpha\)7.2\(\alpha\)a33-transcript in the white matter lesions (Illes et al. 2004). Together with the proinflammatory profile, demonstrated in circulating cells, these observations
suggest that MAIT cells are probably implicated in the development of CNS immune-mediated injury in MS.

CD161\textsuperscript{high}CD8\textsuperscript{+} MAIT cells express CCR6, a receptor that is involved in transmigration of T cells into the central nervous system and in the initiation of EAE (Reboldi et al. 2009). CD161 itself also plays a role in trans-endothelial migration of T cells (Poggi et al. 1997). MAIT cells produce high levels of inflammatory cytokines IFN-\(\gamma\) and TNF-\(\alpha\), and are the highest producers of IL-17; IL-17R is expressed on blood-brain barrier (BBB) epithelial cells in MS lesions and IL-17 increases the permeability of the BBB (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\textsuperscript{high}CD8\textsuperscript{+} MAIT cells in MS 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 MAIT cells. We hence obtained PBMC from patients treated in a protocol using high-dose cyclophosphamide at Johns Hopkins University; and in a protocol of alemtuzumab at the University of Cambridge. There was no post-therapy depletion of MAIT cells in 2 out of 6 analyzed high-dose cyclophosphamide treated patients. PBMC obtained from patients treated with alemtuzumab at different time after their last infusion of the antibody had low frequency of MAIT cells 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 MAIT cells 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 mobilisation, 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 gaining valuable insight into its mechanism of action. Dichotomising 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 8/12 patients. Four patients had a single relapse during the first 2 years post-transplantation (vs. 2 relapses each in the 12 months before transplantation) yet at the end of the ≥ 24 month follow-up post-AHSCT the EDSS scores of all relapsing patients were either improved (n = 3) or unchanged (n = 1) compared to pre-treatment baseline, suggesting that their MS 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 4 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 (ADD REFERENCE Cossburn et al Neurology 2012), 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 MAIT cells, their radical post-therapy depletion of MAIT cells observed after AHSCT and the more variable depletion observed after other treatments we hypothesized that their frequency might align most closely with therapeautic efficay and interrogated the data to detect a potential relationship. Our study had not been designed to examine a correlation of MAIT cell 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 samples with the highest frequency
(1.1%) of residual CD8+ MAIT cells were from two time points of two patients who subsequently had a relapse. Interestingly, CD4+ T cells in the sample associated with relapse at 6-month follow-up showed a greatly enhanced reactivity to myelin basic protein (Supplementary figure 7). In the high-dose cyclophosphamide cohort, one of two patients with high (>10%) CD8+ MAIT cell 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 cell frequency post-therapy (7.4% and 3.6%) both had relapses post-therapy (1 and 4 relapses, respectively). Interestingly, all four patients with high MAIT cell frequency post-therapy in the high-dose cyclophosphamide- and in the alemtuzumab cohorts had high disability levels pre-treatment, 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 (add Jones Brain 2010 reference).

Although these observations are intriguing and support the implication that MAIT cells are active perpetrators of inflammatory disease activity in MS, it should be noted that in all 3 cohorts (AHSCT, high-dose cyclophosphamide and alemtuzumab), there were patients with low circulating MAIT cells who had relapses or MRI activity post-therapy; and there was one patient (HiCy1) who had high circulating MAIT cell frequency yet remained clinically stable. These observations suggest that there is no exclusive association of either low frequency of circulating MAIT cells with disease remission or association of high frequency of circulating MAIT cells with disease relapse. The data, therefore, should be interpreted with caution. Future studies in larger numbers of patients, with prospective enumeration of MAIT cells and clinical monitoring before and during treatment are warranted to establish if a correlation of MAIT cell number and clinical course exists. These studies could address the hypotheses generated from the present study that MAIT cells are implicated as detrimental inflammatory mediators of disease and may represent a biomarker of treatment response in MS.

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 Treg 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, we demonstrate increased numbers of circulating cells with 
regulatory potential as well as the elimination of a pro-inflammatory IL-17-, TNF-α-, and IFN-γ-
producing CD8⁺ cell population which corresponds to the recently described, gut-derived MAIT cell 
population. We further show that CD8⁺ MAIT cells, which express CCR6, actually infiltrate MS 
lesion tissue. Taken together, our results suggest that CD8⁺ MAIT cells might be involved in MS as 
inflammatory mediators and could represent a disease and a treatment biomarker as well as, 
potentially, a therapeutic target.
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REFERENCES


Figure 1
Figure 2

A

B

P = 0.001
Figure 3
Figure 4
Figure 5
Figure 6

A: PreTx, 6 mo, 1 yr, 2 yrs

CD161

Valpha7.2

B: % Vα7.2+CD161+ / CD8+

***

PreTx, 6 mo, 1 yr, 2 yrs

C: % Vα7.2+CD161+ / CD8+

PreTx, 2 yrs

D: % Vα7.2+CD161+ / CD8+

Time since last infusion

E: % Vα7.2+CD161+ / CD8+

AHSCT, HicY, Alen

F: % Vα7.2+CD161+ / CD4+

AHSCT, HicY, Alen

G: % Vα7.2+CD161+ / CD3+

AHSCT, HicY, Alen

F = 0.001

F = 0.004

F = 0.011
Supplementary Figure 1

A. Lymphocytes

B. T cells

C. NK cells

D. B cells

E. CD4+ T cells

F. CD8+ T cells

G. Ratio CD4+/CD8+

* p < 0.001

* p = 0.005

* p = 0.031

p = NS

* p < 0.001

p < NS

p = 0.008
Supplementary Figure 3
Supplementary Figure 4
A

\[
\% \text{Ki-67}^+ / \text{CD4}^+ \text{CD3}^+ \\
\]

\[
\begin{array}{c}
\text{PreTx} \\
\text{6 mo}
\end{array}
\]

\[P = 0.035\]

B

\[
\% \text{Ki-67}^+ / \text{CD4}^+ \text{CD4}^+ \text{Tregs}
\]

\[
\begin{array}{c}
\text{PreTx} \\
\text{6 mo}
\end{array}
\]

\[P = \text{NS}\]