Lymphocyte homeostasis following therapeutic lymphocyte depletion in multiple sclerosis

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Following lymphocyte depletion, homeostatic mechanisms drive the reconstitution of lymphocytes. We prospectively studied this process in 16 patients for 1 year after a single pulse of treatment with Campath-1H, a humanised anti-CD52 monoclonal antibody. We observed two phases of lymphocyte reconstitution. In the first 6 months after treatment the precursor frequency and proliferation index of the patients' autologous mixed lymphocyte reaction increased; the depleted T cell pool was dominated by memory T cells, especially CD4+CD25high T cells, a putative regulatory phenotype; and there was a non-significant rise in peripheral mononuclear cell FoxP3 mRNA expression and fall in constitutive cytokine mRNA expression. In the later phase, from 6-to-12 months after Campath-1H, these changes reversed and there was a rise in ROG mRNA expression. However, total CD4+ numbers remained below 50% of pre-treatment levels at 12 months, perhaps reflecting a failure in homeostasis. This was not due to an impaired IL-7 response, as in rheumatoid arthritis, nor to a lack of IL-7 receptors, which are found on fewer human CD4+CD25high than naive cells. We speculate that CCL21 and IL-15 responses to lymphopaenia may be suboptimal in multiple sclerosis.

Introduction

Campath-1H is a humanised monoclonal antibody that targets the cell surface molecule CD52, the function of which is unknown [1]. CD52 is present on the surface of a number of cell populations including thymocytes, T and B lymphocytes, and monocytes, but not plasma cells or haematological precursors [2]. Campath-1H induced lymphopaenia in patients with multiple sclerosis has been shown to effectively reduce central nervous system inflammation both clinically and radiologically [3]. In this patient group, a single therapeutic course of Campath-1H induces immediate lymphopaenia that lasts for several years [4] despite the fact that Campath-1H does not deplete haematological precursors [2] and has a mean terminal phase half-life of 6.1 days [5].

Similarly prolonged lymphocyte depletion is seen when autologous bone marrow transplantation (BMT) or total lymphoid irradiation is used to treat multiple sclerosis [6, 7] Prolonged lymphopaenia is also seen when rheumatoid arthritis is treated with Campath-1H or autologous BMT [8–10]. This is in contrast with the more rapid and age-dependant reconstitution of lymphocytes seen following treatment for haematopoietic and solid organ tumours using bone marrow transplantation [10–13] or indeed Campath-1H, unless high-dose prolonged courses are used [14, 15]. For instance, among patients with breast cancer, median recovery of
CD4+ cells after autologous bone marrow transplantation took 12 months [10], much more rapid reconstitution than that seen in patients with multiple sclerosis. It is this observation that provides the focus for this paper; we hypothesise that disease specific abnormalities in mechanisms that govern lymphocyte homeostasis are responsible for delayed lymphocyte reconstitution following therapeutic depletion.

The mechanisms that maintain lymphocyte number and diversity include the secretion of cytokines and chemokines such as IL-7, IL-15 and CCL21 and competition for survival factors [16–18]. Many authors suggest that lymphocytes also require continuous engagement of their T cell receptors with self-peptide/MHC-complexes [19], however this may only be true for CD4 T cells in the lymphopaenic environment [20, 21]. These mechanisms are particularly critical in controlling lymphocyte proliferation in response to lymphopaenia - the controlled expansion of the lymphocyte pool that we describe as “homeostatic proliferation”. In patients with rheumatoid arthritis, a failure of the normal rise in IL-7 in response to lymphopaenia seems to underlie the prolonged lymphocyte depletion following both autologous bone marrow transplantation and Campath-1H treatment [9]. IL-7 promotes homeostatic proliferation by directly stimulating thymic output [22], the delivery of anti-apoptotic signals [23], and the expansion and survival of peripheral T cells [24, 25]. Elevated circulating levels of IL-7 have been identified in patients with genetic and acquired lymphopaenia, correlating inversely with the CD4+ count [26, 27].

While IL-7 affects both CD4+ and CD8+ T cells, IL-15 and CCL21 have more selective effects. IL-15 has been identified as being particularly critical for the proliferation and survival of CD8+ memory T cells and NK cells. Mice treated with the soluble IL-15 receptor a chain lack CD8+ memory responses [28] and IL-15 knockout mice had a marked reduction in memory CD8+ cells [29]. In mice, IL-15 enhanced donor CD4+ lymphocyte proliferation, as well as NK cells, after allogeneic bone marrow transplantation [30]. The normal low rate of homeostatic division seen within the CD8+ T cell population is antigen independent and IL-15 dependent [31–33]. In contrast, the chemokine CCL21, normally associated with lymphocyte homing responses, specifically promotes CD4+ T cell regeneration. In CCL21 knockout mice, there is little or no proliferation of the CD4+ subset, whereas CCL21 over-expression caused increased CD4+ T cell proliferation in the absence of lymphopaenia [16].

The aim of this study was to describe the homeostatic response and kinetics of lymphocyte reconstitution over 12 months in a cohort of 16 patients with multiple sclerosis treated with the lymphocyte depleting monoclonal antibody, Campath-1H. We concluded that the depleted lymphocyte pool repopulates in two phases characterised by different dominant lymphocyte subpopulations and induction of differential gene expression. We speculate that a suboptimal response of IL-15 or CCL21 to lymphopaenia may underlie the prolonged delay in lymphocyte reconstitution following Campath-1H treatment of multiple sclerosis.

Results

Campath-1H achieves prolonged depletion of circulating T lymphocytes

A cohort of 16 patients with relapsing-remitting multiple sclerosis was studied intensively. Immediately after the first dose of Campath-1H, neither lymphocytes nor monocytes were detectable in the periphery. Total lymphocyte numbers then rose throughout the 12-month follow up period, to reach 47% of pre-treatment levels by 12 months with a mean of 855 cells/microL compared to 1808 cells/microL pre-treatment (p < 0.00001 at all time points). Amongst the lymphocyte subpopulations, this depletion was attributable to low CD4+ and CD8+ T cell counts, which were 32.9 and 55.4% of pre-treatment values respectively at 12 months (Fig. 1A and B). During this period the ratio of CD4+ to CD8+ lymphocytes dropped significantly from a mean of 2.6 before treatment to 0.2 at 1 month post-treatment (p = 0.0019), suggesting a more rapid repopulation of CD8+ cells compared to CD4+ cells (Fig. 1C). This returned towards the baseline ratio by 12 months. In contrast to the T cell subsets, B cells
returned to pre-treatment levels by 3 months (Fig. 1D). Likewise, the monocyte population recovered within the first month (data not shown). The dose of Campath-1H (72, 100 or 124 mg) did not effect the extent to which lymphocytes were depleted or their rate of recovery (data not shown).

**After Campath-1H treatment, the depleted CD4+ lymphocyte pool is enriched for memory T cells, especially CD4+CD25high cell**

After Campath-1H administration there is a trend towards enrichment of the depleted CD4+ lymphocyte pool for cells with a memory (CD4+CD45RO+) as opposed to a naive (CD4+CD45RA+CD62L+) phenotype. This is significant at month 3, after which the trend reverses (Fig. 2).

Within CD4+CD45RO+ lymphocytes there is a subgroup of lymphocytes believed to be naturally occurring regulatory T cells. These cells are defined in humans by high expression of CD25 and co-expression of CD45RO and CD62L. Hafler et al. [34] have shown previously that human regulatory CD4+ T cells have high CD25 expression, whereas activated CD4+ cells adopt intermediate CD25 expression. Therefore, three levels of CD25 expression were defined in this study: low, intermediate and high. Isotype controls determined the upper limit of CD25low fluorescence. An arbitrary lower limit of "high" expression was defined as that level of APC fluorescence at which between 0.1 and 0.2% of CD4+ lymphocytes were positive for CD25.

Prior to treatment there was no difference between patients and healthy controls in the proportion of CD4+ T cells that were CD4+CD25high. However, after Campath-1H treatment CD4+CD25high cells were significantly over-represented in the depleted CD4+ pool (results from a typical patient are illustrated in Fig. 4A). This was most marked at 3 months (median of 13.5% of CD4+ T cells compared to a baseline of 3.7%, $p < 0.000002$, Fig. 4B). This shift in T cell subpopulations was maintained at 6 months, but by 9 months, the proportion of CD4+ cells that were CD25high had fallen to pre-treatment levels. After Campath-1H, 100% of lymphocytes identified as CD4+CD25high were found to be CD62L positive and CD45RO positive, as compared to 90–95% of CD4+CD25high lymphocytes in healthy controls.
controls and patients prior to treatment. We attempted to demonstrate the functional ability of these cells to suppress stimulated lymphocyte proliferation or cytokine secretion \textit{ex vivo}. However, we could not harvest sufficient cells; at 3 months after Campath-1H, 50 mL of blood from our patients (the maximum volume allowed under our local ethical committee agreement) yielded less than 100 CD4$^+$CD25$^{\text{high}}$ cells. There were no significant changes in the proportions of CD4$^+$ T cells that expressed low or intermediate levels of CD25 (data not shown).

\textbf{All CD4$^+$ lymphocyte subgroups are depleted by Campath-1H \textit{ex vivo}}

In order to explain these observations, the possibility that CD4$^+$CD25$^{\text{high}}$ cells were resistant to depletion by Campath-1H was tested. Using flow cytometric analysis of rat anti-CD52:PE (MCA1642P Serotec) binding to PBMC, we confirmed that all lymphocytes and monocytes, including CD4$^+$CD25$^{\text{high}}$ cells, express CD52, the target antigen of Campath-1H, at similar densities on their cell surface. Furthermore, when whole blood from a healthy volunteer was incubated with a physiological concentration of Campath-1H for four hours \textit{in vitro}, all CD4$^+$ lymphocyte populations were completely depleted, irrespective of CD25 expression (data not shown). This suggests that the CD4$^+$CD25$^{\text{high}}$ T cell enrichment seen following Campath-1H treatment is more likely to be due to preferential expansion of this population, than reduced sensitivity to depletion.

\textbf{Serum IL-7 rises following lymphocyte depletion with Campath-1H}

IL-7 is a pivotal cytokine in the homeostatic control of lymphocyte numbers. Following Campath-1H treatment, patients' median serum IL-7 concentration significantly increased by an average of 508% and was still significantly above pre-treatment levels at 12 months (Fig. 5). The concentration of serum IL-7 did, however, fall by 39% between 1 and 12 months following lymphocyte depletion ($p = 0.009$).

\textbf{The IL-7 receptor is expressed on fewer human CD4$^+$CD25$^{\text{high}}$ cells than other CD4$^+$ subgroups}

After Campath-1H treatment, serum IL-7 increased appropriately in response to lymphocyte depletion. We therefore questioned whether prolonged lymphopaenia in our patients was the result of reduced IL-7 receptor expression. We measured the expression of the IL-7 receptor $\alpha$-subunit (CD127) on various CD4$^+$ cell populations by flow cytometry. This was a cross-sectional study using five healthy controls, seven people with untreated multiple sclerosis, and five who had received Campath-1H 12 months previously. Most CD4$^+$ cells (> 95%) expressed CD127 (Fig. 6). However, when CD4$^+$ subgroups were defined by CD25 expression, significantly fewer cells (70–80%) of the CD4$^+$CD25$^{\text{high}}$ subgroup had detectable cell surface IL-7 receptor. As far as we are aware, this has not been previously described in humans, although it has been found in murine CD4$^+$CD25$^+$ T cells [35]. There was no statistically significant difference in CD127 expression between patients before and after treatment.

\textbf{There is an early transient rise in serum IL-15, and a delayed sustained rise in serum CCL21, after treatment with Campath-1H}

Because of the earlier reconstitution of CD8$^+$ lymphocytes compared to CD4$^+$ lymphocytes, we measured serum IL-15, which is specifically involved in the peripheral expansion and survival of CD8$^+$ memory T cells. There was no significant difference in the serum concentration of IL-15 between untreated people with...
multiple sclerosis and healthy controls (Fig. 7A). However, 1 month after Campath-1H treatment there was a rise in the median serum concentration of IL-15 from 4.2 to 5.6 pg/mL (Fig. 7A, \( p < 0.0005 \)). This significance was lost at 3 months at which time the median IL-15 concentration was 5.00 pg/mL (\( p = 0.066 \) after Bonferroni correction). The early fall in serum IL-15 concentrations was surprising in view of persistently low total numbers of peripheral CD8\(^{\text{+}}\)CD45RO\(^{\text{+}}\) T cells which remained significant to 12 months, \( p = 0.013 \) (Fig. 7B). In contrast, the increase in serum CCL21, a chemokine implicated more specifically with CD4\(^{\text{+}}\) T cell homeostasis, was not apparent until 3 months. Furthermore, this rise was sustained up to and including 12 months after Campath-1H (\( p = 0.006 \) and 0.002 respectively, Fig. 8).

**Th1 and Th2 gene expression is reduced, and immunoregulatory gene expression is increased, following lymphocyte depletion**

We assessed the expression of Tbet and GATA-3 (as transcription factors associated with Th1 and Th2 lymphocyte profiles) as well as of the prototypical cytokines, IFN-\( \gamma \) and IL-5, in unstimulated PBMC from patients, controlling for cell number with CD3 or \( \beta \)-actin expression, from unstimulated PBMC after Campath-1H treatment. The expression of transcription factors and cytokines involved in Th1 and Th2 effector T cells (Tbet, GATA-3, IFN-\( \gamma \) and IL-5) are shown. The median, quartiles and range are shown. \( * = p < 0.05 \).

Figure 7. Box and whiskers plot illustrating (A) the serum concentration of IL-15 (pg/mL) in healthy controls and patients prior to and following lymphocyte depletion; and (B) the median total peripheral CD8\(^{\text{+}}\)CD45RO\(^{\text{+}}\) T cell counts (cells/\( \mu \)L) in patients prior to and following lymphocyte depletion. Median, quartiles and range are shown. \( * = p < 0.05 \).

Figure 8. Box and whiskers plot illustrating the serum concentrations of CCL21 (pg/mL) for patients prior to and following lymphocyte depletion and healthy controls (HC). Medians, quartiles and range of data are shown. \( * = p <0.05 \).

Figure 9. Box and whiskers plots illustrating the semi-quantitative real-time PCR measurement of mRNA expression, controlled for \( \beta \)-actin or CD3 expression, from unstimulated PBMC after Campath-1H treatment. The expression of transcription factors and cytokines involved in Th1 and Th2 effector T cells (Tbet, GATA-3, IFN-\( \gamma \) and IL-5) are shown. The median, quartiles and range are shown. \( * = p <0.05 \).
expression as appropriate (Fig. 9). There was a trend towards reduced expression of these genes for 6 months after Campath-1H treatment, although only the reduction in GATA-3 and IL-5 reached statistical significance. Thereafter, cytokine gene expression drifted back towards normal levels. We went on to assess the expression of transcription factors and cytokines thought to play a role in immunoregulation. The intracellular transcription factor FoxP3 is preferentially expressed in CD4+CD25+ T cells that have regulatory properties and has been shown to be both necessary and sufficient for lymphocyte regulation [36]. FoxP3 expression rose at 3 months, although not reaching statistical significance, and then returned to baseline levels (Fig. 10) following the time course of the relative rise in CD4+CD25high cells in the depleted CD4+ pool (Fig. 4B). A very different pattern was seen in the expression of a repressor of GATA-3 (ROG), an intracellular transcription factor associated with Tr1 cells. ROG expression rose significantly at 9, and still more at 12 months, following treatment (p = 0.009 and 0.015, respectively). Two cytokines often associated with immunoregulation are IL-10 and TGF-β. There was a significant drop in IL-10 expression for the first 3 months following treatment. There was no significant effect of treatment on the expression of TGF-β (Fig. 10).

**Homeostatic lymphocyte proliferation transiently increases after lymphocyte depletion**

Previous groups have used variants of the mixed lymphocyte reaction as surrogate markers of homeostatic proliferation [37, 38]. PBMC from 17 people with multiple sclerosis and 7 healthy controls were left unstimulated, cultured in RPMI supplemented with 10% autologous serum (used to include the patients’ serum factors regulating lymphocyte homeostasis). FACS

**Figure 11.** Box and whiskers plots illustrating the median, quartile and range of (A) the precursor frequency (as a fraction of one), and (B) the proliferation index (a ratio of the sum of the cells in all generations divided by the computed number of original parental cells) of PBMC left unstimulated for 10 days, from healthy controls (HC) and patients prior to and following treatment with Campath-1H. * = p < 0.05.
analysis of CFSE dilution after 10 days of culture allowed specific measurement of the proliferation of lymphocytes, and lymphocyte subgroups. Prior to treatment with Campath-1H there was no significant difference in "precursor frequency" (defined as the proportion of lymphocytes that leave the original parent population to undergo at least two cell divisions) [39, 40] between patients with multiple sclerosis compared to healthy controls (Fig. 11A). The "proliferation index" (the sum of the cells in all generations divided by the computed number of original parental cells) was found to be greater in healthy controls (p < 0.01) compared to patients with multiple sclerosis (Fig. 11B). Following lymphocyte depletion with Campath-1H there was an increase in the precursor frequency of lymphocytes when PBMC were incubated in the absence of stimulus (by 667% at 3 months) which persisted only to 3 months (see Fig. 11A). This was accompanied by an increase in the proliferation index sustained only to 6 months despite continued lymphopaenia (Fig. 11B). This increase in precursor frequency and proliferation index was seen in both the CD4+ and CD8+ subsets (data not shown).

Discussion

General remarks

We have taken advantage of the relatively unusual clinical situation provided by the treatment of people with relapsing remitting multiple sclerosis using Campath-1H, which depletes lymphocytes. We prospectively studied a cohort of 16 patients for 12 months following a single pulse of Campath-1H. This induced complex and dynamic changes in the peripheral T lymphocyte pool. As shown in our previous studies, peripheral B cell numbers returned to normal rapidly after Campath-1H induced depletion, while patients' peripheral CD4+ and CD8+ lymphocyte numbers remained significantly depleted for the 12 months of the study [4]. We propose that the repopulation of T lymphocytes during the first 12 months can be divided into two phases, "early" and "late".

The early phase of peripheral lymphocyte reconstitution after Campath-1H

Initially, and for 3 months after treatment, the depleted T cell pool was dominated by an excess of memory T cells. This is a feature of the reconstitution of lymphocytes from patients with multiple sclerosis following anti-CD4 antibodies [41] and autologous bone marrow transplantation [6], as well as Campath-1H treatment of rheumatoid arthritis [42]. This might reflect homeostatic lymphocyte expansion of residual cells, driven by IL-7, which converts naive and effector T cells into cells with a memory T cell phenotype, as seen in animal models [43]. In a murine adoptive transfer model of lymphopaenia, CD8+ T cells proliferated and differentiated into memory cells in response to IL-7 and endogenous self-peptide, but were independent of the appropriate restricting MHC [44].

As far as we are aware, for the first time we have shown that unselective T cell ablation leads, initially, to a relative excess of T cells with a regulatory phenotype as defined by CD4+CD25high [34] in the residual T cell pool. This coincided with a statistically insignificant increase in expression of FoxP3, a transcription factor both necessary and sufficient for T cells to regulate [36, 45]. However, the profound lymphopaenia at these time points did not allow for sufficient cells to be harvested to establish either that this rise in FoxP3 occurred within the CD4+CD25high cells, nor that they were functional suppressive. This is of particular importance in view of recent work demonstrating that although patients with multiple sclerosis have normal numbers of regulatory T cells (confirmed in this study), they have reduced capacity to suppress effector cell proliferation [46]. We have shown that the relative dominance of CD4+CD25high cells early in reconstitution is not explained by reduced vulnerability of this T cell subgroup to lysis by Campath-1H. We speculate that the higher affinity of regulatory cells for self-antigens may lead to more effective homeostatic expansion within the memory T cell compartment [35]. At the same time peripheral mononuclear cell constitutive cytokine gene expression was reduced. These data provide circumstantial evidence that a single pulse of Campath-1H induces early changes in lymphocyte populations, which would tend to suppress immune responses. We were unable to test the regulatory capacity of CD4+CD25high T cells because so few of these cells could be harvested at these time points.

The late phase of peripheral lymphocyte reconstitution after Campath-1H

From 6-to-12 months after Campath-1H, the T cell subsets within the reconstituting lymphocyte pool return towards a normal distribution. This may reflect naive T cell generation or a more complex process: in an animal model, homeostatic proliferation of naive CD8+ cells converted them to a memory phenotype, but then they reverted to a naive phenotype when proliferation was reduced [47]. We have previously noted an age-dependent increase in T cell receptor rearrangement excision circle (TREC) concentrations in PBMC taken from patients following treatment with Campath-1H compared to pre-treatment samples (unpublished data).
From these data, we infer an increase in thymic function after Campath-1H, despite the expression of CD52 on thymocytes. However, we have been unable to quantify this accurately because of compounding dilution of TREC in proliferating peripheral lymphocytes [48, 49]. At 6 months after Campath-1H treatment, FoxP3 expression (measured in PBMC) falls and constitutive cytokine expression returns. This might be interpreted as a sign of reduced immunoregulation and, indeed, it is during this phase of reconstitution that we have seen the emergence of thyroid autoimmune diseases in previous patients (though none in the cohort under study here) [50]. Until it has been possible study the functional capacity of lymphocytes with a regulatory phenotype, this remains entirely speculative. This interpretation is also complicated by the unexpected finding of a late rise in ROG mRNA expression. ROG suppresses T cell activation and may, through inhibiting GATA-3, also suppress Th2 cytokine expression [51–53]. It is also implicated in a population of inducible regulatory cells, Tr1 cells [54].

**Impaired peripheral lymphocyte reconstitution after Campath-1H**

The reconstitution of lymphocytes after Campath-1H is slow in comparison to other ablative treatments. At 12 months CD4+ T cell numbers are still less than half their pre-treatment levels, despite the fact that Campath-1H does not deplete haematological precursors [2]. This observation suggests impaired lymphocyte homeostasis might be inherent in the pathogenesis of multiple sclerosis. Two defects of lymphocyte homeostasis have been described in rheumatoid arthritis, an autoimmune disease in which therapeutic lymphocyte depletion is also unusually prolonged. First, the production of IL-7 in response to lymphopaenia is impaired [9]. In our patients, however, there was both a prompt and sustained rise in IL-7 in response to lymphocyte depletion. There was no alteration in the proportion of lymphocytes expressing the IL-7 receptor α subunit (CD127) after Campath-1H, although animal experiments suggest that IL-7 receptor expression is reduced on T cells that have received cytokine-mediated survival signals, to avoid unnecessary competition for remaining IL-7 in lymphopaenia [55]. Another anomaly of lymphocyte homeostasis in rheumatoid arthritis is that ex vivo PBMC proliferation is not blocked by anti-MHC class II antibodies, suggesting a drive for lymphocyte proliferation over and above interaction with self-peptide, which was TNF-α [38]. In contrast, proliferation of PBMC cultures from our multiple sclerosis patients, as well as normal controls, was inhibited by an anti-class II antibody (data not shown).

In the late phase of lymphocyte reconstitution after Campath-1H, proliferation of the autologous mixed lymphocyte reaction, an *ex vivo* surrogate of homeostatic proliferation, was reduced despite continued T lymphopaenia and sustained elevation of serum IL-7. We speculated this might be due to an impaired response of other cytokines involved in lymphocyte homeostasis. The first of these, IL-15, has an established role in maintaining the proliferation and survival of CD8+ memory T cells in lymphopaenic [28] and non-lymphopenic conditions [31–33]. It is also involved in the reconstitution of NK cells, whose numbers return to normal rapidly after Campath-1H [50]. The serum level of IL-15 rose briskly at month 1 after Campath-1H, but thereafter fell to baseline levels, despite continued memory CD8+ lymphopaenia. The second serum factor, the chemokine CCL21, which specifically promotes CD4+ T cell regeneration [16], only became elevated at 3 months despite the dramatic T lymphopaenia immediately after Campath-1H. There are insufficient data on the kinetics of the response of IL-15 or CCL21 to a pulse of lymphocyte depletion in man to judge whether those observed in our study explain the prolonged lymphopaenia after Campath-1H and reflect a failure of homeostasis inherent in multiple sclerosis; but we suggest these are reasonable hypotheses to frame further studies.

**Materials and methods**

**Patients**

Patients in this study were participants in one of two clinical trials of Campath-1H for the treatment of multiple sclerosis at Addenbrooke’s Hospital, both approved by the Local Research Ethics Committee (LREC). Separate informed consent was obtained for the additional blood samples required for this study (LREC number 02/263). For the serial analyses described below, a cohort of 16 consecutive patients (10 female and 6 male) were selected with mean age 33.6 years (range 22–48 years), mean disease duration 2.4 years (range 6–54 months), mean annualised relapse rate 1.8 (range 0.9–6 relapses/year) and mean EDSS (expanded disability severity score [56]) 2.2 (range 1.0-6.0). One patient had received IFN-β prior to treatment with Campath-1H, which was stopped for over 1 month prior to inclusion. Venous blood samples were taken from patients prior to and at 1, 3, 6, 9 and 12-month intervals following treatment. As a consequence of the difficulty in obtaining sufficient numbers of T cells after lymphocyte depletion, some cross-sectional studies were performed on an additional cohort (*n* = 17) with comparable disease severity and duration. Controls were healthy volunteers.
Administration of Campath-1H

Patients were admitted to the Wellcome Trust Clinical Research Facility at Addenbrooke’s Hospital for 5 days. They received either 72 mg (6 patients), 100 mg (6 patients) or 124 mg (4 patients) of Campath-1H intravenously, over 5 days in divided doses, depending on which trial of Campath-1H they were participating. The first 3 doses were preceded by 1 g of intravenous methylprednisolone that suppresses the cytokine release syndrome associated with the first dose of Campath-1H [57]. Campath-1H was obtained from ILEXTM Pharmaceuticals L.P.

Flow cytometry

Peripheral blood mononuclear cells were separated from venous blood using a Ficoll density gradient (Ficoll-Paque Plus, Amersham Pharmacia Biotec). Oral of 10⁶ PBMC were used in each assay. Three-colour flow cytometry was performed and analysed using the following monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cytochrome 5 (PE-cy5) or allophycocyanin (APC): anti-CD25:APC (555434 BD PharMingen), anti-CD45RA:PECy5 (555490 BD PharMingen), anti-CD62L:PE (555544 BD PharMingen), anti-CD25:APC (555751 BD PharMingen), mouse IgG 2a:RPE (MCA1642P Serotec), anti-CDw127:PE (555493 BD PharMingen), anti-CD45RO:PE (PE), PE-cytochrome 5 (PE-cy5) or allophycocyanin (APC): mouse IgG 1 (MCA928F Serotec), rat anti-CD52:PE (MCA1642P Serotec). Isotype controls were mouse IgG1:FITC (MCA928F Serotec), rat IgG2a:APC (556924 BD PharMingen), mouse IgG1:APC (555751 BD PharMingen), mouse IgG2a:RPE (555574 BD PharMingen) and mouse IgG1:RPE-Cy5 (555750 BD PharMingen). Fluorescence was detected using a FACScalibur flow cytometer (Becton Dickinson) and gated initially on lymphocytes on the basis of forward and side scatter. A minimum of 10 000 events were collected within the lymphocyte gate. Analysis was performed using CELLQuest™ and WinMDI 2.8 software.

Real-time semi-quantification of mRNA expression

At each time point at least 10⁶ unstimulated PBMC were frozen at −70°C in RNAlater (Ambion) according to the manufacturer’s instructions. Total mRNA was subsequently extracted with RNeasy Mini Kit (Qiagen) and converted to cDNA using Pro-STAR First Strand RT-PCR Kit (Stratagene). PCR primers and probes were designed using the Primer Express software (PE Biosystems, Foster City, CA, USA), based on GenBank sequences and purchased from Oswel DNA Service. Real-time expression of various mRNA sequences was measured using the PerkinElmer ABI Prism 7900HT Sequence Detection System using PCR Mastermix containing ROX (Eurogentec RT-QP2X-03). The primers and probes used were: FoxP3: For 5'-CCC ACA AGG CAG GCT CAT G-3', Rev 5'-GCC GAT CCA CGG GTA CT-3'; FAM-labelled Probe: 5'-TTT CTG TCA GTT CAC TTC ACC AAG CCT G-3'; β-Actin: For 5'-CCT GCC ACC CAG CAC AAT-3', Rev 5'-GCG GAT CCA CGG GTA CT-3'; JOE-labelled Probe: 5'-TCA AGA TCA TTG CTC TCT CTC AGC GC-3'; ROG: FOR 5'-GCC ACC CAG CAC AAT-3', Rev 5'-TGG TCA TGG CCG AGA AGT C-3'; JOE-labelled Probe: 5'-TCT CCT GTA GCC TTT GTC CTC AGC GCT-3'; Tbet: For 5'-ACT GAG GGT CGC GCT CAA C-3', Rev 5'-TCC CTG CTT GGT GAT GAT CAT-3'; FAM-labelled Probe: 5'-ACC ACC TGG TGT GGT CCA AGT TTA ATC AGC A-3'; IL-5: For 5'-GAC GCA GTT TTG TAC TAT GCA CTG TCT-3', Rev 5'-AGA AGC ATC ATG TGT CTC CTG A-3'; JOE-labelled Probe: 5'-TTG CCA AAG GCA AAC GCA GAA C-3', TGF-β For 5'-CGA GCC TGA GGC CGA CTA C-3', Rev 5'-CTT GAA CTT GTC ATG ATG GCC ATT TTG TAG CTG-3'; JOE-labelled Probe: 5'-CAT CCA AGT GAT GGC TGA ACT GTC GCT-3', CD3: For 5'-GGC TCG CAT CTT AGT AA-3', Rev 5'-GCG GCC GAA GAA CTC TCC AGT GA-3'; JOE-labelled Probe: 5'-AAC AGT CCC ATG AAA CAA AGA TGC AGT CG-3'; IL-7 receptor α chain (CD127): For 5'-TCT ATC CAG CAC AAA GCT GAC ACT-3', Rev 5'-TGG ATC GAA CCT TAA TCT CAT ACA TTG-3'; FAM-labelled Probe: 5'-CTG CAG AGA AAG CTC CAA GCG GCA-3'; IL-10: For 5'-TGA GAA CAG CTG CAC CCA CTT-3', Rev 5'-GCT GAA GGC ATC TCG GAG GAT-3'; FAM-labelled Probe: 5'-CCA AGG AAC ATC GCT CGG TCG CAT A-3', Rev 5'-TGG GAC GAC TCC ATG CCT AGA CT-3'; FAM-labelled Probe: 5'-AGG TGC TCC TCC CCG ACA GCT-3'. Primers were used at 3 mM concentration and probes at 10-mM concentrations. Gene expression was normalized against either β-actin (IL-10, IL-7R, TGF-β, ROG and IFN-γ), or CD3 (Tbet, GATA-3, FoxP3, IL-5) where expression was known to be limited to T cells.

Whole blood in vitro assay

The whole blood (20 mL) was incubated at 37°C for 4 h with Campath-1H (MabCampath/Alemtuzumab, Ilex), creating a final concentration of 10 mcg/mL, estimated to represent the circulating concentration of Campath-1H in vivo [58]. The remaining PBMC were then separated over a Ficoll density gradient and interrogated using the range of conjugated monoclonal antibodies previously described.

Quantification of serum IL-7, IL-15 and CCL21 concentrations

At each time point, patients’ sera were frozen within 24 h of collection and stored at −70°C until used. The concentrations of IL-7, IL-15 and CCL21 in the serum were measured using the Quantikine Human IL-7, IL-15 and 6Ckine/CCL21 immunoassay kits (R & D HS750, D1500 and D6C00, respectively). The plates were read using a microplate reader (model 680, Bio-Rad) and results were compared to those from six healthy volunteers.

Autologous mixed lymphocyte reaction proliferation assays

PBMC were isolated as described and labelled with the intracellular cell division tracking dye (CFSE; Vybrant CFDA SE Cell Tracer Kit V-12883, Molecular Probes) [39]. CFSE was added to PBMC suspended in RPMI without serum, to a final concentration of 5 mM, incubated at room temperature for 15 min and then quenched with ice-cold RPMI containing 10% serum. Then, 10⁶ PBMC were incubated unstimulated in RPMI...
1640 medium with 1% penicillin, 1% streptomycin, 1% L-glutamine and 10% autologous serum in 12-well flat bottom plates for 10 days at 37°C. In a subset of assays a further 106 PBMC were incubated in the absence of antigen, but in the presence of an anti-HLADR monoclonal antibody to a final concentration of 0.1 mcg/mL (Cat # 247360, BD Biosciences), in order to block the presentation of antigen. CFSE fluorescence was measured using a FACScalibur flow cytometer (Becton Dickinson) and CELLQuest™ software. Precursor frequency and proliferation index were calculated using Modfit LT 3.0 (Verity Software) [40].

Statistical analysis

All statistical analyses were performed using SPSS 10.0 for Windows. Following assessment of normality, parametric (Student’s t-test) or non-parametric (Wilcoxon or Mann Whitney) tests were performed. Bonferroni corrections for multiple comparisons were made where applicable.

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