Depletion of CD52-positive cells inhibits the development of central nervous system autoimmune disease, but deletes an immune-tolerance promoting CD8 T-cell population. Implications for secondary autoimmunity of alemtuzumab in multiple sclerosis

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Summary
The objective was to determine whether CD52 lymphocyte depletion can act to promote immunological tolerance induction by way of intravenous antigen administration such that it could be used to either improve efficiency of multiple sclerosis (MS) inhibition or inhibit secondary autoimmunities that may occur following alemtuzumab use in MS. Relapsing experimental autoimmune encephalomyelitis was induced in ABH mice and immune cell depletion was therapeutically applied using mouse CD52 or CD4 (in conjunction with CD8 or CD20) depleting monoclonal antibodies. Immunological unresponsiveness was then subsequently induced using intravenous central nervous system antigens and responses were assessed clinically. A dose–response of CD4 monoclonal antibody depletion indicated that the 60–70% functional CD4 T-cell depletion achieved in perceived failed trials in MS was perhaps too low to even stop disease in animals. However, more marked (~75–90%) physical depletion of CD4 T cells by CD4 and CD52 depleting antibodies inhibited relapsing disease. Surprisingly, in contrast to CD4 depletion, CD52 depletion blocked robust immunological unresponsiveness through a mechanism involving CD8 T cells. Although efficacy was related to the level of CD4 T-cell depletion, the observations that CD52 depletion of CD19 B cells was less marked in lymphoid organs than in the blood provides a rationale for the rapid B-cell hyper-repopulation that occurs following alemtuzumab administration in MS. That B cells repopulate in the relative absence of T-cell regulatory mechanisms that promote immune tolerance may account for the secondary B-cell autoimmunities, which occur following alemtuzumab treatment of MS.

Keywords: autoimmunity; experimental autoimmune encephalomyelitis/multiple sclerosis; neuroimmunology; tolerance/suppression/energy.

Introduction
Multiple sclerosis (MS) is the major demyelinating disease of the central nervous system. Although the aetiology is obscure, genetic susceptibility, pathology and response to therapy indicate that the disease is immune-mediated. Although MS appears to be uniquely human, clinical and pathological similarities between MS and experimental autoimmune encephalomyelitis (EAE) have resulted in MS being viewed as a T-cell-mediated autoimmune disease targeting oligodendrocytes. Although the innate immune system and B cells can contribute to the disease process in EAE, it is clear that T-cell activity is central to pathogenesis. This is indicated by the ability to adoptively

Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; SCH, spinal cord homogenate
transfer disease and via T-cell inhibition. In many cases, disease is mediated by CD4 T cells, although pathogenic CD8 T-cell models have been developed to mirror the CD8 predominance in some MS lesions. However, supportive data for a CD4, T helper type 17-mediated pathogenesis in MS is largely circumstantial and not supported by the perceived failure of CD4-depleting monoclonal antibodies (mAb).

In animals, the disease course is predictable allowing optimized treatment to achieve maximal inhibition and CD4 depletion can control most T- and B-cell (T-dependent) immune responses. However, at the time of the initial CD4 mAb trials, AIDS was becoming prevalent as a result of HIV. Therefore, long-term depletion below 250 CD4 cells/mm$^3$ (about 70% depletion) was felt to be contra-indicated and trials aimed to maintain CD4 T-cell numbers above this limit. This is substantially less than the > 85% depletion used to inhibit EAE. However, alemtuzumab, which is a CD52 lymphocyte-depleting mAb, produces a long-term and marked (>90%) depletion of CD4 T cells and effectively inhibits relapsing MS. However, secondary B-cell autoimmune diseases often occur as a delayed side-effect of alemtuzumab treatment in people with MS. In addition, generalized immunosuppression may result in infections and other adverse effects that may limit the wide adoption of the treatments.

Antigen-specific immunotherapy has the advantage of controlling pathogenic T cells while leaving the rest of the immune system to fight infection and cancers. Although there are many ways to induce antigen-specific tolerance, a consistently robust method has been achieved by intravenous antigen delivery following transient T-cell deletion. This combination, and not the individual treatments, eliminates relapsing EAE in animals with established disease. Similarly, depletion of CD4 T cells and depletion of intravenous oligodendrocyte-directed antigens have been tried and so far failed to eliminate relapses in MS, despite some efficacy. However, these data indicate that such combinations could be safe and feasible in MS. No CD4 mAb is currently licensed for MS so it was hypothesized that alemtuzumab could be used as a T-cell-depleting agent for tolerance induction. This could perhaps be used in combination with oligodendrocyte-associated autoantigens to control MS or other autoantigens, such as thyroid antigens to treat the alemtuzumab-induced autoimmune diseases that are highly prevalent in MS. This hypothesis was investigated using a mouse CD52-specific mAb in relapsing EAE.

**Materials and methods**

**Animals**

Male or female Biozzi ABH mice were from stock bred at Queen Mary University of London, these were housed and fed with RM1(E) chow and water ad libitum as described previously. They were used according to the United Kingdom, Animals (Scientific procedures) Act 1986, incorporating review by the local Animal Welfare and Ethical Review Body and the United Kingdom Home Office.

**Antibodies**

Purified and fluorescent mouse CD4 (mCD4) -specific mAb were used: rat IgG2b clone YTS191.1 mAb (Bio X cell, West Lebanon NH; AbD Serotec Kidlington, UK); rat IgG2b RM4-5 (AbD Serotec); rat IgG2b clone YTA3.1 (Dr S. Cobbold, University of Oxford), rat IgG2b GK1.5 (AbD Serotec); rat IgG2c KT174 (AbD Serotec and Dr K. Tomonari, Fukui Medical School, Japan) or rat IgG2a KT6 (Dr K. Tomonari) were obtained.

In vivo mAb treatment

Mice were injected intraperitoneally or subcutaneously with various amounts of, typically 250 µg, mCD4-depleting rat IgG2b (YTS191.1) mAb. This was purchased from Bio X cell. Genzyme Corporation (Framingham MA) supplied the 250 µg mouse CD52 (mCD52) -depleting mouse IgG2a mAb. This was injected subcutaneously over 5 consecutive days. Mouse 18B12 CD20-depleting mouse IgG2a mAb was a kind gift of Biogen Idec Inc. (Cambridge, MA). Irrelevant rat IgG2b and mouse IgG2a mAbs do not influence the course of EAE in ABH mice.

**Flow cytometry**

Erythrocyte-free splenocytes generated using erythrocyte lysis buffer (eBiosciences Ltd, Hatfield, UK) were counted and then 1 x 10$^6$splenocytes were incubated with 1–5 µg/ml fluorescent mouse-specific antibodies. CD4 and CD8 T cells were identified using CD4-FITC and CD8a-phycocerythrin-Cy7 conjugates, respectively. Allophycocyanin-conjugated CD45RA (B220, RA3-6B2) was used to identify B cells, CD35 Peridinin chlorophyll protein-Cy5 identified natural killer cells and neutrophils were stained using GR-1 allophycocyanin-Cy7. These were purchased from Becton Dickinson (Oxford, UK). Monocytes/macrophages were characterized by the binding of F4/80 FITC (eBiosciences). Regulatory T cells were detected using: CD4 FITC, CD25 allophycocyanin-Cy7 (both Becton Dickinson) and intracellular Forkhead box P3-phycocerythrin (FJK-16s; eBiosciences). Staining of regulatory T cells was performed according to the manufacturer’s protocol. Briefly, following the extracellular staining, cells were incubated with a fixation/permeabilization working solution for at least 30 min at 4º in the dark. The cells were then centrifuged at 300 g for 3 min, washed with permeabilization buffer (prepared from a
10× stock solution) and centrifuged once more. Intracellular antibodies, including isotype controls, were added at appropriate dilutions in permeabilization buffer with 5% mouse serum and incubated for 30 min at 4°C in the dark. The cells were then washed and resuspended in FACS buffer before flow cytometric analysis. The lymphocyte population was gated on forward, side-scatter characteristics. In some instances, splenocytes were pre-incubated with saturating 20 μg/ml amounts of unconjugated CD4-specific mAb, for 30–60 min before incubation with conjugated CD4-specific mAb.

Induction of experimental autoimmune encephalomyelitis

Six- to eight-week-old adult ABH mice were subcutaneously injected with 1 mg mouse spinal cord homogenate (SCH) emulsified in Freund’s complete adjuvant containing 60 μg Mycobacterium tuberculosis H37Ra and Mycobacterium butyricum (8 : 1) in the flank on days 0 and 7 as described previously. Clinical disease was scored: Normal = 0; Fully flaccid tail = 1; Impaired righting reflex = 2; Hindlimb paresis = 3; Complete hindlimb paralysis = 4 and Moribund/death = 5. Details of randomization, blinding and sample size calculations and other experimental details relevant to the ARRIVE guidelines have been reported previously. Use of SCH as immunogen precludes ex vivo ery have been described previously. The data are typically plotted as a Kaplan–Meier curve to allow animals to be removed from the study, rather than remain with disability and hence offers advantage in the Refinement, Reduction and Replacement (3Rs) of animals in research.

Induction of unresponsiveness

Erythrocyte-free splenocytes were prepared from ABH mice and SCH was chemically coupled to splenocytes using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for 1 hr as described previously and 2.5 × 10⁷ SCH–antigen coupled spleen cells (SCH-SC) in 0.1–0.2 ml of PBS were injected intravenously into the tail vein of each mouse. This was administered 1–3 weeks after CD4 T-cell depletion. To assess the development of unresponsiveness, animals were rechallenged with a further set of injections of SCH in Freund’s incomplete adjuvant typically 2 weeks after tolerance induction.

Statistical analysis

Results represent the mean maximum ± SEM clinical score or day of onset ± SD, and were analysed using non-parametric statistics using SIGMAPLOT V11.8

Results

Repopulation kinetics and immune inhibitory function following CD4 T-cell depletion

Previously it has been reported that physical depletion of CD4 T cells can inhibit disease and 250 μg of YTS191.1 antibody silenced CD4 T-cell activity for approximately 3 weeks following detection of the same CD4 epitope used for depletion. Cross-blocking experiments indicated that there are a number of distinct CD4 epitopes that can be used to monitor T-cell depletion with YTS191.1 rat IgG2b monoclonal antibody (see Supplementary material, Fig. S1, Table S1). Following administration of 250 μg rat IgG2b CD4-depleting (YTS191.1) mAb there was rapid receptor blockade of ~90% cells within 24 h (Fig. 1a). However, by 14 days lymphocytes had recovered to about 50–70% of their original values at a time when there was limited rat immunoglobulin detected. This is achieved by rapid expansion of the memory T-cell pool. The level of receptor blocked with 25 μg and 50 μg YTS191.1 CD4-specific mAb was largely comparable and created a functional depletion of 60–70% (Fig. 1a), but 5 μg CD4-depleting mAb had minimal impact and reduced the level of CD4⁺ T cells by ~30% for 1 week (Fig. 1a). The pattern of depletion as identified using antibodies targeting the RM4.4 antigen was similar to the YTS191.1 epitope, except that it was clear that physical depletion was taking longer than the receptor blockade (Fig. 1b). At 1 day after administration of 250 μg YTS191.1 intraperitoneally 15.4 ± 0.9% of the splenocytes exhibited surface rat immunoglobulin but this dropped to 1.84 ± 0.1% at 3 weeks after administration, when CD4 had already begun to recover. Physical depletion of CD4 T cells, as indicated by the number of cells expressing the RM4.4 epitope was ~90%, but by 3 weeks the number of cells had recovered to be about 30% of the splenic population. Both 25 μg and 50 μg YTS191.1 CD4-specific mAb induced a similar level of depletion that peaked with about a 60% depletion within 3 days and with a 20–30% depletion at 2 weeks following antibody administration. There was essentially no depletion 27 days after antibody administration. Five micrograms of CD4-depleting mAb had minimal impact and reduced the level of CD4⁺ T cells by a maximum of ~30% (Fig. 1b). This level of depletion failed to influence the development of EAE when animals were randomized to treatment following the onset of EAE when they were exhibiting a paralysed tail and weight loss (Fig. 1c) and the development of neurological disease (Fig. 1d). Inhibition of EAE-associated weight loss was dose-dependent and it was clear that both 500 μg and 250 μg YTS191.1 mAb controlled weight loss and animals began to recover within 24 h following injection. Although weight loss was stabilized following injection of 50 μg YTS191.1 mAb, neurological
disease continued to deteriorate. Likewise although there was a small amelioration of disease activity, animals treated with 25 µg YTS191.1 mAb continued to develop disease (Fig. 1d). Therefore, in this highly optimized system, depletion of CD4 numbers by about 50–60% was insufficient to effectively control disease in EAE and about 30% CD4 T-cell depletion exhibited no control of EAE. Similar inhibition of neurological EAE was achieved following injection of 250 µg YTA3.1 (Fig. 1d).

CD4 T-cell depletion can inhibit relapsing EAE

Two hundred and fifty micrograms YTS191.1 mAb could inhibit the development of EAE in Biozzi ABH mice when administered as a single injection on day 10 post-inoculation (p.i.) (Fig. 2a). Control mice developed EAE with high incidence and mice that developed disease exhibited a mean maximum neurological score of 3.7 ± 0.2 compared with CD4-T-cell-depleted mice that exhibited no disease, Score 0.0 ± 0.0 (P < 0.001) up to day 24 p.i. Freedom from disease was maintained until animals were rechallenged with a further injection of antigen in Freund’s incomplete adjuvant on day 28 p.i. where the majority of animals (n = 14/20) developed disease (Fig. 2a). This procedure can reactivate previously primed cells in an antigen/peptide-specific manner and breaks unresponsiveness induced by either CD4 T-cell depletion or intravenous antigen alone but not the combination of CD4 depletion and intravenous antigen. In this experiment, control animals relapsed and exhibited mean maximal score of 3.9 ± 0.1. Increasing the number of doses did not improve this whereas five daily subcutaneous CD4-mAb administrations of...
YTS191.1 could again completely inhibit the development of EAE. However, it again failed to limit the capacity to re-induce EAE, indicating that robust immune unresponsiveness, which can resist antigen rechallenge, via multiple mechanisms,\textsuperscript{4,18,21} was not induced (Fig. 2b). Similar to inhibition of the initial acute EAE attack, relapsing disease was inhibited when 250 µg CD4-depleting mAb was injected during the post-acute remission phase with 0/8 animals relapsing up to day 60 p.i. compared with 8/8 in untreated controls (Fig. 2c). However, with time, disease began to develop and by day 90 p.i. it was found that 3/8 CD4-treated animals had relapsed (mean day of onset 66.7 ± 15.6 compared with controls day 36.7 ± 5.3 \( (P < 0.01) \) (Fig. 2c). However, when CD4 T-cell depletion was combined with intravenous antigen in the form of 2.5 \( \times 10^7 \) SCH-coupled splenocytes (SCH-SCH), robust unresponsiveness was induced that could largely \( (n = 2/10 \ P < 0.001) \) resist antigen rechallenge (Fig. 2d).

Repopulation kinetics and immune inhibitory function following CD52 cell depletion

The pattern of splenic lymphocyte repopulation following CD52 depletion was assessed in ABH mice by flow cytometry. Surprisingly, following a single injection of 250 µg (10 mg/kg) mouse CD52-specific mAb on day 10 there was only about a 30% CD4 T-cell depletion \( (n = 2/10 \ P < 0.001) \) resist antigen rechallenge (Fig. 2d).
CD52 depletion in CNS autoimmunity

It was found that consistent with the low level (~30%) of CD4 T-cell depletion induced by the CD52 mAb (Fig. 3a), a single injection of 250 µg CD52 mAb failed to prevent the development of EAE (Fig. 4a). In contrast, repeated subcutaneous administrations of 250 µg CD52 mAb, which depleted splenic CD4 T cells by ~90% (Fig. 4a–c), completely inhibited the development of disease \( n = 0/10 \) \( (P < 0.001; \) Group Score 0·0 ± 0·0 \( P < 0.001 \) compared with control mice that developed \( n = 6/7 \) severe EAE \( (\text{Group score} 3·5 ± 0·6 \) and EAE Score 4·1 ± 0·3 and Day of onset 16·7 ± 2·5) up to day 24 p.i. \( (\text{Fig. 4b}) \). However, as with the depletion induced by CD4-specific mAb, disease could be reactivated following a further antigen-rechallenge with SCH in Freund’s incomplete adjuvant administered within about 2 weeks of cessation of antibody treatment \( (\text{Fig. 4a},\text{b}) \). This indicates that disease can be induced despite significant T-cell depletion. Therefore, mCD52d does not appear to reinduce immunological unresponsiveness to autoimmune central nervous system disease as shown here. This effect was compatible with the efficacy of a single injection of 250 µg mCD4d mAb, reported previously.3,4 It was found that control animals relapsed \( (n = 5/5 \) as two animals were culled during the initial acute attack) and exhibited severe disease \( (\text{Group score} 4·0 ± 0·0 \) that developed 9·0 ± 2·8 days after antigen rechallenge. This was not significantly different for mCD52-depleting mAb where all animals developed disease \( (n = 10/10 \) with a Group Score of 3·6 ± 0·1 that developed 11·6 ± 3·0 days after rechallenge \( (\text{Fig. 4b}) \). In addition to inhibition of the initial acute phase of EAE \( (\text{Fig. 4b}) \), likewise, following the establishment of EAE, daily subcutaneous administration of 250 µg mCD52d mAb from day 27–31 p.i. inhibited the incidence \( (P = 0.002 \) and development \( (P = 0.001 \) of relapse. As such, no animal \( (n = 0/8 \) had developed a relapse by day 50 p.i. compared with 7/8 controls \( (P = 0.002 \) \( (\text{Fig. 4c}) \). However, this inhibitory effect was transient and spontaneous relapse eventually developed \( (n = 5/8 \) CD52-specific mAb versus controls \( n = 8/8 \), maximum relapse score 3·4 ± 0·4 versus 4·0 ± 0·0 in

Inhibition of relapsing EAE with CD52-specific mAb

T cells were now depleted by approximately 80–90% within 7 days of antibody delivery and this level of depletion persisted during an observation period of 28 days \( (\text{Fig. 3b}) \). CD4, CD25, FoxP3 positive T regulatory cells were relatively unaffected by CD52-mAb. There, was also marked depletion of CD45RA/B220 B cells within the spleen \( (70 ± 0·6\% \) \( (\text{Fig. 3b}) \). However, there was substantially \( (P < 0·05 \) more B-cell depletion \( (95·9 ± 0·1\% \; n = 3 \) within the peripheral blood, than within the spleen, within 7 days of initiating antibody treatment \( (\text{Fig. 3c}) \). This compared to 88·0 ± 0·1% \( (\text{spleen}) \) and 88·1 ± 0·2\% \( (\text{blood}) \) depletion of CD8 T cells.

Figure 3. Depletion of leucocyte subsets by a mouse CD52-specific monoclonal antibody (mAb) in ABH mice. Animals were injected with either: (a) a single injection \( (n = 3 \) or (b, c) five consecutive daily injections \( (n = 3/\text{group}) \) of 250 µg mouse CD52-specific mAb. (a, b) Splenocytes or (c) peripheral blood leucocytes were prepared and stained with directly conjugated fluorescent CD4 (clone RM4.4); CD8, CD45RA/B220 or a combination of CD4, CD25 and Fox3P-specific antibodies. Cells were analysed by flow cytometry. The results represent the mean ± SEM per cent of cells compared with the absolute cell number at baseline. [Colour figure can be viewed at wileyonlinelibrary.com] according to instructions from the manufacturers. It was found that the relative percentages of both CD4 and CD8...
controls) about 3 weeks after the last antibody administration, which was later (Day of relapse onset 56.4 ± 5.4 days in mCD52-treated animals versus 36.7 ± 5.3 days in controls) than observed in untreated controls representing a significant (P < 0.001) delay in the onset of spontaneous relapse when observed until day 80 p.i. Therefore, although the mCD52d mAb was immunosuppressive, it did not induce robust immunological unresponsiveness.

In MS binding and inhibitory responses to alemtuzumab can develop within a month of infusion.22,23 Although the mCD52-specific mAb is of mouse origin, as ABH mice do not express the Igh1a allotype associated with production of IgG2a isotype and largely produce antibodies of the IgG1 and IgA isotypes,24,25 the generation of IgG allotypic/idiotypic antibodies was examined, but was not detected when examined up to 2 months after administration (Fig. 4d).

Mouse CD52-specific mAb inhibits the generation of immunological unresponsiveness

To determine whether mCD52d mAb could facilitate intravenous antigen-specific tolerance induction as
showed with CD4-depleting mAb (Fig. 2d), EAE was inhibited following daily subcutaneous administrations of 250 μg mCD52-depleting from day 10 to day 14 p.i. (Fig. 5a). Intravenous antigen in the form of 2.5 × 10^7 SCH coupled to splenocytes (SCH-SC i.v.) was administered 1 week after the initiation of mAb treatment on day 17 and then animals were rechallenged with SCH 2 weeks after SCH-SC i.v. to assess tolerance induction. The majority of animals (8/10) developed disease (Group score 2.9 ± 1.8 and EAE score 3.8 ± 0.1 in CD52-specific mAb-treated animals) compared with 10/10 animals developing EAE (Group score/EAE 3.6 ± 0.1) about a week after rechallenge (Day of onset 11.6 ± 3.0 in CD52-specific mAb-treated animals versus 10.8 ± 2.0). To counter the argument that insufficient cell numbers were present at the time of SCH-SC i.v. administration to allow unresponsiveness to develop, tolerance induction was therefore assessed at 1 (day 22 p.i.), 2 (day 29 p.i.), and 3 (day 36 p.i.) weeks after the final dose of CD52-specific mAb (Fig. 5a). Animals treated with CD52-specific mAb typically displayed no clinical signs of disease until disease re-induction was attempted at day 43 (Fig. 5a). Although such treatment delayed disease development (P < 0.001) (Table 1), by the termination of the experiment, the majority of mice had developed clinical EAE, regardless of treatment strategy; with 70–90% of animals treated with CD52-specific mAb alone or in combination with intravenous myelin antigens developing EAE (Fig. 5a, Table 1). Furthermore, there was no significant difference in either the mean maximum disease score between groups or the animals that specifically developed within each group (Table 1). This suggests that this CD52-specific mAb was not able to induce immune unresponsiveness, and in comparison to CD4-specific mAb, it could perhaps be blocking tolerance induction by deleting a regulatory cell population.

The induction of unresponsiveness was assessed by supplementing the CD4-specific mAb-induced depletion using CD8 (YTS169.4) and CD20 (18B12) depleting mAb. As shown previously, 250 μg CD8-depleting antibody induces marked depletion and fails to prevent the development of EAE, such that it behaves as the untreated control in terms of incidence, onset and severity. Although CD4 mAb and intravenous antigen induced unresponsiveness that could largely resist an antigen rechallenge as shown previously, it was found that depletion of CD4- and CD8-positive T cells or CD4, CD8 and CD20 cells, developed disease that was comparable to that seen with CD52-mediated depletion (Fig. 5b). The majority (70–90%) of animals treated with CD8-specific mAb developed paralytic disease, supporting the concept that a population of regulatory CD8 T cells were controlling unresponsiveness during EAE. This population could be inhibited as a consequence of CD52-mediated depletion and so could

Figure 5. The combination of CD52-mediated depletion and intravenous antigen does not induce myelin-specific immune unresponsiveness in ABH mice. Experimental autoimmune encephalomyelitis (EAE) was induced in ABH mice by injection of spinal cord homogenate (SCH) in Freund’s complete adjuvant on days 0 and 7. (a) On day 12 post-inoculation (p.i.), mice were untreated or were injected subcutaneously with 250 μg mouse CD52-specific monoclonal antibody (mAb) daily for 5 consecutive days. CD52 mAb animals were untreated or injected intravenously with 2.5 × 10^7 antigen-coupled splenocytes (SCH-SC) at 1, 2 and 3 weeks after the last CD52 mAb injection. Disease was re-induced in all animals on day 43 p.i. with SCH in Freund’s incomplete adjuvant. Results represent the time of onset of disease for each animal from initial immunization. (n = 7–10 mice per group) (b). ABH mice were inoculated with SCH in CFA on days 0 and 7. At day 10 p.i., mice were injected with either 250 μg mouse CD52-specific mAb days 10–14, or a single injection of 250 μg mouse YTS191.1 CD4-specific mAb, or a combination of either 250 μg CD4 and 250 μg CD8-specific (YTS169.4) mAb, or 250 μg CD4, 250 μg CD8 and 250 μg CD20-specific (18B12) mAb intraperitoneally. On day 17 p.i., mice were injected intravenously with 2.5 × 10^7 SCH-coupled splenocytes (SCH-SC). Disease was re-induced at day 28. Untreated animals are shown in black. Results represent the time of onset of disease from initial immunization for each animal (n = 7–10 mice per group). [Colour figure can be viewed at wileyonlinelibrary.com]
Experimental autoimmune encephalomyelitis (EAE) was induced in ABH mice by injection of spinal cord homogenate (SCH) in Freund’s complete adjuvant on days 0 and 7. On day 12 post-inoculation (p.i.), mice were untreated or were injected subcutaneously with 250 μg mouse CD52-specific monoclonal antibody (mAb) daily for 5 consecutive days. CD52 mAb animals were untreated or injected intravenously with 2.5 × 10^7 antigen-coupled splenocytes (SCH-SC) at 1, 2 and 3 weeks after the last CD52 mAb injection. Disease was re-induced in all animals on day 43 p.i. with SCH in Freund’s incomplete adjuvant. Results represent the number of animals that developed disease, the mean day of onset ± SD for each animal from initial immunization for the first attack, the mean ± SEM maximum neurological score of the first attack of all animals in each group (Group Score), the mean ± SEM maximum neurological score of the first attack of animals that developed EAE in each group (EAE Score) and the mean ± SD day of onset of disease (***(< 0.001 between treatment and control).

perhaps contribute the occurrence of secondary autoimmune disease that occurs following use of alemtuzumab in MS.

**Discussion**

This study further supports the view that central nervous system autoimmunity is mediated by the action of CD4 T cells. This molecule appears to contain at least four different domains that can be detected by distinct CD4 mAb clones.26,27 These were used to demonstrate that CD4 mAb can both functionally, by blocking the receptor, and physically deplete T cells to inhibit disease. In young adult mice, CD4 T-cell depletion lasted about 2–3 weeks before returning to about 30% of their original levels, which was not sufficient to control autoimmunity.3,4 It was found that a functional depletion about 70% of baseline levels is needed to inhibit disease in an optimized animal system. In humans studies, CD4 depletion mAb also only induced a transient depletion and T-cell numbers also rapidly repopulated within a month10 followed by a persistent lymphopenia for many months.10,25,28 Furthermore, as found in mice also,4 this treatment targeted the naive CD4 T-cell subsets and there was particularly rapid repopulation of the CD40RO memory T-cell subset,28,29 which would contain the pathogenic T cells. It was found that depletion of T cells by about 50–60% failed to stop EAE from developing in this optimized system. This contrasts with the marked 70–95% depletion of CD4 T cells induced by alemtuzumab.11 This level of depletion by CD4-specific mAb, including the lack of targeting of CD45RO+ T cells, suggests that the trials in MS were unlikely to have succeeded.6 This perceived failure questioned the role of CD4 T cells in MS and also supported the devaluing of the use of animal models of MS.8 However, such a failure could have been predicted if a few simple experiments had been performed before embarking on a clinical trial programme. This represents a further example of how human activity and trial design, rather than animal studies, probably contributed to the failure to translate.2 Interestingly, however, CD4-specific mAb were not inert and there was a modest and significant reduction in relapse rate in MS.6 However, despite the suggestion that efficacy was related to T-cell number; radiation maintenance of depleted levels of T cells were not attempted further, until alemtuzumab was used.

Alemtuzumab targets CD52-expressing cells and produces rapid, marked (< 200 cells/mm^3) and sustained CD4 and CD8 T-cell depletion lasting many months, whereas there is rapid repopulation of CD19+ B cells to levels above baseline.11 Importantly, this resulted in the inhibition of lesion formation and relapsing disease and alemtuzumab is currently one of the most effective licensed treatments of MS.12 The effectiveness of alemtuzumab is used to support the concept that T cells drive relapsing disease, which can be clearly shown in animal models. It is therefore of interest that it has been reported that disease activity can sometimes be associated with CD4+ T-cell reconstitution,30,31 such that counts below 390 CD4 T cells/mm^3 (about 55–60% depletion) predicted stability.30 This is consistent with the hypothesis that a threshold of T-cell depletion is required to effectively target the pathogenic T-cell pool. The data obtained from CD4-depleting mAb trials in MS may therefore provide only a weak argument against T cells being important in MS and indeed long-term and marked (95–70%)

| Group                              | No.EAE/Total | Group max. score ± SEM | EAE max. score ± SEM | Mean day of onset ± SD |
|------------------------------------|--------------|------------------------|----------------------|------------------------|
| Untreated                          | 10/10        | 3.9 ± 0.1              | 3.9 ± 0.1            | 16.3 ± 3.9             |
| CD52 mAb                           | 9/10         | 3.2 ± 0.4              | 3.6 ± 0.1            | 50.2 ± 1.5***          |
| CD4mAb + 1 week SCH-SC             | 5/7          | 2.5 ± 0.7              | 3.5 ± 0.3            | 54.0 ± 1.4***          |
| CD4mAb + 2 weeks SCH-SC            | 9/10         | 3.2 ± 0.4              | 3.5 ± 0.2            | 53.1 ± 1.7***          |
| CD4mAb + 3 weeks SCH-SC            | 6/8          | 2.8 ± 0.6              | 3.7 ± 0.2            | 48.7 ± 8.4***          |
| Untreated                          | 10/10        | 3.9 ± 0.1              | 3.9 ± 0.1            | 16.4 ± 2.2             |
| CD4mAb + SCH-SC                    | 2/10         | 0.6 ± 0.4***           | 3.0 ± 0.5            | 34.0 ± 0.0***          |
| CD4 and CD8 mAb + SCH-SC           | 7/10         | 2.7 ± 0.6              | 3.9 ± 0.1            | 27.3 ± 6.4***          |
| CD4,CD8 and CD20 + SCH-SC          | 9/10         | 3.2 ± 0.4              | 3.5 ± 0.2            | 28.4 ± 6.9***          |

**Table 1.** The combination of CD52-mediated depletion and intravenous antigen does not induce immune unresponsiveness in ABH mice
depletion of CD4- and CD8-positive T cells with alem-
tuzumab are associated with inhibition of the develop-
ment of relapses in MS.12

In this study mCD52-depleting mAb also depleted T
cells and inhibited the development of relapsing EAE,
which is associated with reduced immune infiltration and
consequent reduced demyelination and neuroprotec-
tion.2,19 The depletion profiles appeared relatively similar
to that seen in hCD52 transgenic CD-1 mice injected
with alemtuzumab and SJL and C57BL/6 mice injected
with the mCD52-depleting mAb.19,32,33 However in con-
trast to proteolipid protein-induced relapsing EAE in SJL
mice,19 inhibition of EAE was more transient and disease
often returned, as has been seen after cessation of treat-
ment with immunosuppressive agents.4,34 Furthermore,
disease onset could be accelerated following reactivation
of antigen-specific primed cells with an antigen rechal-
genge, which can occur in both athymic and euthymic
mice following CD4 T-cell depletion.4 Therefore, there is
some variability in responsiveness of individual mouse
models related to depletion status and it is clear that
there is some variability in the responsiveness of people
to alemtuzumab.13

The use of animals also permits the investigation of tis-
ues typically inaccessible in humans, and differences were
found between the peripheral blood and secondary lym-
phoid organs. Consistent with other studies,32,33 there
was less marked depletion of B cells within the lymphoid
tissues compared with the peripheral blood compared
with T cells.32,33 This may relate to the mechanism of
depletion of alemtuzumab that centres on antibody-
dependent cellular cytotoxicity rather than complement
fixation.32 Although ABH mice express normal comple-
ment components, unlike some laboratory mice, and can
promote complement-mediated lysis by antibodies,35,36
ABH mice have abnormal Fc receptors that could influ-
ence function.37 This may account for subtle differences
in depletion kinetics between mouse strains.19 However,
if humans do not show marked depletion of B cells in
their lymphoid tissue, and possibly bone marrow, this
could facilitate the rapid (hyper)repopulation of the
peripheral blood after alemtuzumab treatment.11 This
may contribute to the development of secondary B-cell
autoimmune diseases that are common following treat-
ment of people with MS, who are genetically prone to
autoimmunity, with alemtuzumab.11,13 This does not
occur with similar frequency in people with cancer, when
they are treated with alemtuzumab.38

We have shown also that a transient depletion of CD4
T cells followed by tolerogenic delivery15 of pathogenic
antigen can induce robust unresponsiveness that effect-
tively silences established, relapsing disease.4 This acts via
multiple mechanisms that involve central and peripheral
depletion, anergy and also active suppression of T- and
B-cell responses, as shown previously.4,15,21 CD52

lymphocyte depletion was used to determine whether
CD52-specific mAb had value as a depleting agent, either
as a prelude to antigen-specific therapy to inhibit MS, or
as a method to induce antigen-specific tolerance to limit
secondary autoimmune diseases. However, it appeared
that rather than facilitating the induction of unrespon-
siveness, CD52 depletion was actually inhibiting tolero-
genic mechanisms. This appeared to occur through
inhibition of a CD8 T-cell function as additional deple-
tion of CD8+, but not CD20+, cells largely inhibited the
tolerogenic potential of CD4-specific depleting antibodies.
Although there is much interest in the pathogenic poten-
tial of CD8+ T cells in MS, it is well established that this
subset has regulatory activity that may control autoim-

munity.38

Furthermore, although it has been reported that alem-
tuzumab induces a relative sparing of Fox3P positive, T
regulatory cells as shown here, which favours the regu-
lation of CD4 T cells.41,42 in MS there is a substantial reduc-
tion in the absolute number of regulatory T cells and
CD8+ T cells.11,14 Hence, following alemtuzumab treat-
ment, B cells repopulate in the relative absence of CD4 T
regulatory cells and CD8 T suppressor cells. These T regu-
latory cells can control silencing of autoreactive immature
B cells exiting the bone marrow43,44 and furthermore T
cells control the affinity maturation of antibodies within
lymphoid tissue that may lead to B-cell autoactivity.35,46

The loss of immune-tolerance and B-cell hyper-repopu-
lation,11 may underlie the high prevalence (about 50% within
a median of 7 years13) of B-cell autoimmune dis-
es occurring as a consequence of alemtuzumab treat-
ment.13,14 Furthermore, it may contribute also to the
marked occurrence of alemtuzumab-specific antibodies
that occur in > 80%, higher than seen with other human-
ized antibodies,47 of people treated with alemtuzumab.32,23
Although these do not appear to eliminate immune deple-
tion,23 the development of binding or neutralizing anti-
bodies could impact the therapeutic activity of the
antibody in people requiring repeated infusions that are
needed in some people with MS.13 In this study, B-cell
hyper-proliferation and anti-globulin responses to the
mCD52 mAb were not detected; however, although ABH
mice are susceptible to a variety of different induced
autoimmune diseases and can produce high titre autoanti-
bodies,48 performing studies in animals that are genetically
prone to spontaneous B-cell autoimmune diseases, may be
more suitable to try and model mCD52 mAb-induced sec-
ondary autoimmune illness. Likewise, further studies are war-
ranted to define the CD8 T-cell regulatory mechanisms.

Interestingly, B-cell hyper-repopulation and marked
loss of T cells does not occur following B-cell depletion
with hCD20-specific antibodies or cladribine in people
with MS, both of which control relapsing MS, but are
not associated with the development of secondary
autoimmune diseases.49,50 They do however occur in
non-alemtuzumab-induced, non-ablative haematopoietic stem cell therapy, which is also associated with delayed onset of B-cell autoimmune diseases.\textsuperscript{51} Therefore, B-cell repopulation and perhaps auto sensitization occur in an environment that is deficient in T-cell regulation and allows autoimmunity to be initiated. This can then become manifest once CD4\textsuperscript{+} T-cell help is repopulated to levels that can facilitate autoantibody production and so account for a relative delay in the development of autoimmune responses relative to the B-cell repopulation kinetics.\textsuperscript{11–13} As people taking alemtuzumab require regular monthly blood sampling as part of their care package, monitoring the development of antigen-specific (such as thyroid or, more easily, alemtuzumab-specific) B-cell and T-cell function may provide a useful model system to study the development of human autoimmunity. Furthermore, methods to limit B-cell hyper-population or promote reconstitution of CD8\textsuperscript{+} T-cell regulation may provide a method to limit the unwanted adverse effects of this very effective treatment of multiple sclerosis.

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References

1 Compton A, Coles A. Multiple sclerosis. Lancet 2008; 372:1502–17.
2 Baker D, Amor S. Experimental autoimmune encephalomyelitis is a good model of multiple sclerosis if used wisely. Mult Scler Relat Disord 2014; 3:55–84.
3 O’Neill JK, Baker D, Davison AN, Allen SJ, Butter C, Waldmann H et al. Control of immune-mediated disease of the central nervous system with monoclonal (CD4-specific) antibodies. J Neuroimmunol 1993; 45:1–14.
4 Pyce G, O’Neill JK, Crawford JL, Amor S, Hankey DJ, East E et al. Autoimmune tolerance eliminates relapse but fails to halt progression in a model of multiple sclerosis. J Neuroimmunol 2005; 168:41–52.
5 Sun D, Whitaker JN, Huang Z, Liu D, Coleclough G, Wyrkeley H et al. Myelin antigen-specific CD8\textsuperscript{+} T cells are encephalitogenic and produce severe disease in C57BL/6 mice. J Immunol 2001; 166:7579–87.
6 van Oosten BW, Lai M, Hodgkinson S, Barakh F, Miller DH, Mosley IF et al. Treatment of multiple sclerosis with the monoclonal anti-CD4 antibody CM-T142: results of a randomized, double-blind, placebo-controlled, MR-monitored phase II trial. Neurology 1997; 49:351–7.
7 Rumbach L, Racadot E, Armpach J, Namer D, Bonville IF, Wijdemans J et al. Biological assessment and MRI monitoring of the therapeutic efficacy of a monoclonal anti-T CD4 antibody in multiple sclerosis patients. Mult Scler 1996; 2:107–12.
8 Strom A, Steiner I. Experimental allergic encephalomyelitis: a misleading model of multiple sclerosis. Ann Neurol 2005; 58:839–45.
9 Wofsy D. Regulation of immunity by anti-T cell antibodies. West J Med 1990; 153:265–8.
10 Lindsey JW, Hodgkinson S, Mehta R, Siegel RC, Mitchell DJ, Lim M et al. Phase 1 clinical trial of chimeric monoclonal anti-CD4 antibody in multiple sclerosis. Neurology 1994; 44:413–9.
11 Coles AJ, Wing M, Smith S, Corradu F, Greer S, Taylor C et al. Pooled monoclonal antibody treatment and autoimmune thyroid disease in multiple sclerosis. Lancet 1999; 354:1691–5.
12 Cohen JA, Coles AJ, Arnold DL, Confavreux C, Fox EJ, Hartung HP et al. Alemtuzumab versus interferon \(\beta\)1a as first-line treatment for patients with relapsing– remitting multiple sclerosis: a randomized controlled phase 3 trial. Lancet 2012; 380:1819–28.
13 Tuohy O, Costeloe L, Hill-Cawthorne G, Bjornson I, Harding K, Robertson N et al. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy. J Neurol Neurosurg Psychiatry 2015; 86:208–15.
14 Willis MD, Harding KE, Pickersgill TP, Wardle M, Pearse OR, Saldling NJ et al. Alemtuzumab for multiple sclerosis: long term follow-up in a multi-centre cohort. Mult Scler 2016; 22:1215–23.
15 Lanoue A, Bona C, von Boehmer H, Sarukhan A. Conditions that induce tolerance in mature CD4\textsuperscript{+} T cells. J Exp Med 1997; 185:405–14.
16 Lutterotti A, Yousef S, Spurtzik A, Stürner KH, Stellmann JP, Breiten P et al. Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. Sci Transl Med 2013; 5:180ra75.
17 van Noort DM, Baby M, Nicken PJ, Verbeek R, Vennema H. Therapeutic intervention in multiple sclerosis with \(\beta\)-Crystallin: a randomized controlled phase IIa trial. PLOS ONE 2013; 10:e0143366.
18 Al-Izki S, Pryce G, O’Neill JK, Butter C, Giovannoni G, Amor S et al. Practical guide to the induction of relapsing progressive experimental autoimmune encephalomyelitis in the B10.AH mouse. Mult Scler Relat Disord 2012; 1:29–38.
19 Turner MJ, Pang PT, Chertien N, Havari E, Lalor-McE N, Oliver J et al. Reduction of inflammation and preservation of neurological function by anti-CD52 therapy in murine experimental autoimmune encephalomyelitis. J Neuroimmunol 2015; 285:4–12.
20 Aloja A, Shape J, Dunn R, Kasigian M, Kehry MR, Shlomchik MJ. Depletion of B cells in murine lupus: efficacy and resistance. J Immunol 2007; 179:3351–61.
21 Smith PA, Morris-Dowm M, Heijmans N, Pryce G, Arter S, O’Neill JK et al. Epitope spread is not critical for the relapse and progression of MOG 8-21 induced EAE in Biozzi ABH mice. J Neuroimmunol 2005; 164:76–84.
22 Sommerfield J, Hill-Cawthorne GA, Lin A, Zandi MS, McCarthy C, Jones JL et al. A novel strategy to reduce the immunogenicity of biological therapies. J Immunol 2010; 185:763–8.
23 Ziemsen T, Arnold DL, Cohen JA, Coles AJ, Fox EJ, Hartung HP et al. Immunogenicity of alemtuzumab does not impact safety and efficacy in relapsing remitting multiple sclerosis patients in the CARE-MS I study. J Mult Scler 2013; 19 (Suppl. 1):212–3.
24 Sant’Anna OA, Mouton D, Danese OM, Bouthillier Y, Mesli JC, Reis MH et al. Basal immunoglobulin serum concentration and isotype distribution in relation to the polygenic control of antibody responsiveness in mice. Immunogenetics 1985; 22:131–9.
25 Martin RM, Brady JL, Low AM. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. J Immunol Methods 1998; 212:187–92.
26 Vignali DA, Vignali KM. Prolonged enhancement of T cell activation mediated by the interaction between the TCR and the D3 domain of CD4. J Immunol 1999; 162:1431–9.
27 Dianzani U, Shaw A, al-Ramadi BK, Kubo RT, Inawae CA. Physical association of CD4 with the T cell receptor. J Immunol 1992; 148:678–80.
28 Renton MJ, van Oosten BW, Roos MT, Adair HE, Polman CH, van Lier RA. Treatment with depleting CD4 monoclonal antibody results in a preferential loss of circulating naïve T cells but does not affect IFN-\(\gamma\) secreting TH1 cells in humans. J Clin Invest 1997; 99:2225–31.
29 Llewellyn-Smith N, Lai M, Miller DH, Rudge P, Thompson AJ, Cutzner ML. Effects of anti-CD4 antibody treatment on lymphocyte subsets and stimulated tumor necrosis factor \(\alpha\) production: a study of 29 multiple sclerosis patients entered into a clinical trial of CM-T142. Neurology 1997; 48:810–6.
CD52 depletion in CNS autoimmunity

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Figure S1. CD4-binding epitopes. 
Table S1. CD4-binding epitopes.