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B-Cell Reconstitution and BAFF After Alemtuzumab (Campath-1H) Treatment of Multiple Sclerosis

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Abstract
Introduction Treatment with alemtuzumab is highly effective in relapsing–remitting multiple sclerosis; however, 30% of patients develop autoimmunity. Alemtuzumab (previously called Campath 1-H) induces a prolonged T-cell lymphopenia with memory cells dominating the reconstituting T-cell pool for at least 3 months.
Results Here we show that B-cell recovery is rapid, returning to baseline by 3 months and rising to 165% of baseline by 12 months after treatment. Immature transitional 1 B cells are the predominant cell type 1 month after treatment. This coincides with a surge in serum B-cell activating factor (BAFF), which remains elevated by 33% for at least 12 months after alemtuzumab. BAFF is critical for transition to the mature naive B-cell phenotype, which dominates from 3 months after alemtuzumab. Differentiation to memory B cells is slow so there are radical and prolonged alterations to the B-cell pool after alemtuzumab.

Keywords BAFF: B-cell activating factor · B cells · autoimmunity · reconstitution · T1 B cell: transitional type I B cell

Introduction

The homeostatic response to lymphocyte depletion has been difficult to study in humans because lymphocyte-depleting agents have traditionally been used in complicated clinical situations, such as autologous hematopoetic stem-cell transplantation, compounded by infections and multiple immunotherapies. However, in recent years, selected cohorts of fit immunotherapy-naive patients have been exposed to lymphocyte-depleting monoclonal antibodies. They provide an opportunity to study human lymphocyte homeostasis. Here, we report for the first time on B-lymphocyte subgroup reconstitution after repeated lymphocyte depletion by alemtuzumab.

The specific context is alemtuzumab treatment of multiple sclerosis (MS). Alemtuzumab, previously known as Campath-1H, causes prolonged lymphopenia and is a highly effective treatment of early relapsing–remitting multiple sclerosis, reducing both the risk of relapse and of accumulation of disability by over 70% when compared to the standard licensed therapy, interferon beta [1]. We speculate that this efficacy may be due not only to depletion of T lymphocytes, classically considered pathogenic in multiple sclerosis [2], but also to B-cell depletion. A role for B cells in the pathogenesis of the multiple sclerosis is suggested by the universal presence of oligoclonal immunoglobulin bands in the cerebrospinal fluid of patients, the partial efficacy of rituximab [3], and the identification of B-cell lymphoid follicles in the meninges of some patients with MS [4–6].

Alemtuzumab is a humanized monoclonal antibody directed against CD52, a glycoprotein found on the surface of all differentiated lymphocytes and monocytes but importantly not on hematological precursors [7]. Therefore, the reconstitution of lymphocytes after alemtuzumab indirectly indicates the ability of precursors to regenerate the mature lymphocyte repertoire. We have previously shown that treatment with a single dose of alemtuzumab leads to a rapid and prolonged T-lymphopenia, with complex kinetics of T-cell reconstitution. CD4+ T cells

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are particularly slow to recover, remaining depleted for at least 5 years. Initially, the reconstituting CD4+ T-cell pool is dominated by CD45RO CD4+ memory T cells, and within the first 3 months following alemtuzumab, the pool is enriched for cells with a regulatory cell phenotype, i.e., CD25high CD4+ cells [8]. In contrast, we showed that the recovery of total B cell number is rapid, indeed at times superseding baseline numbers, to fill the "empty space" left by T cells [8].

Now for the first time, we show that alemtuzumab induces profound and prolonged alterations in the reconstituted B lymphocyte pool, which might contribute to the efficacy and adverse effects of alemtuzumab treatment. We also show that these changes correlate with alterations in serum B-cell activating factor (BAFF, also known as BLyS) but not other factors thought to be involved in B-cell survival and differentiation.

Materials and Methods

Patients

This was a cross-sectional study of 78 patients (53 female; mean age 31.8 years, range 17–48 years) and 13 healthy controls (12 female; mean age 31.8 years, range 17–55 years). All patients had active relapsing–remitting multiple sclerosis and were participants in one of two clinical trials, CAMMS-223 and CAMMS-224 (REC 02/315 and 03/078), in which alemtuzumab is given by intravenous infusion of 12–24 mg/day for 5 days, followed by retreatment with a reduced dose (12–24 mg/day for 3 days) at 12 and 24 months. Serum was collected from a subcohort of ten patients followed longitudinally. Patients and controls consented to venesection for research purposes (LREC 02/263), and all were free from exposure to other disease-modifying agents, including steroids, for at least 1 month at the time of blood sampling. Venous blood samples were taken prior to treatment and at 1, 3, 6, 9, and 12 months following each alemtuzumab treatment. For cross-sectional studies, at least ten patients were studied at each time point.

Cell Surface Staining

Peripheral blood mononuclear cells were isolated from heparinized blood by centrifugation over a density gradient (Ficoll-Paque Plus, Amersham Pharmacia Biotec). T1 B lymphocytes (CD19+/CD27−/CD23−), mature naive or mature naïve B lymphocytes (CD19+/CD27−/CD23+), and memory B lymphocytes (CD19+/CD27+) were identified by staining with the following conjugated mouse antihuman monoclonal antibodies: CD19 APC (BD Pharmingen 555415), CD27 R-PE (BD Pharmingen 555441), CD23 FITC (Serotec MCA1931F), and CD23 R-PE (Serotec MCA1931PE). Isotype controls were IgG1 FITC (Serotec MCA928F), IgG2a FITC (Serotec MCA929F), IgG2a PE (Serotec MCA929PE), IgG1 PE (Serotec MCA928PE), and IgG1 κ APC (BD Pharmingen 555751). Fluorescence was detected using a FACScalibur Becton Dickinson flow cytometer. A minimum of 30,000 events were collected within a lymphocyte gate drawn on the basis of forward and side scatter. Data were analyzed using WinMDI 2.8 (http://facs.scripps.edu/software.html).

Enzyme-Linked Immunosorbent Assays

Sera collected from ten patients before and at 1, 3, 6, 9, and 12 months after alemtuzumab were assayed for soluble BAFF, B-cell maturation factor (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and a proliferation-inducing ligand (APRIL) by sandwich-enzyme immunoassay according to the manufacturer's instructions (R & D Systems: DBLYS0, DY193, DY174 and Bender Medsystems: BMS2008, respectively). Data were compared to ten healthy controls.

Statistics

Data were analyzed using SPSS 12.0.1 for Windows. Following assessment for normality, parametric (Student's *t* test) or nonparametric (Wilcoxon Mann–Whitney) tests were performed. *p* values are stated throughout the text, a value of $p<0.05$ was considered as statistically significant, modified by a Bonferroni correction where indicated.

Results

Alemtuzumab Induces a Prolonged T-Cell Lymphopenia

T cells, B cells, and monocytes were undetectable in peripheral blood immediately following each treatment with alemtuzumab (data not shown). Cell numbers recovered but at varying rates. Monocytes recovered to 81% of baseline by month 3, then remained at this level, with a nonsignificant trend towards incomplete recovery, following further cycles of alemtuzumab treatment (Fig. 1). As previously shown, CD4+ T cells were slow to reconstitute, reaching 8.8% of baseline by month 3 and only 36% of baseline by month 12 (0.893×10^9/L at baseline vs. 0.078×10^9/L at month 3 and 0.322×10^9/L at month 12; $p<0.001$ for all comparisons; Fig. 1). CD8+ T cells recovered more rapidly, reaching 13% of baseline by month 3 and 71% by month 12 (0.340×10^9/L at baseline vs. 0.044×10^9/L at month 3, $p<0.0001$ and 0.242 at month 12, $p=0.13$; Fig. 1).

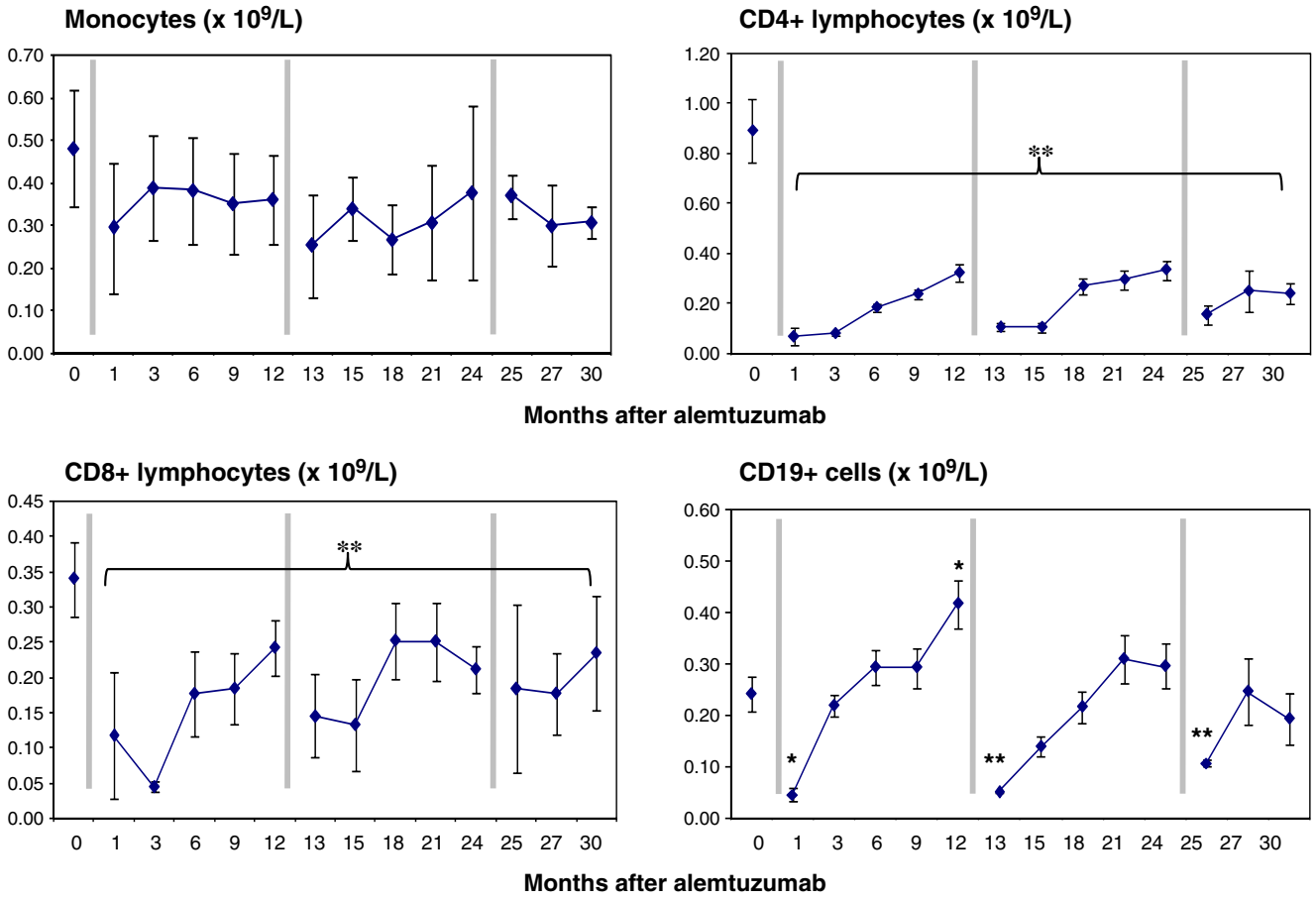

Fig. 1 Monocyte, CD4+ T cell, CD8+ T cell, and CD19+ B-cell reconstitution following repeated cycles of alemtuzumab. Immediately following each cycle of alemtuzumab treatment (indicated by *vertical gray bars*), monocytes and lymphocytes were undetectable in peripheral blood. Cell numbers recovered over time but at varying rates, with CD4+ T cells being particularly slow to recover. Repeated dosing did not significantly affect cell recovery after alemtuzumab. Statistical test used was Mann–Whitney *U*, using $p<0.0036$ and $p<0.0007$, and Bonferroni corrections of $*p<0.05$ and $**p<0.01$, respectively. *Error bars* indicate standard deviation

The rate and pattern of and T-cell reconstitution was similar following each dose of alemtuzumab (Fig. 1).

Recent Bone Marrow Emigrants Dominate the Early Reconstituting B-Cell Pool

There was no difference in B-cell subset numbers between untreated patients with multiple sclerosis and healthy controls (data not shown). After alemtuzumab, total B-cell number returned to baseline by month 3 (0.24×10^9/L at baseline vs. 0.22×10^9/L at 3 months; Fig. 1) and then continued to rise further, reaching 165% of baseline by 12 months (0.24×10^9/L at baseline vs. 0.39×10^9/L at 12 months; $p=0.002$), although numbers never reached this supernormal level after subsequent treatments.

At 1 month after alemtuzumab, the depleted B-cell pool was dominated by recent bone marrow emigrants, that is, cells with a T1 phenotype (CD19+/CD23−/CD27−) (53.8% of the CD19+ B-cell pool at month 1 vs. 7.3% at baseline, $p=1.1\times10^{-6}$; Fig. 2). Absolute T1 numbers tended to be lower at month 1 when compared to baseline, but this was not statistically significant (0.01 at month 1 vs. 0.02 at baseline). By 3 months, absolute T1 cell numbers increased, but the proportion of T1 cells within the B-cell pool fell as other B-cell phenotypes emerged (53.8% at month 1 compared to 19% at month 3, 15% at month 6, 18% at month 9, and 13% at month 12; $p<1.5\times10^{-4}$ for all comparisons).

Mature Naive B Cells Dominate Late Reconstitution of B Cells After Alemtuzumab

We defined a subset of B cells as "mature naïve," which were intermediate between T1 and memory by virtue of CD19 and CD23 positive staining but CD27 negative. At 1 month posttreatment, mature naive B-cell numbers were significantly reduced compared to baseline (0.002×10^9/L at month 1 vs. 0.148×10^9/L at baseline; $p=5.7\times10^{-7}$; Fig. 2), representing 18% of the circulating B-cell pool. By month

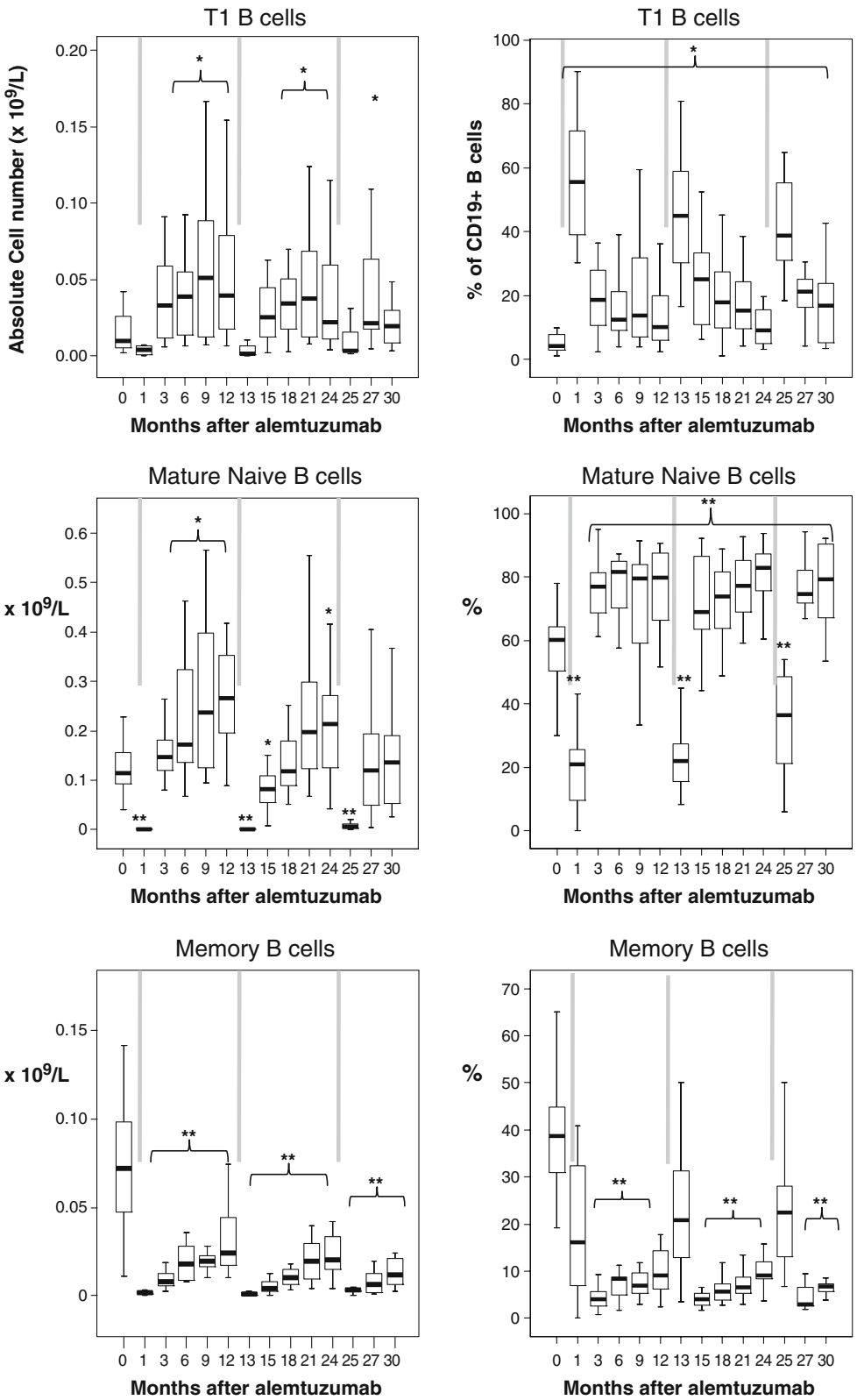

Fig. 2 Absolute number of circulating cells ($\times 10^9$/L) and percentage of circulating CD19+ B cells with three phenotypes: T1, mature naive, and memory B cells. Statistical test used was Mann–Whitney *U*, using $p<0.0036$ and $p<0.0007$, and Bonferroni corrections of $*p<0.05$ and $**p<0.01$, respectively. *Error bars* indicate standard deviation

3, mature naive cell numbers had returned to just above baseline numbers (0.17×10^9/L at month3 vs. 0.15×10^9/L at baseline), then continued increasing, dominating the B-cell pool (0.28×10^9/L at month6; $p=0.016$; 0.26×10^9/L at month9; $p=0.006$ and 0.28×10^9/L at month12; $p=0.0002$). As a result, mature naive B-cell numbers had expanded to 165% of baseline, representing ~75% of the total B-cell pool at all time points beyond month3. After

the second treatment, there was an increase in mature naïve B-cell numbers but only significantly at 24 months (month 24, 0.228×10^9/L vs. 0.15×10^9/L at baseline; $p=0.023$). Proportionally, this subgroup significantly dominated the pool 3 to 12 months after all subsequent retreatments.

Alemtuzumab Induces Prolonged Depletion of Memory B Cells

At month 1 post-alemtuzumab CD27+, memory cell numbers were significantly reduced compared to baseline (0.0014×10^9/L at month 1 vs. 0.92×10^9/L at baseline; $p=9.5\times10^{-9}$; Fig. 2), representing 19% of the circulating B-cell pool. CD27+ memory cell numbers continued to increase slowly but remained low even 12 months later (0.038×10^9/L at month 12 vs. 0.092×10^9/L at baseline), reaching only 25% of baseline at month 12. At month 1, due to a relative absence of mature naive cells, memory B cells remained a significant proportion of the B-cell pool. But over subsequent months, as mature naive cells increased proportionally, memory B cells became the least common constituent of the depleted B-cell pool.

There was no difference in the frequency of T1 cells, mature naive cells, or memory B cells with repeated cycles of alemtuzumab treatment (e.g., T1 cells, 15% vs. 20% vs. 16% of total B-cell pool at 6 months following the first, second, and third doses of alemtuzumab; mature naive cells, 78% vs. 72% vs. 79%; and memory B cells, 7% vs. 7% vs. 6% of the total B-cell pool at the same time points).

Serum BAFF Is Increased Following Alemtuzumab

Serum BAFF increased threefold by month 1 posttreatment (4,595 pg/mL vs. 1,278 pg/mL at baseline; $p=4.3\times10^{-5}$), and, despite falling from months 1 to 3, remained significantly elevated compared to baseline (Fig. 3). This was true for all subsequent time points (baseline BAFF 1,278 pg/mL vs. 2,068 pg/mL at month 3; $p=0.002$; 1,931 pg/mL for month 6; $p=0.003$; 1,872 pg/mL for month 9; $p=0.001$; 1,704 pg/mL for month 12; $p=0.017$). A similar pattern was observed after repeat courses of alemtuzumab with levels even higher than baseline from months 15 to 24 ($p<0.007$). There was no significant difference in the serum level of BAFF, APRIL, BCMA, or TACI between healthy controls and untreated patients with multiple sclerosis. Serum levels of APRIL, BCMA, and TACI were unchanged following treatment with alemtuzumab (data not shown).

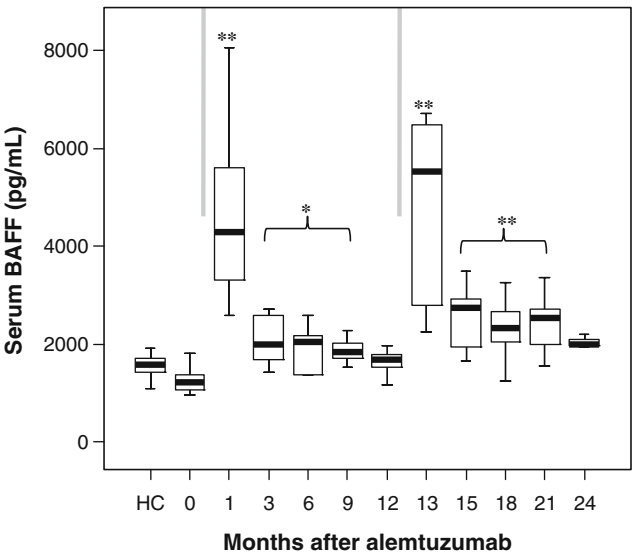

Fig. 3 Levels of serum BAFF in ten patients followed longitudinally before and after treatment with alemtuzumab. Statistical test used was Mann–Whitney *U*, using $p<0.0042$ and $p<0.00083$, and Bonferroni corrections of $*p<0.05$ and $**p<0.01$, respectively. *Error bars* indicate standard deviation

Discussion

Treatment of multiple sclerosis with alemtuzumab provides a rare opportunity to study the kinetics of lymphocyte reconstitution in humans, free from confounds of infections and concomitant immunotherapies of more complex clinical contexts such as hematopoietic stem cell transplantation. Here, we show that B-cell reconstitution recapitulates the early stages of B-cell development after alemtuzumab but then arrests prematurely.

We have replicated our previous observation that a single dose of alemtuzumab induces a prolonged T-cell lymphopenia, with memory T cells dominating the reconstituting T-cell pool for at least 3 months following treatment [8,9]. We have gone on to show that repeated cycles of alemtuzumab do not have a significant effect on the kinetics of T cell, B cell, or monocyte reconstitution, nor did repeated dosing influence the pattern of regeneration seen within the B-cell pool.

In the bone marrow, B-cell precursor cells proliferate and progress through a series of tightly regulated steps culminating in the production of immature, surface immunoglobulin expressing B cells. These newly produced, immature cells, termed transitional type-1 cells (T1), leave the bone marrow and migrate to the spleen where they differentiate first into transitional type-2 cells and then into mature naive B cells (follicular or marginal zone) before migrating to secondary lymphoid tissue where, in response to antigenic stimulation, they differentiate into effector cells.

Here, using the cell surface markers CD19, CD23, and CD27 (a marker for memory B cells [10]), we explored B-cell reconstitution following repeated dosing with alemtuzumab. Immediately after treatment, the majority of B

cells detectable in the periphery are T1 cells, trafficking from bone marrow to spleen, at an unchanged rate from before treatment. Their phenotype closely resembles that of immature B cells within the bone marrow, and they can be distinguished from mature naive cells on the basis of low CD23 expression [11]. Over subsequent months after alemtuzumab, peripheral T1 numbers increase, presumably reflecting a homeostatic response to B-cell depletion. But from 3 months after alemtuzumab, the reconstituting B-cell pool becomes dominated proportionally by mature naive cells (intermediate between T1 and memory, by virtue of CD19 and CD23 positive staining, but CD27 negative). This could be driven by survival signals from BAFF [12,13], levels of which are greatly increased 1 month after treatment and then reduced to a level above baseline for at least 12 months after each alemtuzumab cycle, as mature naive numbers continued to expand. BAFF (also known as BLyS, TALL-1, THANK, and zTNF4) and APRIL have emerged as crucial factors for B-cell survival, differentiation, germinal center formation, and immunoglobulin production. They are produced by monocytes, macrophages, and dendritic cells. They bind to B cells through three different receptors: BAFF-R, BCMA, and TACI. Previous work has shown that BAFF mediates the survival of peripheral immature B cells. BAFF-deficient mice have B cells arrested at the T1 stage of development with a loss of T2, follicular and marginal zone (MZ) B cells undergoing rapid apoptosis [13]. This can be reversed with anti-μ antibodies providing the B-cell receptor (BCR) signaling enabling T2 cells to survive and differentiate into mature B cells [12]. BAFF transgenic mice have increased B-cell numbers and develop a systemic lupus erythrematosis (SLE)-like syndrome [14]. In contrast to BAFF, we have demonstrated that serum levels of APRIL (an important factor in immunoglobulin class switching [15–17] and plasma cell survival [18]) and two generic BAFF/APRIL receptors, BCMA and TACI, are unchanged after alemtuzumab (data not shown; there is no established assay for serum levels of the selective BAFF receptor, BAFF-R).

In contrast to immature cells, CD27+ memory B-cell recovery after alemtuzumab is slow, reaching only 25% of baseline by month 12. T cell help is normally required for the development of a memory B cell. The slow recovery of T cells after alemtuzumab could in part explain the delay in memory B-cell reconstitution; however, a similar "maturation arrest" has also been described following B-cell depletion with rituximab [19–21] and in the context of hematopoietic stem cell transplantation [22]. Normal differentiation of mature naive to mature B cells requires BCR-mediated diacylglycerol and calcium signals via a BTK- (Bruton's tyrosine kinase) and PLC-2-dependent (Phospholipase C) mechanism [23]. Prolonged memory B-cell lymphopenia may, in part, explain alemtuzumab's efficacy in multiple sclerosis; in the context of rituximab treatment of rheumatoid arthritis, B-cell memory reconstitution was associated with increased disease activity [21]. However, there is no indication of lost immune competence after alemtuzumab: total serum immunoglobulin concentrations are unaffected [24], and there are surprisingly few infections after alemtuzumab [1], suggesting the retained capacity to respond to a variety of new and old pathogens. We are currently examining the ability of patients to respond to novel and recall antigens in a formal clinical study.

In fact, patients after alemtuzumab are at greater risk of autoimmunity than serious infection, with 20% developing autoimmune thyroid disease, 3% developing autoimmune thrombocytopenia, and single cases of other autoimmune diseases [1,24]. We note that elevated levels of BAFF have been associated with antibody-mediated autoimmunity in animals [25] and in patients with B cell related autoimmune diseases including SLE [26], idiopathic thrombocytopenic purpura [27], Sjogren's syndrome [28], and Wegener's granulomatosis [29]. Additionally, a predominance of T2 B cells in the germinal centers of salivary glands, with up-regulated BAFF levels locally, is a feature of Sjogren's syndrome [30]. Although the mechanism by which BAFF precipitates autoimmunity is not fully understood, it is likely to include the rescue of self-reactive B cells from deletion during the later stages of maturation [31]. The predilection for thyroid disease seen after alemtuzumab and why autoimmunity does not include a return of multiple sclerosis is currently unexplained.

In summary, we have shown that repeated lymphocyte depletion with alemtuzumab does not impair the capacity of the immune system for regeneration. And we demonstrate that, in contrast to our previous observations on T cells, B-cell reconstitution after alemtuzumab results in a pool skewed towards immature phenotypes, driven by a brisk BAFF response, with a slow recovery of B memory cells.

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