Immune status following alemtuzumab treatment in human CD52 transgenic mice

Michael J. Turner *, Michael J. LaMorte, Nathalie Chretien, Evis Havari, Bruce L. Roberts, Johanne M. Kaplan, William M. Siders

Neuroimmunology Research, Genzyme, a Sanofi Company, 49 New York Avenue, Framingham, MA 01701, United States

ABSTRACT

Alemtuzumab is a monoclonal antibody against the CD52 antigen present at high levels on the surface of lymphocytes. While treatment of multiple sclerosis patients with alemtuzumab results in marked depletion of lymphocytes from the circulation, it has not been associated with a high incidence of serious infections. In a human CD52 transgenic mouse, alemtuzumab treatment showed minimal impact on the number and function of innate immune cells. A transient decrease in primary adaptive immune responses was observed post-alemtuzumab but there was little effect on memory responses. These results potentially help explain the level of immunocompetence observed in alemtuzumab-treated MS patients.

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1. Introduction

Alemtuzumab is a humanized monoclonal antibody that specifically binds human CD52 (huCD52), a 12 amino acid GPI-anchored protein (Hale et al., 1990; Hale, 2001). CD52 is expressed at high levels on the surface of T and B lymphocytes and at lower levels on natural killer (NK) cells, monocytes, and macrophages (Hale et al., 1990; Rao et al., 2012). There is little or no CD52 on neutrophils, plasma cells, or bone marrow stem cells (Hale et al., 1990; Gilleece and Dexter, 1993). While the exact function of CD52 is currently unknown, reports have suggested that CD52 may play a role in cell–cell interactions as well as T cell migration or co-stimulation (Rowan et al., 1995; Masuyama et al., 1999; Watanabe et al., 2006).

Clinical trials have recently been completed comparing alemtuzumab to interferonβ-1a (IFNβ-1a) therapy in treatment naive relapsing/remitting MS (RRMS) patients (CAMMS223, CARE-MS I) and in RRMS patients who experienced a relapse while on prior therapy (CARE-MS II). In these randomized, rater-blinded trials, alemtuzumab was administered intravenously at 12 or 24 mg/day for 5 consecutive days at the start of the study and for 3 days one year later. IFNβ-1a (44 μg) was administered subcutaneously 3 times per week for the duration of the study. Patients in CAMMS223 may have received an optional third course of treatment at the investigator's discretion 24 month after their first dose of alemtuzumab. Using the co-primary

endpoints of relapse rate and progression of disability, alemtuzumab showed overall greater clinical efficacy than IFNβ-1a (Coles et al., 2008; Cohen et al., 2012; Coles et al., 2012a, 2012b). In addition, long term follow-up of patients from the Phase II study (CAMMS223) suggested that alemtuzumab provides a long-lasting clinical benefit as it lowered the risk of sustained accumulation of disability by 72% and the rate of relapse by 69% compared to IFNβ-1a out to four years after the last alemtuzumab treatment (Coles et al., 2012a).

The mechanism by which alemtuzumab exerts its therapeutic effect in MS is not fully understood but may involve rebalancing of the immune system through the depletion and repopulation of lymphocytes. The administration of alemtuzumab results in rapid depletion of lymphocytes from the circulation which may reduce the inflammatory processes associated with MS. This is followed by a prolonged period of lymphocyte repopulation with B cells returning to baseline levels within 6 months and T cell counts rising more slowly, generally approaching the lower limit of normal levels by 12 months post-treatment. Research findings suggest that alemtuzumab alters the number, proportions and properties of lymphocyte subsets during repopulation which may contribute to its long term treatment effect (Cox et al., 2005; Jones et al., 2010; Thompson et al., 2010). Multiple sclerosis patients treated with alemtuzumab also display an increase in the frequency of secondary autoimmune conditions, primarily thyroid disorders and, less frequently, immune thrombocytopenia (Cohen et al., 2012; Coles et al., 2012b). The factors responsible for development of secondary autoimmunity in a subset of patients are poorly understood and remain an active area of investigation (Jones et al., 2009; Cossburn et al., 2011; Costelloe et al., 2012).

An understanding of the relationship between the observed lymphopenia, immune status, and protective immunity post-alemtuzumab has also been lacking. In spite of the marked

* Corresponding author. Tel.: +1 508 271 2705; fax: +1 508 271 4694.
E-mail address: michael.turner@genzyme.com (M.J. Turner).

lymphocyte depletion and prolonged period of repopulation observed after treatment, MS patients did not experience a high incidence of serious infections (Coles et al., 2008; Cohen et al., 2012; Coles et al., 2012a, 2012b). An increase in the overall incidence of infection was observed in alemtuzumab-treated *vs.* IFNβ-1a-treated patients (66–77% *vs.* 45–66%, respectively, across the Phase II and III trials) but most were mild to moderate in severity and responded to conventional treatment. The most common infections were upper respiratory and urinary tract infections. The highest incidence of infection occurred during the first month following alemtuzumab treatment. Acyclovir prophylaxis was implemented during that period to decrease the incidence of herpes infection. Importantly, infection rates did not increase after the second or, in some cases, third course of alemtuzumab (Coles et al., 2008; Cohen et al., 2012; Coles et al., 2012a, 2012b).

In order to better understand the impact of alemtuzumab on the immune status of the host beyond the phenotypic analysis conducted on peripheral blood from MS patients, a huCD52 transgenic mouse model was used to systematically characterize the effect of treatment on the functionality of various immune system components. The results showed a minimal impact of alemtuzumab treatment on the number and function of innate immune cells. There was also little effect of alemtuzumab on memory B and T cell responses while primary immune responses were transiently decreased. Overall, the level of preserved immunocompetence measured post-alemtuzumab may help explain why there was not a high incidence of serious infections in alemtuzumab-treated MS patients.

2. Materials and methods

2.1. Mice and tissue collection

Human CD52 transgenic mice were generated and maintained as described previously (Hu et al., 2009). Briefly, huCD52 transgenic mice were created by microinjecting CD1 embryonic mouse stem cells with a bacmid construct containing ~145 kilobases of genomic DNA from human chromosome 1 and the entire huCD52 gene and promoter region. All experimental protocols were approved by the Genzyme Institutional Animal Care and Use Committee and studies were conducted in Genzyme's Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. All studies were performed using a single intravenous injection of alemtuzumab or Remicade™ (Hanna Pharmaceutical, Willmington, DE) at 1 mg/kg or an equivalent volume of vehicle prior to the initiation of the experimental procedures.

2.2. Quantitation of cellular populations by flow cytometry

Quantitation of immune cell populations in the spleen, thymus, and pooled lymph nodes (including inguinal, axillary, brachial, cervical, and mesenteric nodes) was performed by producing single cell suspensions from these organs. Bone marrow cells were obtained by flushing the tibiae and femurs with HBSS. The total number of immune cells in each organ was measured using a Coulter Counter (Beckman Coulter, Indianapolis, IN). Polychromatic flow cytometry was performed to quantify various cell types following alemtuzumab administration. Staining of immune cells was performed by incubating 1×10^6 cells with fluorescently-labeled antibodies for mouse cell surface markers including CD4 (RM4-5), CD8 (53–6.7) CD44 (1M7), CD62L (Mel-14), CD25 (PC61.5), FR4 (12A5), B220 (RA3-6B2), CD93 (AA4.1), NK1.1 (PK136), F4/80 (8M8), CD11b (M1/70), and GR-1 (RB6.8C5) purchased from Ebioscience (San Diego, CA) or BD Bioscience (San Jose, CA). Vβ analysis was performed by staining splenocytes from vehicle- or alemtuzumab-treated animals with a panel of fluorescein-labeled anti-Vβ specific antibodies as described by the manufacturer (BD Bioscience, San Jose, CA). Peripheral blood was evaluated by staining 50 μl of whole blood with the indicated antibodies followed by removal of contaminating red blood cells with FACS lysis buffer (BD Bioscience, San Jose, CA) as described by the manufacturer. Fluorescence intensities

were determined using an LSR-II flow cytometer (BD Bioscience, San Jose, CA) and data analysis was performed using Flowjo software (Treestar Inc., Ashland, OR).

Quantification of absolute numbers of specific cell populations in the spleen, lymph nodes, thymus, and bone marrow was determined by multiplying the percentage of the FACS-identified cellular population by the total number of cells present in each organ. Numbers of specific cell populations per microliter of peripheral blood were quantified using Countbright Absolute Counting Beads (Invitrogen Life Technologies, Grand Island, NY) as described by the manufacturer (# cells/μl = number of cells counted/number of beads counted × number of beads added). Absolute numbers of cell populations in the peripheral blood of each animal was calculated by multiplying the number of cells/μl for each specific population by the estimated volume of blood (3000 μl) in these animals (40 g average body weight/mouse). Percent control for all tissues was calculated by dividing the number of total cells for each population by the mean total number of the same cellular population from the vehicle-treated control group [total cells in test animal / mean of total cells in control group × 100].

2.3. Chromium release assay

NK cell function was evaluated by performing a standard chromium release assay on purified NK cells. Briefly, NK cells from the spleens of alemtuzumab- or vehicle-treated animals were isolated by negative selection using antibody-coated magnetic beads as described by the manufacturer (Miltenyi Biotec, Auburn, CA). NK cells were cultured with ^{51}chromium-labeled YAK-1 target cells at various effector:target ratios. After 5 h of incubation, the amount of ^{51}chromium released in the supernatant was measured in a MicroBeta Trilux Scintillation Counter (Wallac, Gaithersburg, MD). The amount of ^{51}chromium spontaneously released was obtained by incubating target cells alone in medium. Spontaneous release was typically below 20%. The total amount of ^{51}chromium incorporated by the target cells was determined by adding 1% Triton X-100 in distilled water, and the percent specific lysis was calculated as follows: [(sample cpm − spontaneous cpm) / (total cpm − spontaneous cpm)] × 100.

2.4. Antibody responses

T-independent antibody responses were evaluated by immunizing huCD52 transgenic mice with 100 μg of DNP-conjugated Ficoll (Biosearch Technologies, Novato, CA) intraperitoneally either 21 or 3 days after alemtuzumab treatment. Five days later, serum was collected and anti-DNP titers determined by an anti-DNP IgM-specific ELISA. Briefly, DNP-KLH or KLH alone were coated in phosphate-buffered saline (PBS) on high protein binding ELISA plates. Antibodies present in serially diluted serum samples were detected with an HRP-labeled anti-IgM specific secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) followed by incubation with TMB substrate.

Similarly, T-dependent antibody responses were evaluated following subcutaneous immunization with 100 μg of DNP-conjugated chicken gamma globulin (CGG) (Biosearch Technologies, Novato, CA) emulsified in Complete Freund's Adjuvant (Chondrex Inc., Redmond, WA) either 21 or 3 days following alemtuzumab treatment. Fourteen days later, serum samples were collected and evaluated for DNP reactivity using an ELISA assay similar to that described above with a secondary antibody specific for IgG antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA).

2.5. Peritonitis assay

Peritonitis was induced by injecting thioglycolate intraperitoneally (Becton Dickenson, Franklin Lakes, NJ) three days following

alemtuzumab treatment. Peritoneal exudate cells were isolated by flushing the peritoneal cavity with PBS 24 or 48 h after thioglycolate injection. Samples of exudate cells from individual mice were analyzed by flow cytometry (see above) and CD11b positive cells were negatively purified from the remaining population by MACS isolation according to the manufacturer's recommendations (Miltenyi Biotec, Auburn, CA). CD11b-enriched cells were then tested for phagocytic activity using a colorimetric assay (Cell Biolabs Inc., San Diego, CA) and for cytokine production. CD11b-enriched exudate cells (5×10^5) were cultured overnight in complete RPMI medium and stimulated with 10 μg/ml of LPS for an additional 24 h. The levels of cytokines released in the culture medium were then quantified by cytometric bead array (CBA) as described by the manufacturer (BD Bioscience, San Jose, CA).

2.6. Functional T cell assay

Functional activity of T cells was evaluated three days after alemtuzumab treatment. Cellular proliferation was tested by culturing splenocytes in the presence of plate-bound anti-CD3 (3 μg/ml) for 72 h followed by an additional 24 h with 1 μCi of ^{3}H-thymidine. In parallel cultures, supernatant was collected after 24 h of incubation and evaluated for cytokine levels using a mouse Th1/Th2 cytometric bead array as described by the manufacturer (BD Bioscience, San Jose, CA).

Anti-adenovirus T cell responses were evaluated by immunizing huCD52 transgenic mice subcutaneously with 1×10^9 infectious units of recombinant adenovirus serotype 2 (Ad2). Immunizations were performed as described in the Results section. Anti-Ad2 specific T cells were identified by IFNγ ELISPOT assay. Briefly, multiscreen-IP plates were coated with 5 μg/ml of rat anti-muIFNγ antibody (RMMG-1, Biosource, Grand Island, NY) for 1 h at 37 °C. The plate was blocked for 1 h with complete RPMI at humidified 37 °C. Splenocytes were added in the presence or absence of 10 μg/ml of heat-killed Ad2 virus. Twenty-four hours later, splenocytes were removed and bound IFNγ was detected with 2 μg/ml of biotinylated rat anti-muIFNγ antibody (XMG1.2, BD Bioscience, San Jose, CA) diluted in PBS. Two hours later, excess secondary antibody was removed and bound antibody was detected with a 1:800 dilution of streptavidin-AP in PBS and developed with BCIP reagent (Kirkegaard & Perry Laboratories, Gaithersburg, ML).

2.7. Statistical analysis

Analysis was performed on GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). Data are presented as the mean +/− standard error; p values were determined by Student's t test.

3. Results

3.1. Differential depletion of immune cells by alemtuzumab in human CD52 transgenic mice

Alemtuzumab is a humanized monoclonal antibody that specifically recognizes human CD52 (huCD52) and does not cross-react with murine CD52 due to sequence differences between the human and mouse proteins. Therefore, a huCD52 transgenic mouse model was used to investigate the impact of alemtuzumab treatment on immune function. As described previously, the tissue distribution and levels of huCD52 expression on various immune cell populations in these mice are similar to those observed in humans (Hu et al., 2009; Rao et al., 2012) and treatment with alemtuzumab results in a similar pattern of immune cell depletion (Hu et al., 2009). In this study, the nature and functionality of immune cell subsets remaining post-alemtuzumab treatment were investigated in greater detail. First, the distribution of major immune cell populations was evaluated in mice treated with a single dose of alemtuzumab approximating the total dose administered to MS patients (1 mg/kg). Analysis conducted at 3 days post-treatment, when maximal lymphocyte depletion occurs (supplemental Fig. 1),

showed a dramatic reduction in the number of immune cells in the blood with a lesser impact in lymphoid organs. Importantly, only a small percentage of total cells are actually found in the blood and significant numbers of immune cells were still present in the bone marrow, lymph nodes, thymus and spleen of animals post-alemtuzumab (Fig. 1a). These results suggest that measurement of circulating blood cell counts alone does not accurately reflect the totality of the immune system and may overestimate the degree of immune cell depletion post-alemtuzumab.

Examination of the immune cell populations remaining in the spleen 3 days after alemtuzumab treatment showed that T and B lymphocytes, which express high levels of CD52 (Hu et al., 2009), were most susceptible to depletion while innate immune cells including natural killer (NK) cells, macrophages and polymorphonuclear leukocytes (PMNs), which express little CD52 (Hu et al., 2009), were relatively unaffected or even proportionally increased (Fig. 1b). Similarly, in the blood, T and B lymphocytes were strongly depleted (Fig. 1c) while innate immune cells underwent a significantly lower level of depletion with a rapid recovery to baseline (Fig. 1d). Lymphocytes underwent a gradual repopulation over 100 days with B lymphocytes recovering faster than T lymphocytes (Fig. 1c). This pattern of peripheral blood lymphocyte depletion and repopulation with relative sparing of innate immune cells mirrors that observed in MS patients treated with alemtuzumab (Coles et al., 2010).

3.2. Minimal impact of alemtuzumab treatment on the function of innate immune cells

The number of innate immune cells did not appear to be greatly affected by alemtuzumab treatment and we next sought to assess their functionality. NK cells isolated from spleens at 3 days post-alemtuzumab treatment were tested for their ability to lyse target cells in a standard chromium release assay. As illustrated in Fig. 2a, the lytic activity of NK cells from vehicle- and alemtuzumab-treated mice was comparable at all effector:target (E:T) ratios tested. These results indicate that after alemtuzumab treatment, remaining NK cells are functionally intact.

The activity of macrophages and polymorphonuclear leukocytes (PMNs) was evaluated in vivo using a peritonitis model of inflammation. Human CD52 transgenic mice were treated with vehicle or alemtuzumab and were injected intraperitoneally 3 days later with thioglycolate to induce local inflammation. The ability of innate immune cells to respond to this inflammation and migrate into the peritoneal cavity was assessed 24 and 48 h later by measuring the total number of cells recovered by peritoneal lavage and by characterizing the nature of the cells present in the peritoneal exudate by polychromatic flow cytometry. As demonstrated in Fig. 2b, animals that were not injected with thioglycolate displayed a relatively small number of resident cells in the peritoneal cavity. Animals receiving thioglycolate showed an approximately 3 fold increase in the number of cells isolated from the peritoneal cavity at 24 and 48 h. Importantly, there was no statistically significant difference in the number of infiltrating cells in animals treated with vehicle or alemtuzumab demonstrating an equivalent recruitment of immune cells at the site of inflammation. In addition, the composition of the cellular exudate was comparable in both groups as determined by staining for B cells, eosinophils, mast cells, macrophages, monocytes and PMNs at the 48-hour time point (Fig. 2b). These results indicate that alemtuzumab treatment has little impact on the magnitude and quality of the inflammatory response in this model.

The functional activity of the macrophages recruited into the peritoneal cavity was next assessed by measuring their ability to phagocytose particles and secrete cytokines in response to stimulation with bacterial lipopolysaccharide (LPS). As illustrated in Fig. 2c, CD11b $^+$ macrophages enriched from the peritoneal exudate of vehicle-treated (purity 91.6 +/− 2.5%) and alemtuzumab-treated (purity 94.2 +/− 2.1%) animals showed equivalent levels of phagocytosis of labeled particles
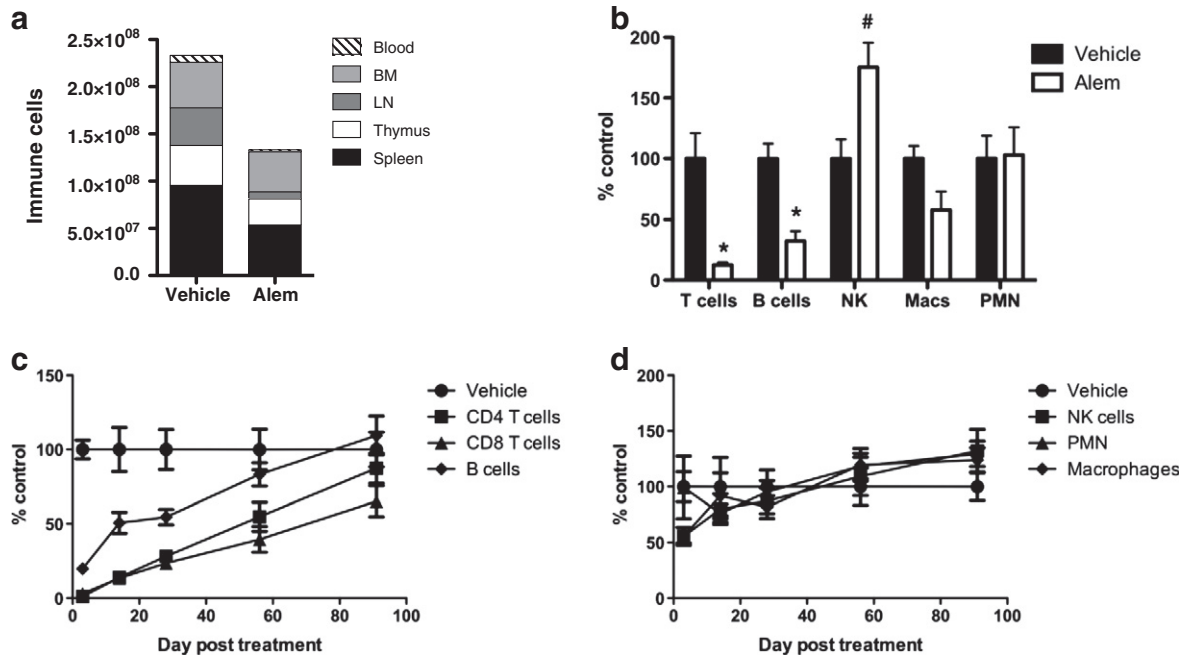
Fig. 1. Alemtuzumab-mediated depletion and repopulation of immune cells in huCD52 transgenic mice. Immune cell depletion was evaluated in huCD52 transgenic mice three days following intravenous treatment with vehicle or alemtuzumab (Alem). (A) Total numbers of immune cells were obtained from a single cell suspension of the primary and secondary lymphoid organs indicated. (B) Cellular subsets were quantified by polychromatic flow cytometry in the spleen three days following treatment with vehicle or alemtuzumab. (T cells: CD4+ and CD8+; B cells: B220+; NK cells: NK1.1+; PMN: Gr-1+) *p < 0.01; #p < 0.05 by Student's t test. Repopulation of lymphocyte (C) or innate immune cell subsets (D) following alemtuzumab treatment of huCD52 transgenic mice: absolute numbers of the indicated cell populations were periodically quantified in the peripheral blood after alemtuzumab treatment. Error bars indicate the standard error of the mean of 4 to 5 animals/group. In panels (C) and (D), statistically significant differences compared to control were observed for CD4+ and CD8+ T cells from day 3 to day 56, for B cells from day 3 to day 40, and day 3 for NK cells. BM: bone marrow, LN: lymph nodes, NK cells: natural killer cells, PMN: polymorphonuclear leukocytes, Macs: macrophages. Experiments were performed three times with similar results.
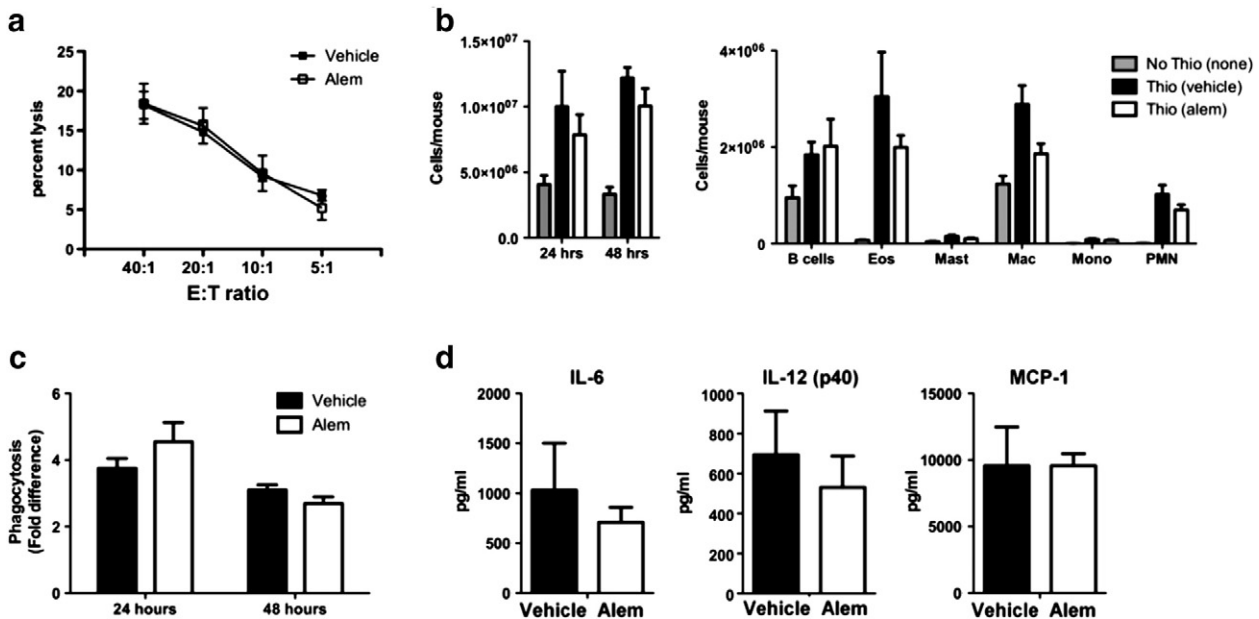
Fig. 2. Characterization of innate immune cell function following alemtuzumab treatment. (A) The lytic activity of NK cells isolated from the splenocytes of huCD52 transgenic mice three days following alemtuzumab (Alem) treatment was tested at various effector:target (E:T) ratios in a ^{51}Cr release assay. (B) Enumeration of peritoneal exudate cells following alemtuzumab treatment and thioglycolate (thio) injection. HuCD52 transgenic mice were treated with alemtuzumab and 3 days later injected intraperitoneally with thioglycolate. The total numbers of exudate cells (left) and specific cellular subsets (right) were quantified either 24 or 48 h after thioglycolate injection (gray-untreated, black-vehicle control, white-alemtuzumab). Eos: eosinophils, Mast: mast cells, Mac: macrophages, Mono: monocytes, PMN: polymorphonuclear leukocytes. (C) Phagocytic activity of enriched CD11b exudate cells was evaluated at 24 and 48 h post-injection of thioglycolate. (D) Release of IL-6, IL-12 (p40) and MCP-1 by CD11b-enriched exudate cells was evaluated following 24 h *in vitro* stimulation with LPS. Also measured but not shown: G-CSF, GM-CSF, IFN-γ, MIP1α and RANTES. No statistically significant differences were observed between alemtuzumab and vehicle control groups by Student's t test for any of the read-outs. Error bars indicate the standard error of the mean of 4 to 5 animals/group. Studies were performed twice with similar results.

in a colorimetric assay. Similarly, CD11b$^+$ cells from both groups released similar levels of proinflammatory cytokines when stimulated with LPS (Fig. 2d). Overall, these data indicate that the ability of innate immune cells to respond to inflammation *in vivo* was not significantly impacted by treatment with alemtuzumab.

3.3. Modest impact of alemtuzumab treatment on primary B cell responses

As shown in Fig. 1c and as observed in MS patients, administration of alemtuzumab results in a marked decrease in the number of circulating B lymphocytes suggesting that the development of antibody responses may be reduced after treatment. However, as described above, even when most lymphocytes are depleted from the circulation, significant numbers of B and T cells are still present in lymphoid organs and are presumably still available to mount an immune response (Fig. 3a, Hu et al., 2009). The ability of alemtuzumab-treated huCD52 transgenic mice to develop antibodies in response to immunization with model T-dependent and T-independent antigens was therefore investigated. Animals were immunized with DNP-conjugated Ficoll (DNP-Ficoll; T-independent) or DNP-conjugated chicken gamma globulin (DNP-CGG; T-dependent) either 3 days (maximal lymphocyte depletion) or 21 days after alemtuzumab treatment. Levels of DNP-specific IgM antibodies in the serum of animals immunized with the T-independent DNP-Ficoll antigen were measured by ELISA 5 days after immunization. As demonstrated in Fig. 3b, mice treated with alemtuzumab developed IgM antibody responses similar to those of vehicle-treated animals when challenged 3 or 21 days post-alemtuzumab. Levels of DNP-specific IgG antibodies were also measured by ELISA 14 days after immunization with the T-dependent DNP-CGG antigen. In this instance, a decrease in the level of serum antibodies to DNP was observed when immunization occurred 3 days after alemtuzumab treatment but antibody responses were back to the levels obtained in vehicle control animals by 21 days post-alemtuzumab (Fig. 3c). Overall, these results suggest a modest impact of alemtuzumab treatment on B cell responses, as illustrated by no measurable effect of alemtuzumab on the development of a *de novo* antibody response to a T-independent antigen but a decrease in T-dependent antibody at the nadir of lymphocyte depletion.

3.4. Preservation of function and diversity in residual T cells post-alemtuzumab

We next investigated whether T cells remaining after alemtuzumab treatment still exhibited normal functional activity. In these studies, huCD52 transgenic mice were treated with alemtuzumab or vehicle and three days later splenocytes were evaluated for their ability to proliferate and produce cytokines in response to polyclonal stimulation with plate-bound anti-CD3. The actual number of T cells present in the splenocyte cultures was determined by flow cytometry and the results are expressed on a per cell basis. As shown in Fig. 4a and b, T cells from alemtuzumab- or vehicle-treated mice proliferated at a similar rate and released comparable levels of Th1, Th2, and Th17 type cytokines indicating retention of function in residual T cells post-alemtuzumab.

In addition, an analysis of T cell receptor Vβ expression was conducted to determine whether particular Vβ families might be more susceptible to alemtuzumab-mediated depletion thereby potentially causing a skewing in the T cell repertoire. Using a flow cytometry-based analysis of Vβ expression by splenic CD4 and CD8 T cells, no significant differences were observed in the distribution of Vβ families in alemtuzumab- and vehicle-treated mice for either T cell subset (Fig. 4c, d) except for variations in some minor populations (*e.g.* Vβ2, Vβ3 and Vβ17a in CD4 T cells). Similarly, Vβ analysis of central memory, effector memory, naïve and T regulatory cells failed to show any overall differences in diversity following treatment with alemtuzumab *vs.* vehicle (data not shown). Taken together, these data indicate that T cells present post-alemtuzumab treatment retained normal functional
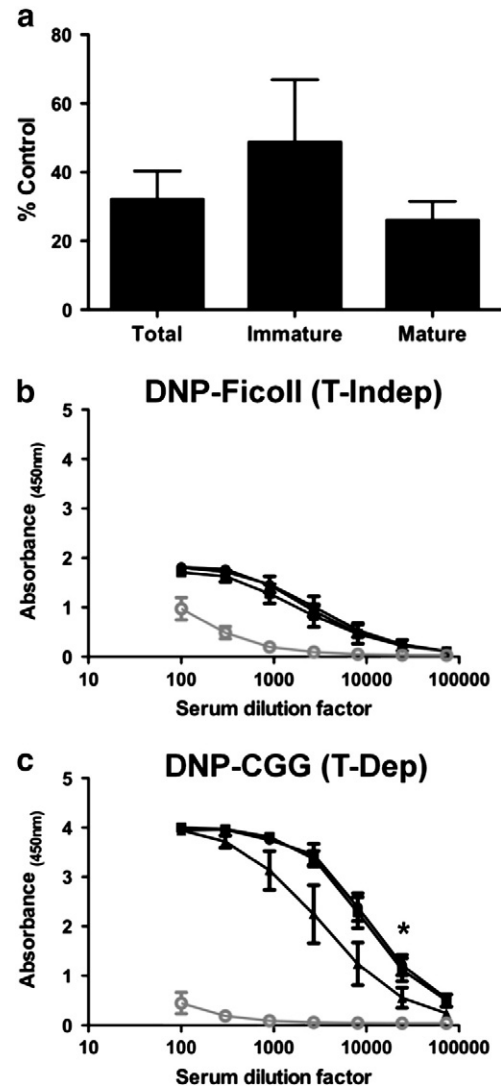
Fig. 3. Characterization of B cell responses following alemtuzumab therapy. (A) Mature (B220+, CD93^{lo}) and immature B cell (B220+, CD93^{hi}) subsets were quantified in the spleens of huCD52 transgenic mice three days after alemtuzumab treatment. (B, C) Evaluation of T-independent and T-dependent antibody responses. Human CD52 transgenic mice were treated with vehicle (circle) or alemtuzumab at 21 (squares) or 3 (triangles) days prior to immunization with DNP-Ficoll (B) or DNP-CGG (C). Anti-DNP antibody titers were then evaluated by ELISA either 5 or 14 days after immunization, respectively. T-independent antibody responses were performed by evaluating the binding of IgM anti-DNP antibodies to DNP conjugated to KLH (black symbols) or KLH alone (gray symbols). T-dependent antibody responses were quantified by an IgG anti-DNP specific ELISA against the same antigens. Error bars indicate the standard error of the mean of 3–5 animals/group. *p < 0.05 by Student's *t* test. Studies were performed twice with similar results.

activity and that alemtuzumab treatment did not introduce a bias in the diversity of the T cell receptor repertoire.

3.5. Adenovirus-specific T cell responses in alemtuzumab-treated mice

The previous data indicate that, while alemtuzumab treatment reduced the overall number of T lymphocytes, the cells that remained were capable of responding functionally and the overall spectrum of T cell receptor diversity was maintained. This suggests that a certain level of immune reactivity in response to immunization or infection may also be maintained post-alemtuzumab. Further analysis of the T cell populations present in the spleen after alemtuzumab treatment showed that naïve CD4$^+$ and CD8$^+$ T cells were the most susceptible to depletion while memory T cells (central and effector memory), as well T regulatory cells, were depleted to a lesser extent (Fig. 5a, b)
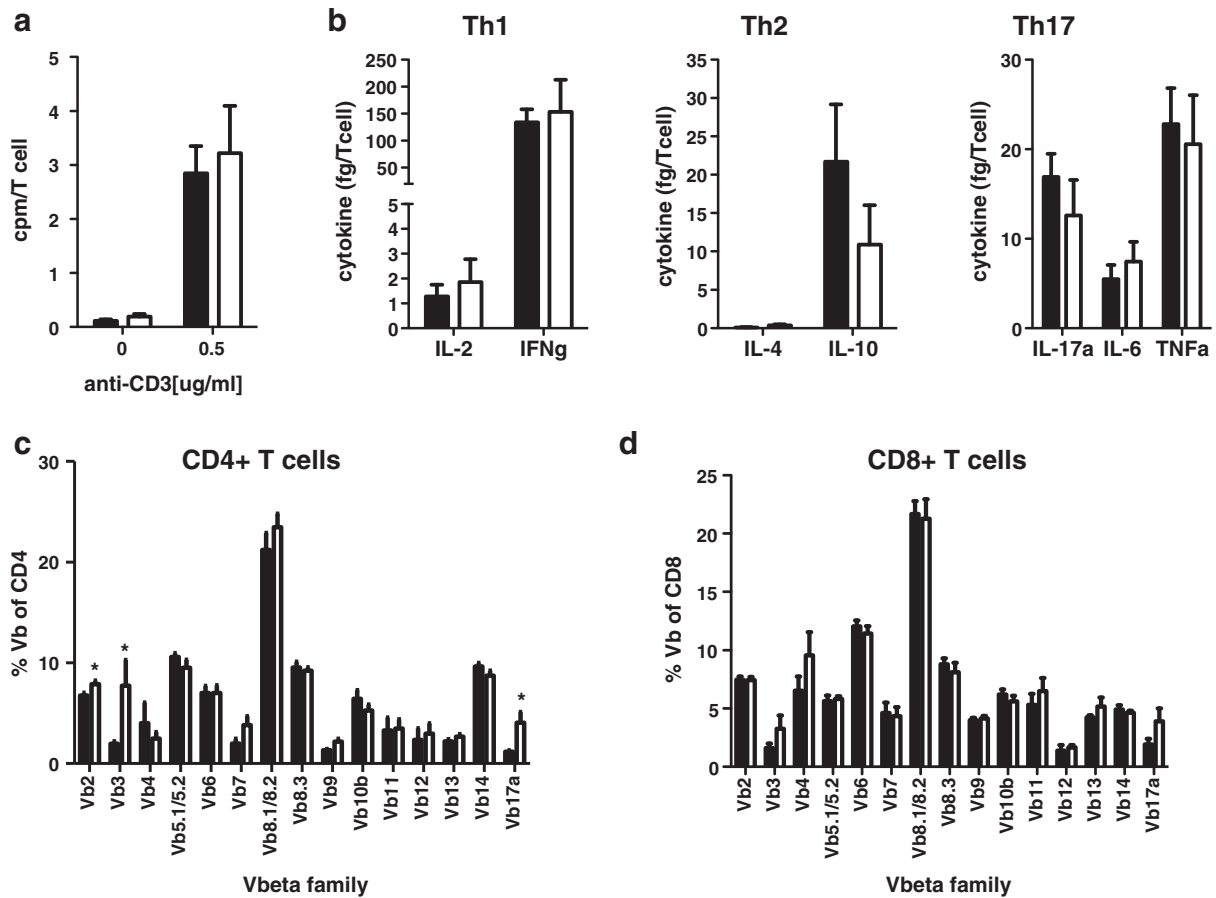
Fig. 4. T cell function and repertoire are unchanged following alemtuzumab treatment. T cell function was assessed in huCD52 transgenic mice three days following alemtuzumab (white bars) or vehicle (black bars) treatment. Splenic cultures were evaluated for proliferative capacity (A) and cytokine production (B) following anti-CD3 stimulation *in vitro*. Overall responses were normalized to the total number of T cells within the assay following flow cytometric evaluation. CD4 (C) and CD8 (D) T cell repertoire diversity was evaluated for Vβ expression by flow cytometry three days after alemtuzumab treatment. Error bars indicate the SEM of 5 animals/group. *p < 0.05 by Student's *t* test. Studies were performed twice with similar results.

suggesting that memory T cell responses may be preserved. This possibility was investigated in huCD52 transgenic mice challenged with an adenovirus vector (Ad2).

To study the effect of alemtuzumab on primary T cell responses, huCD52 transgenic mice were immunized with Ad2 either 7, 21 or 35 days following treatment with alemtuzumab or vehicle as a control. The frequency of Ad2-specific T cells was measured by IFNγ ELISPOT 14 days after immunization as described in Materials and methods. As demonstrated in Fig. 6a, the magnitude of the Ad2-specific T cell response was reduced at 7 and 21 days following alemtuzumab

treatment and returned to control levels by 35 days. Interestingly, the recovery in T cell responses was observed well before the numbers of circulating T cells had returned to baseline (Fig. 1a). These data indicate that alemtuzumab treatment resulted in a transient reduction of the primary T cell response to Ad2.

As described above, memory T cells appeared to be less susceptible to alemtuzumab-mediated depletion compared to naïve T cells. We therefore evaluated whether this observation might translate into the preservation of memory immune responses post-alemtuzumab. Human CD52 transgenic mice were immunized with Ad2 and allowed to develop a
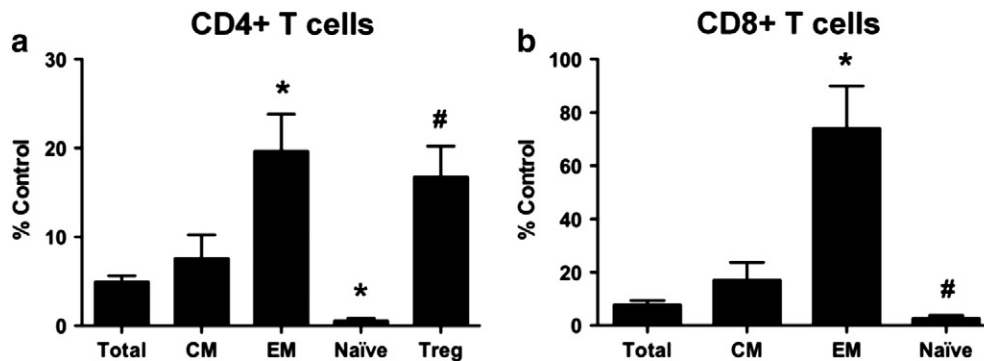
Fig. 5. Alemtuzumab-mediated depletion of T lymphocytes. Splenic CD4 (A) and CD8 (B) T cell subsets were quantified in huCD52 transgenic animals three days following treatment with alemtuzumab. Cellular subsets were identified by multiparameter flow cytometry and compared to vehicle-treated animals. Error bars indicate the SEM of 5 animals/group. *p < 0.01; #p < 0.05 by Student's *t* test. CM: central memory (CD62L^hi, CD44^hi), EM: effector memory (CD62L^lo, CD44^hi), Naïve: (CD62L^hi, CD44^lo), Treg: (CD4+, CD25^hi, FR4^hi). Studies were performed three times with similar results.

memory response over 30 days. The animals were then treated with alemtuzumab or vehicle and were rechallenged with Ad2 vector 3 days later. The frequency of Ad2-specific T cells was again measured by IFNγ ELISPOT but this time at 7 days post-challenge to capture the more rapid memory response. Control groups included animals that did not receive any immunization with Ad2 as well as animals that did not receive the second rechallenge. As shown in Fig. 6b, animals immunized a second time with Ad2 developed a response significantly greater than that obtained with a single immunization, demonstrating the increased potency of the recall response generated. Importantly, the magnitude of the Ad2-specific memory T cell response was comparable in alemtuzumab- and vehicle-treated animals suggesting that memory T cell responses are relatively unaffected by alemtuzumab.

4. Discussion

Treatment of MS patients with 2 courses of alemtuzumab, at initiation of treatment and 1 year later, has shown superior efficacy compared to s.c. IFNβ administered 3 times weekly in Phase II and III clinical trials (Coles et al., 2008; Cohen et al., 2012; Coles et al., 2012a, 2012b). Administration of alemtuzumab gives rise to a robust depletion of CD52$^+$ lymphocytes from the circulation and, while B lymphocyte counts typically return to baseline within 6 months, T cell recovery is slower and generally reaches the lower limit of normal by 11–12 months (Coles et al., 2012a). In spite of this observed lymphopenia, only 3% of alemtuzumab-treated MS patients in clinical trials developed serious infections. An increase in the overall incidence of infection was observed in alemtuzumab-treated *vs.* IFNβ-treated patients but the vast majority of infections were mild to moderate in severity and responded to conventional treatment (Coles et al., 2008; Cohen et al., 2012; Coles et al., 2012a, 2012b). These findings suggest that the perceived risk of infection suggested by low lymphocyte blood counts may not reflect the actual immune status of the host. Results obtained in this study using huCD52 transgenic mice mirror some of the observations made in MS patients and provide additional information that may help explain the level of immune protection observed in alemtuzumab-treated patients. First, analysis of lymphoid tissues, which were not sampled in the human clinical trials, indicates that the loss of immune cells from the circulation post-alemtuzumab represents only a small percentage relative to the totality of the immune system. This suggests that many immune cells are still present in the remainder of the body and are available to participate in an immune response. In addition, very little depletion was observed in the bone marrow and thymus, the primary lymphoid organs that are essential to lymphocyte repopulation. The low degree of alemtuzumab-mediated depletion in these organs may be explained by the absence or minimal expression of CD52 by bone marrow stem cells and early thymocyte populations and/or by reduced access of the antibody to these sites (Hale et al., 1990; Gilleece and Dexter, 1993; Hu et al., 2009). Clark et al. have also recently reported that, in alemtuzumab-treated cutaneous T cell lymphoma patients, the paucity of neutrophil-mediated antibody-mediated cellular cytolysis (ADCC) in the skin resulted in preservation of skin resident effector memory T cells (Clark et al., 2012). A low abundance of effector mechanisms in organs such as the thymus may also play a role in lymphocyte preservation post-alemtuzumab.

Although the actual number of lymphocytes is reduced post-alemtuzumab, functional analysis in mice indicated that the activity of the remaining lymphocytes is unimpaired. Splenic T lymphocytes from alemtuzumab-treated mice showed normal proliferation and cytokine production in response to anti-CD3 stimulation (Fig. 4a, b). In addition, Vβ analysis of T cell receptors from bulk CD4 and CD8 T cells (Fig. 4c, d) as well as central memory, effector memory, naïve and T regulatory cells (data not shown) indicated that T cell receptor diversity was maintained post-alemtuzumab and that treatment with the antibody did not introduce a bias toward any particular Vβ family.

Another important finding is the limited impact of alemtuzumab on the innate immune system. In both humans and huCD52 transgenic mice, innate immune cells including NK cells, macrophages and PMNs express only low levels of CD52 and undergo limited depletion with a rapid return to baseline (Fig. 1d; Coles et al., 2010). In this study, the functionality of innate immune cells was also shown to be preserved post-alemtuzumab. Natural killer cells from alemtuzumab-treated mice showed normal cytolytic activity *ex vivo* and normal innate immune responses to peritonitis were observed *in vivo*. Equivalent numbers and types of cells migrated into the peritoneal cavity of alemtuzumab-treated and control mice in response to thioglycolate-induced inflammation and the macrophages recovered from the peritoneal cavity displayed normal phagocytic and cytokine-producing abilities. These results suggest that the innate immune system, which represents the first line of defense against infection, is minimally impacted by alemtuzumab treatment.
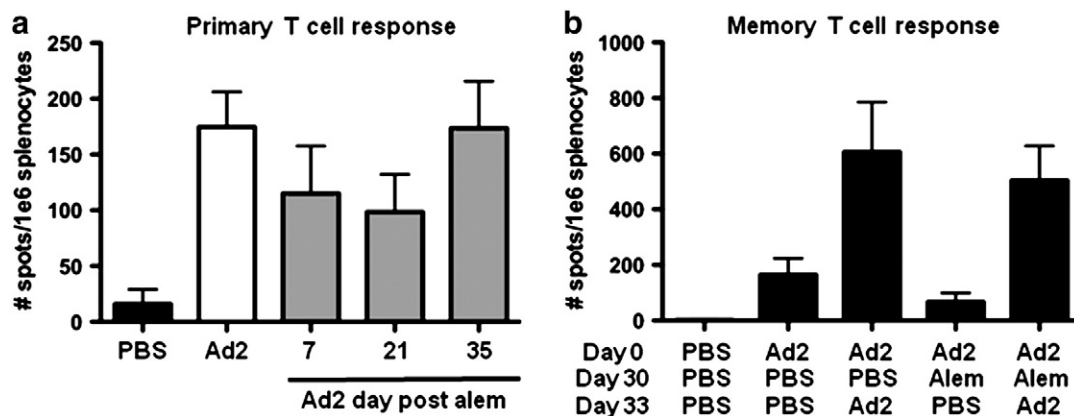
Fig. 6. Characterization of primary and memory T cell responses in alemtuzumab-treated huCD52 transgenic mice. (A) Human CD52 transgenic mice were evaluated for the development of Ad2-specific T cells following treatment with alemtuzumab (Alem) 7, 21, or 35 days prior to immunization with recombinant Ad2 or PBS. Fourteen days following the immunization, antigen-specific T cells were evaluated by IFNγ ELISPOT assay using heat-killed Ad2 vector. Error bars are the SEM of 4–5 animals per group. No statistically significant differences were observed between animals treated with vehicle or alemtuzumab. (B) Ad2-specific memory T cell responses were evaluated in animals immunized with Ad2 virus and rechallenged with Ad2 three days after alemtuzumab treatment. Seven days following the second immunization, splenocytes were evaluated for the frequency of Ad2-specific T cells by IFNγ ELISPOT assay. Error bars are the SEM of 4–5 animals per group. No statistically significant differences were observed between animals receiving single Ad2 immunizations in the presence of vehicle or alemtuzumab, or between animals receiving secondary immunizations following vehicle or alemtuzumab. Experiments were performed twice with similar results.

Assessment of specific acquired immune responses was also conducted. In studies of B cell function, the antibody response to a model T-independent antigen was not significantly affected by alemtuzumab treatment suggesting that remaining B lymphocytes were sufficient to mount a normal antibody response. By comparison, there was a transient decrease in the antibody response to a model T-dependent antigen when immunization occurred soon after alemtuzumab treatment (day 3) with a recovery to control levels by day 21 post-alemtuzumab. An impact on primary T cell responses was also observed in mice immunized with adenovirus. Compared to control, there was a decrease in the IFNγ ELISPOT T cell response to Ad2 when immunization occurred 7–21 days post-alemtuzumab, although the difference was not statistically different in this study (Fig. 6a). Importantly, the T cell response to adenovirus immunization returned to control levels by day 35 post-alemtuzumab, at a time when peripheral T lymphocyte counts were still below normal (Fig. 1c). These results suggest that protective levels of T cell immunity against infection can still be present in the face of lymphopenia. This may be due, at least in part, to the recovery of sufficient numbers of lymphocytes in the lymphoid organs that can participate in the development of immune responses. The animal data are also in agreement with the MS clinical data showing that the highest incidence of infection occurs in the first month post-alemtuzumab treatment and declines thereafter even though blood T cell counts remain below the normal range (Coles et al., 2012a).

Interestingly, subset analysis of the T cell populations remaining in huCD52 transgenic mice post-alemtuzumab indicated that T cells with a central memory, effector memory and regulatory phenotype were proportionally depleted to a lesser extent than naïve T cells (Fig. 5a, b). A similar pattern has also been observed in the peripheral blood of MS patients post-alemtuzumab (Cox et al., 2005). This finding suggested that memory recall responses may be relatively unaffected following alemtuzumab treatment. Testing of this possibility in the huCD52 transgenic mouse indicated that there was in fact little or no impact of alemtuzumab treatment on the memory response to adenovirus (Fig. 6b). Animals immunized with adenovirus 30 days prior to alemtuzumab treatment developed T cell (Fig. 6b) and antibody (not shown) responses comparable to those of control animals when rechallenged with adenovirus. These results suggest that memory responses developed as a result of prior infections or immunizations may not be dramatically impacted by alemtuzumab treatment.

Overall, our results indicate that alemtuzumab treatment of huCD52 transgenic mice within the dose range used in MS patients allows for maintenance of a level of immune competence characterized by largely unaffected innate immunity, functionality of remaining lymphocytes, and a transient decrease in the ability to respond to novel antigens with little impact on memory responses. These animal data may help explain why alemtuzumab-treated MS patients have not experienced a high incidence of serious infections.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2013.04.018.

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