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Differential Reconstitution of T Cell Subsets following Immunodepleting Treatment with Alemtuzumab (Anti-CD52 Monoclonal Antibody) in Patients with Relapsing–Remitting Multiple Sclerosis

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Alemtuzumab (anti-CD52 mAb) provides long-lasting disease activity suppression in relapsing–remitting multiple sclerosis (RRMS). The objective of this study was to characterize the immunological reconstitution of T cell subsets and its contribution to the prolonged RRMS suppression following alemtuzumab-induced lymphocyte depletion. The study was performed on blood samples from RRMS patients enrolled in the CARE-MS II clinical trial, which was recently completed and led to the submission of alemtuzumab for U.S. Food and Drug Administration approval as a treatment for RRMS. Alemtuzumab-treated patients exhibited a nearly complete depletion of circulating CD4+ lymphocytes at day 7. During the immunological reconstitution, CD4+CD25+CD127low regulatory T cells preferentially expanded within the CD4+ lymphocytes, reaching their peak expansion at month 1. The increase in the percentage of TGF-β1–, IL-10–, and IL-4–producing CD4+ cells reached a maximum at month 3, whereas a significant decrease in the percentages of Th1 and Th17 cells was detected at months 12 and 24 in comparison with the baseline. A gradual increase in serum IL-7 and IL-4 and a decrease in IL-17A, IL-17F, IL-21, IL-22, and IFN-γ levels were detected following treatment. In vitro studies have demonstrated that IL-7 induced an expansion of CD4+CD25+CD127low regulatory T cells and a decrease in the percentages of Th17 and Th1 cells. In conclusion, our results indicate that differential reconstitution of T cell subsets and selectively delayed CD4+ T cell repopulation following alemtuzumab-induced lymphopenia may contribute to its long-lasting suppression of disease activity. The Journal of Immunology, 2013, 191: 000–000.

Alemtuzumab is a humanized mAb directed against human glycosylated GPI-linked CD52 cell surface protein (1). As an effective lymphocyte-depleting therapy, it has been evaluated as a treatment for rheumatoid arthritis (2), chronic lymphocytic leukemia (3), T cell prolymphocytic leukemia (4), and as an conditioning agent prior to bone marrow (5) and kidney transplantation (6). Anti-CD52 mAb treatment has been studied during the past years as one of the medications with the longest lasting effect against multiple sclerosis (MS) (7). It depletes T and B lymphocytes and, to a lesser extent, monocytes, macrophages, dendritic cells, and NK cells via Ab-dependent cellular cytotoxicity, complement-induced cell lysis (8–11), and the induction of apoptosis (12). Although effectively depleting all CD52-bearing cells, this immunodepleting therapy does not affect hematopoietic stem cells (13), and thus the potential for an immune response reconstitution is preserved. The long-lasting clinical disease suppression and an improvement in disability measures (14) have led to the hypothesis that in addition to the immune cell depletion, the subsequent repopulation of CD4+ cells may contribute to decreased new brain lesion formation, which has been consistently demonstrated by brain magnetic resonance imaging scans in phase II and III clinical trials (15). Although several studies have demonstrated predominant memory cell reconstitution, there are no available data on the reconstitution of individual T cell subsets, which may contribute to the prolonged therapeutic effect.

In a phase II, randomized clinical trial (14), alemtuzumab was given in an initial 5-d infusion, followed at 12 mo by a 3-d infusion. However, its efficacy persisted during the 36 mo of the trial, and for a subset of patients as long as 5 y (16). Based on the positive results of the recently completed phase III studies (15, 17), an application for the approval of alemtuzumab as a treatment for relapsing–remitting MS (RRMS) has been submitted to the U.S. Food and Drug Administration.

In contrast to its documented efficacy, alemtuzumab’s mechanisms of action are not fully understood. In the present study, using blood samples from patients enrolled in the CARE-MS II phase III clinical trial, we have examined the cellular and molecular mechanisms of alemtuzumab-induced long-lasting immunomodulatory effects.

Materials and Methods

Study subjects

Ten RRMS patients who were randomized to alemtuzumab and four to control IFN-β1a therapy in the CARE-MS II clinical trial were enrolled in this laboratory study. An additional four RRMS patients were enrolled in the extension study. Alemtuzumab was administered via i.v. infusion (12...
mg/d) for 5 consecutive days, and again after 12 mo for 3 d, whereas IFN-β1a treatment was given subcutaneously (44 μg three times a week). The study duration was 24 mo. In the extension phase of the study, four patients initially randomized to the IFN-β1a treatment were treated with alemtuzumab using the same regimen as in the initial 2-y study. All patients signed an Institutional Review Board–approved consent form.

Twenty-five untreated RRMS patients were enrolled in the in vitro study. The inclusion criteria consisted of a RRMS diagnosis (18); the exclusion criteria were treatment with immunomodulatory or immunosuppressive therapy as reported previously (19).

Flow cytometry

Peripheral blood samples were collected from 10 RRMS patients randomized to alemtuzumab and 4 to control IFN-β1a therapy in the CARE-MS II clinical trial at baseline and months 1, 3, 6, 12, and 24 after therapy. In an extension study, peripheral blood samples were collected from four patients at baseline and at day 7 as well as at months 1, 2, 3, and 6 after alemtuzumab therapy. For these ex vivo studies, fresh PBMCs were separated using Ficoll gradient. Surface markers were stained with fluorescein-conjugated Abs against CD52 (Santa Cruz Biotechnology), CD4, CD8, CD45RA, CD45RO, CD25, CD127, GITR (BD Biosciences), and CD39 (eBiosciences) on the nonmanipulated cells. Intracellular staining was performed after stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) for 2 h, and brefeldin A (1:1000 dilution) (eBioscience) was added for an additional 3 h. Cells were fixed, permeabilized, and stained with fluorescein-conjugated Abs against IL-17A, IFN-γ, IL-4, FOXP3, granzyme B, perforin (eBioscience), TGF-β1 (LifeSpan BioSciences), IL-10, and CD4 (BD Biosciences). The percentages of cells expressing each molecule in gated T cells were determined using a BD FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

ELISA

Serum samples were collected from four patients in the extension phase of the study at baseline and at day 7 and months 1, 2, 3, and 6 after alemtuzumab treatment. IL-17A, IL-17F, IL-21, IL-22, IL-9, IL-23, IL-7, IL-15 (eBioscience), IFN-γ, IL-4, TGF-β1, IL-10, TNF-α, IL-1β, IL-6, IL-12p70, IL-2 (BD Biosciences), IL-11, and IL-27 (R&D Systems) were measured in duplicate by ELISA. The sample incubation was for 24 h at 4°C, and the detection Ab incubation was for 2 h at room temperature.

Western blotting

CD4+ T cells were isolated from PBMCs collected from three untreated RRMS patients using CD4 microbeads (Miltenyi Biotec) and cultured in the absence or presence of IL-7 (100 ng/ml) for 30 min, 1, 2, 4, and 24 h. Cell lysates were denatured in SDS, resolved by 7.5% or 10% SDS-PAGE, and incubated with Abs against pSTAT1, STAT1, pSTAT3, STAT3, pSTAT5, STAT5, pSTAT6, STAT6 (Cell Signaling Technology), T-bet, FOXP3 (Santa Cruz Biotechnology), and tubulin (Sigma-Aldrich), followed by secondary Ab incubation (20). The protein bands were quantified using an Odyssey infrared imaging system (LI-COR Biosciences).

Statistics

For the longitudinal data analysis, where each individual provided blood samples for repeated measurements over time, we applied the generalized estimating equation (GEE) $E(y_{ij}) = \beta_0 + \beta_1T_{Time_k} + \ldots + \beta_jT_{Time_k}$ for $i = 1, \ldots, N$ and $j = 1, \ldots, T$, where $y_{ij}$ is the measurement of the $i$th subject at the $j$th time point, $N$ is the number of subjects, $T$ is the number of repeated measurements for each subject, and $T_{Time_k}$ is an indicator variable that is 1 if $j = k$ and otherwise is 0 for $k = 1$. In this study, we have compared the difference between baseline and the next time points. To test whether the change was significantly different, we carried out the tests of the parameters $\beta_j$ in the GEE formulation. The GEE analyses were implemented using SAS 9.3 software (SAS Institute, Cary, NC) using the GENMOD procedure.

Results

Alemtuzumab treatment effectively depletes lymphocytes, with delayed reconstitution of CD4+ T cells

To characterize the cellular and molecular mechanisms of the long-lasting immunomodulatory effects induced by alemtuzumab, we studied PBMCs from 10 RRMS patients enrolled in the CARE-MS II clinical trial who were randomized to alemtuzumab and 4 patients randomized to IFN-β1a control therapy. Flow cytometry studies were performed on fresh cells to determine the expression of multiple surface markers and intracellular cytokines at baseline and at months 1, 3, 6, 12, and 24 after therapy.

Alemtuzumab significantly decreased the absolute numbers of lymphocytes, both CD4+ and CD8+, with a significant depletion persisting up to 12 mo after treatment (Fig. 1A). Following treatment, the percentage of lymphocytes within the PBMCs had significantly decreased at months 1–6 and gradually returned to baseline at month 12 (Fig. 1B). The percentage of CD4+ and CD8+ T cells in gated lymphocytes was decreased at month 1 and remained significantly decreased in the case of CD4+ cells for 24 mo (Supplemental Fig. 1), whereas the relative number of CD8+ T cells normalized at month 3 (Fig. 1B). These results reveal alemtuzumab’s effective depletion of lymphocytes, with significantly decreased absolute numbers of both CD4 and CD8 cells up to 12 mo, but with selectively delayed reconstitution of only the CD4+ cell percentage within the lymphocytes.

To identify the nadir of alemtuzumab-mediated depletion and the onset of the reconstitution phase, we enrolled an additional four RRMS patients participating in the CARE-MS II extension study. We found that at day 7 after alemtuzumab treatment, the percentage of CD4+ and CD8+ T cells in lymphocytes had decreased by 99.8 and 98.5%, respectively, with the onset of reconstitution evident at month 1 (Fig. 1C).

Alemtuzumab treatment induces nearly complete depletion of memory T cells, which is followed by their preferential expansion during the immune reconstitution

We next examined the alemtuzumab-induced depletion and subsequent reconstitution of naive and memory T cells. One month after treatment, there was a significant decrease in the percentage of naive CD45RA+ cells in both CD4+ and CD8+ lymphocytes, accompanied by a predominant expansion of CD45RO+ memory cells (Fig. 2A, 2B).

To address whether CD52+ memory cells are preferentially lysed and subsequently expanded, or are spared during the alemtuzumab-mediated T cell lysis, we determined their relative percentages at an earlier time point (day 7 after therapy) in four patients enrolled in the extension phase. Our results confirm that the memory cells were depleted at day 7 and then preferentially expanded during the homeostatic proliferation, whereas the percentage of residual naive T cells remained elevated on day 7, followed by a rapid decrease at month 1 after therapy (Fig. 2C).

Immunological reconstitution following alemtuzumab treatment is characterized by relative expansion of CD4+ CD25+CD127low regulatory T cells and subsequent expansion of TGF-β and IL-10-producing CD4+ cells

We further examined the effects of CD4+CD25+ CD127low (T-regulatory cell [Treg]) expressing Th1, Th2, and Th17 T cell subsets, which displayed a similarly high baseline expression of CD52 (data not shown). Following nearly complete depletion of all CD4+ T cells at day 7, individual T cell subsets showed differential reconstitution patterns. We observed a significant increase in the percentage of CD4+CD25+CD127low Treg cells, which peaked at month 1 (Fig. 3A, 3B). Because CD4+CD25+
CD127<sub>low</sub> cells are not detectable at 7 d after alemtuzumab treatment, we conclude that their relative percentage increase at month 1 reflects their homeostatic proliferation. The in vivo–expanded CD4<sup>+</sup>CD25<sup>+</sup>CD127<sub>low</sub> Treg cells following alemtuzumab treatment in patients in the extension phase (baseline and day 7 to month 6 after treatment) maintained an immunosuppressive FOXP3<sup>+</sup>CD39<sup>+</sup>granzyme<sup>+</sup>TGF-β<sub>1</sub> phenotype (Fig. 3C), which has a documented regulatory function in human in vitro studies (21–23). However, owing to a low CD4<sup>+</sup>CD25<sup>+</sup>CD127<sub>low</sub> cell number in alemtuzumab-treated lymphodepleted patients, we did not examine their function, and thus their suppressive capacity was not confirmed.

Our study for the first time demonstrates that alemtuzumab treatment induces an incremental increase in the percentage of TGF-β1 and IL-10–producing CD4<sup>+</sup> T cells (Fig. 3D, 3E), which

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**FIGURE 1.** Alemtuzumab treatment significantly depletes lymphocytes, with a long-lasting depletion of CD4<sup>+</sup> T cells. (A) Absolute numbers of lymphocytes, CD4<sup>+</sup> cells, and CD8<sup>+</sup> cells at baseline and at indicated time points following alemtuzumab therapy. Graphs present median values ± SD at each time point. (B) Changes in the percentages of lymphocytes within the PBMCs and CD4<sup>+</sup> and CD8<sup>+</sup> cells within the gated lymphocytes from 10 RRMS treated patients at baseline and at indicated time points following alemtuzumab treatment. (C) Percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were determined in a lymphocyte-gated population in four RRMS patients enrolled in the extension phase of the study at baseline and at day 7 and months 1, 2, 3, and 6 following alemtuzumab treatment. Statistical analysis was performed using GEE. *p < 0.05, ***p < 0.001.

**FIGURE 2.** Alemtuzumab depletes CD45RO<sup>+</sup> memory T cells, which is followed by their preferential expansion. (A) PBMCs from 10 RRMS patients at baseline and at indicated time points following alemtuzumab treatment were stained with CD45RA-FITC, CD45RO-PE-Cy5, and CD4- and CD8-allophycocyanin mAbs for gating. Representative staining from 1 of the 10 treated patients is shown. (B) Graphs present percentages of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> cells in gated CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from alemtuzumab-treated patients. (C) Percentages of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> cells in gated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from four RRMS patients in the extension phase of the study at baseline and at day 7 and at months 1, 2, 3, and 6 following alemtuzumab treatment. Statistical analysis was performed using GEE. *p < 0.05, **p < 0.01, ***p < 0.001.
peaked at 3 mo, as well as an increase in the percentage of CD8+ cells producing TGF-β1 and IL-10, which peaked at month 12 and month 6, respectively (data not shown). Changes in the percentage of CD4+CD25+CD127low Treg cells positively correlated with the percentage of TGF-β1–producing CD4+ T cells during the 12-mo treatment period ($r = 0.404$, $p = 0.004$).

We determined that a control treatment with IFN-β1a, which did not induce changes in the absolute lymphocyte numbers or the relative expansion of Treg or TGF-β+CD4+ cells, induced a significant increase in the percentage of IL-10–producing CD4+ and CD8+ T cells (data not shown). However, the magnitude of increase of IL-10–secreting CD4+ T cells was at all time points lower than in the alemtuzumab-treated patients, consistent with a significantly higher efficacy of the alemtuzumab treatment.

Immune reconstitution following alemtuzumab treatment is characterized by changes in serum cytokine profiles

To test how alemtuzumab-induced lymphopenia changes the serum cytokine profiles that may contribute to the immune reconstitution, we measured the serum levels of multiple cytokines in patients enrolled in the extension study at baseline and day 7 to month 6 after treatment. We detected gradually increasing levels of the homeostatic cytokine IL-7, which reached significance at day 7 and maintained it up to month 6 in comparison with baseline. Consistent with the Th2 cell reconstitution, we observed a significant increase in serum IL-4, which also peaked at month 3. Additionally, serum cytokine measurements revealed a decrease in Th17 cytokines IL-17A, IL-17F, IL-21, and IL-22, Th1 cytokine IFN-γ, as well as of Th17-polarizing IL-11, IL-1β, IL-6, and IL-23 and Th1-polarizing IL-27 and IL-12 (Fig. 5). Cytokine measurements for IL-9, TGF-β1, IL-10, TNF-α, and IL-2 did not reveal significant changes over the examined time points.

Linear correlation analyses revealed that the serum IL-7 level was positively correlated with the percentage of CD45RO+ memory cells ($r = 0.4403$, $p = 0.0313$) and the IL-4 level ($r = 0.693$, $p = 0.0002$), whereas it was negatively correlated with IL-17F ($r = -0.4980$, $p = 0.013$) and IL-11 ($r = -0.473$, $p = 0.02$), suggesting IL-7

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**FIGURE 3.** CD4+CD25+CD127low Treg cells expand during immunological reconstitution, followed by an expansion of TGF-β and IL-10–secreting CD4+ cells. (A) Representative CD4+CD25+CD127low Treg cell staining at baseline and multiple time points after alemtuzumab therapy from one treated patient. (B) Graph presents percentages of Treg cells within the CD4+ lymphocytes at baseline and multiple time points after therapy in 10 patients. (C) Phenotype of CD4+CD25+CD127low Treg cells at baseline and month 1 after therapy. The figure represents one of four similar experiments. (D) Representative intracellular cytokine staining for TGF-β1 and IL-10 from 1 of 10 treated patients. (E) The graphs present percentages of TGF-β1 and IL-10+ CD4+ cells at baseline and indicated time points after therapy. Statistical analysis was performed using GEE. *$p < 0.05$, ***$p < 0.001$. 

Although IFN-β1a control treatment did not change the absolute number of lymphocytes, it induced a significant decrease in the percentage of CD4+ Th1 cells at months 6 and 12 and Th17 cells at months 12 and 24 (data not shown), consistent with a previous report (24). Immune reconstitution following alemtuzumab treatment is characterized by changes in serum cytokine profiles...
involvement in the differential reconstitution of T cell subsets in
the setting of lymphopenia (25).

**IL-7 in vitro treatment induces the expansion of CD4+CD25+
CD127<sup>low</sup> Treg cells and inhibition of Th17 and Th1 cells**

To determine to what extent the increased IL-7 levels in lym-
phopenic patients following alemtuzumab therapy contribute
to CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> Treg cell expansion (26), we cultured
PBMCs derived from six untreated RRMS patients in the ab-
sence or presence of IL-7, or TGF-β<sub>1</sub> and IL-2, cytokines with
an established role in Treg cell differentiation and expansion.

IL-7 induced a significant dose-dependent CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>
Treg cell expansion (Fig. 6A), which reached a peak at day 10
(Supplemental Fig. 3A). We further confirmed that IL-7 induced
the proliferation of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>FOXP3<sup>+</sup> Treg cells
(Fig. 6B). IL-2 also induced a dose-dependent CD4<sup>+</sup>CD25<sup>+</sup>
CD127<sup>low</sup> cell expansion, but to a lesser extent than did IL-7,
whereas TGF-β1 failed to induce this cell subset expansion in
the dose range tested (Supplemental Fig. 3B). Therefore, our
findings suggest that IL-7 may contribute to the CD4<sup>+</sup>CD25<sup>+</sup>
CD127<sup>low</sup> cell expansion in the setting of alemtuzumab-induced
lymphopenia.

**FIGURE 4.** The percentages of Th2 cells
increased whereas the percentages of Th17 and
Th1 cells decreased in CD4<sup>+</sup> cells during the
immune reconstitution following alemtuzumab
treatment. (A) Representative intracellular cyto-
kinic staining for IL-4 and IFN-γ from 1 of 10
treated patients. (B) The graph presents per-
centages of IL-4<sup>+</sup>CD4<sup>+</sup> cells at baseline
and indicated time points after treatment. Statistical
analysis was performed using GEE. **p < 0.01,
***p < 0.001. (C) Representative intracellular cyto-
kinic staining for IL-17A and IFN-γ in
CD4<sup>+</sup> cells from 1 of 10 treated patients. (D)
Graphs present percentages of IL-17A and IFN-
γ<sup>+</sup>CD4<sup>+</sup> cells at baseline and month 12 after
treatment.

**FIGURE 5.** Changes in serum cytokine profiles occur during the immune reconstitution following alemtuzumab treatment. The production of the in-
dicated cytokines in serum samples from four RRMS patients at baseline, day 7, and months 1, 2, 3, and 6 after alemtuzumab therapy is shown. Statistical
analysis was performed using GEE. *p < 0.05, **p < 0.01, ***p < 0.001.
From six untreated RRMS patients were cultured in the absence or presence of IL-7 (1–200 ng/ml) for 10 d. The percentage of CD4+CD25+CD127low Treg cells was determined in gated CD4+ T cells. Statistical analysis was performed using GEE.

Gated CD4+CD25+ cells. The figure represents one of six similar experiments. (A) PBMCs from six untreated RRMS patients were cultured in the absence or presence of IL-7 (1–200 ng/ml) for 10 d. The percentage of CD4+CD25+CD127low Treg cells was determined in gated CD4+ T cells. Statistical analysis was performed using a paired t test. (B) Representative staining for IL-17A and IFN-γ in CD4+ cells, cultured in the absence or presence of IL-7. (C) CD4+ T cells from three RRMS patients were cultured in the absence or presence of IL-7 (100 ng/ml) for 10 d. Statistical analysis was performed using a paired t test. (D) Representative staining for FOXP3 in CD4+ cells, cultured in the absence or presence of IL-7. (E) CD4+ T cells from three RRMS patients were cultured in the absence or presence of IL-7 (100 ng/ml) for the indicated times. Western blotting results represent one of three similar experiments.

To clarify the role of IL-7 in the regulation of the reconstitution of T cell subsets following alemtuzumab-induced lymphodepletion, PBMCs from 10 untreated RRMS patients were cultured in the absence or presence of IL-7. Our results indicate that IL-7 significantly decreased the percentages of Th17 and Th1 cells in gated CD4+ cells (Fig. 6C, 6D), which replicated the ex vivo findings following alemtuzumab treatment.

To uncover the signaling events involved in the IL-7–mediated regulation of the reconstitution of T cell subsets, CD4+ T cells from three untreated RRMS patients were cultured in the absence or presence of IL-7. Western blotting studies showed that IL-7 induced early (30 min) STAT5 phosphorylation, which persisted until the last time point tested (24 h). FOXP3 levels were also increased at all time points. IL-7 induced STAT1 phosphorylation at 24 h, but T-bet expression was decreased at all time points. At 24 h, IL-7 also induced the phosphorylation of STAT6 Th2 transcription factor, whereas it inhibited STAT3 phosphorylation, which is involved in Th17 cell differentiation (27), (Fig. 6E).

**Discussion**

Our study suggests that alemtuzumab’s early therapeutic effect is mediated by a nearly complete depletion of lymphocytes from the peripheral circulation, and that differential reconstitution of individual T cell subsets may contribute to the delayed repopulation of CD4+ cells (14). Whereas alemtuzumab efficiently depletes all CD52+ cells, the delayed reconstitution of CD4+ cells, resulting from deficient CD4+ cell homeostatic proliferation, susceptibility to apoptosis (28), and a relative expansion of immunoregulatory CD4+CD25+CD127low Treg, TGF-β, IL-10+, and IL-4+CD4+ cells, mediates its long-lasting clinical effect. Treg cells preferentially proliferate in the setting of lymphopenia, which can contribute to the suppression of CD4+ homeostatic proliferation and a prolonged reconstitution of CD4+ cells. To our knowledge, our findings demonstrate for the first time that alemtuzumab induces a significant expansion of TGF-β+CD4+ cells. TGF-β inhibits Ag presentation and T cell proliferation and induces the expansion of inducible Treg cells. Deficient thymic output in adults (29), which is even more prominent in MS patients (30), may also contribute to the prolonged CD4+ cell decrease and to the resetting of long-term CD4+ cell counts to numbers that are lower than at baseline (31).

At day 7, the earliest time point so far examined after this treatment, the depletion of circulating lymphocytes was nearly complete (28). Because the percentages of CD4+ cells within the lymphocytes increased at month 1, we conclude that this time point represents an early immune reconstitution phase.

We found that CD4+ and CD8+ memory cells were almost completely depleted at day 7. However, they subsequently underwent a rapid homeostatic proliferation, which at month 1 led to an increased percentage of CD4+ and CD8+ memory cells in comparison with baseline. Following nearly complete depletion, memory cells preferentially expanded owing to their homeostatic proliferation, the conversion of naïve cells to the memory phenotype, decreased requirements for high Ag dose and costimulatory signals for activation, as well as preferential survival in peripheral lymphoid organs from which they can repopulate the peripheral circulation (32, 33).

This study focused on the reconstitution of regulatory and pathogenic T cell subsets (24, 34), which may underlie the long-lasting disease activity suppression. We did not detect a differential CD52 expression on CD4+CD25+CD127low Treg, Th1, Th2, and Th17 CD4+ cells, which were indiscriminately depleted at day 7. However, these T cell subsets exhibited a differential reconstitution pattern. We identified a significant relative expansion of CD4+CD25+CD127low Treg cells with a peak at month 1, which has also been reported in alemtuzumab-treated transplant patients.
(23), being thus related to the lymphopenia and not to the underlying disease.

In contrast to documented CD4+CD25+CD127low Treg cell expansion (35), the reconstitution of other T cell subsets is largely unexplored. To our knowledge, our study has for the first time identified a significant posttreatment incremental increase in the percentages of the TGF-β1–, IL-10–, and IL-4–producing CD4+ cells, with the highest increase at month 3 (3.4–, 5.6–, and 2.9-fold increase in comparison with baseline, respectively), as well as a subsequent decrease in the IL-17A–producing and IFN-γ–producing CD4+ cells at months 12 and 24. The most dramatic relative expansion was noted in TGF-β–producing CD4+ cells, which reached 48.9% of CD4+ cells at month 3. We found that most CD4+CD25+CD127low Treg cells secreted TGF-β (Fig. 3C), suggesting that Treg cells have an inducible phenotype (36). TGF-β is one of the key negative regulators of immune homeostasis, suppressing autoreactive T cell expansion via the inhibition of MHC class I and II expression on APCs (37) and via the induction of FOXP3 expression and conversion of CD4+CD25– suppressor cells (38). Furthermore, TGF-β promotes T cell production of IL-10 through direct activation of the IL-10 promoter (39). These immunoregulatory cytokines may contribute to deficient CD4+ cell reconstitution in alemtuzumab-treated RRMS patients. Additional delayed effects of alemtuzumab treatment include the suppression of IL-17A and IFN-γ–producing CD4+ cells at months 12 and 24, which may also be related to the increased numbers of TGF-β–, IL-10–, and IL-4–secreting CD4+ cells. Future studies are needed to characterize the immunosuppressive effects of expanded CD4+CD25+CD127low Treg cells and their role in the induction of immunoregulatory cytokine secretion and the suppression of inflammatory Th1 and Th17 T cell subsets following alemtuzumab treatment.

Although we did not study the B cell reconstitution, which may mediate secondary autoimmunity in ~25–30% of alemtuzumab-treated patients, IL-4, TGF-β, and IL-10 produced by the reconstituting T cells may contribute to the B cell maturation (40), regulation of Ab isoforms (41), and the induction of Ab production (42). This may contribute to the induction of secondary autoimmune diseases, which was in our study detected in only one patient who developed immune thrombocytopenia 9 mo after second infusion of alemtuzumab. Measurements of serum BAFF and APRIL levels were positively correlated with the percentage of CD4+CD45RO+ memory cells, consistent with the induction of memory cell homeostatic proliferation in lymphopenia by IL-7 (35). Additionally, we found increased serum IL-4, which peaked at 3 mo after therapy. The Th17-polarizing cytokines (IL-1β, IL-6, IL-23, and IL-11) and Th1-polarizing cytokines (IL-27 and IL-12) were decreased during immunoreconstituted, as were Th17 effector cytokines IL-17A, IL-17F, IL-21, IL-22, and Th1 IFN-γ.

A single-nucleotide polymorphism in the gene encoding IL-7R is associated with susceptibility for MS (43); however, the role of IL-7R signaling in the pathogenesis of the disease has not been elucidated (44). Consistent with previous reports (45), our study reveals that IL-7 signaling induces STAT5 phosphorylation, which directly binds to the FOXP3 promoter (46). Interestingly, Treg cells are characterized by low CD127 (IL-7Rα) expression, implying that inducible Treg cells could have received an IL-7 signal at an early stage of immune reconstitution (47). In our in vitro study, we found that IL-7–induced CD4+CD25+CD127low Treg cell expansion and inhibited the percentages of Th17 and Th1 cells, which is consistent with our ex vivo results. Studies regarding the effect of IL-7 on the development of Th17 and Th1 cells are controversial. Ishizu et al. (48) found significantly decreased cerebrospinal fluid IL-7 levels in MS patients, implying that decreased IL-7 levels may be associated with proinflammatory cytokine changes. Indeed, Lee et al. (49) found that in RRMS patient serum, the IL-7 levels were inversely correlated with IL-17F. However, in the same study, IL-7 (at 10 ng/ml) was reported to promote Th1 cell differentiation of naïve CD4+ cells. Thus, our 10-d cultures of PBMCs in the presence of high-dose IL-7 (100 ng/ml) may reflect an IL-7 dose effect, or an IL-7–mediated induction of the CD4+CD25+CD127low Treg cell expansion, which may subsequently decrease Th1 and Th17 cell numbers, as determined in the ex vivo studies of alemtuzumab-treated patients. We emphasize that the in vitro experiments demonstrating IL-7–mediated FOXP3 induction used a high dose of IL-7 (100 ng/ml; Fig. 6E), which in our study, similar to previous reports (50), induced expansion of CD4+CD25+CD127low Treg cells (Fig. 6A). Therefore, the mechanistic in vitro results provide a proof of principle but do not reflect the IL-7 levels measured in treated RRMS patients (Fig. 5). In conclusion, our study has identified novel mechanisms of immune tolerance reconstitution following alemtuzumab immunodepleting therapy and demonstrated that during the immune reconstitution dominated by transient early CD4+CD25+CD127low Treg cell expansion, patients subsequently exhibited expanded TGF-β–, IL-10–, and IL-4–secreting immunoregulatory CD4+ cells, followed by the suppression of Th1 and Th17 cells. These results may advance our understanding of immunological reconstitution in alemtuzumab-treated MS patients.

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Disclosures

The authors have no financial conflicts of interest.

References


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