Regulatory T Cells Are Reduced During Anti-CD25 Antibody Treatment of Multiple Sclerosis

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Objective: Maintenance therapy with anti-CD25 antibody has emerged as a potentially useful treatment for multiple sclerosis (MS). Constitutive CD25 expression on CD4+ CD25+ regulatory T cells (Treg) suggests that anti-CD25 antibody treatment may potentially target a subset of T cells that exhibit immune suppressive properties. We examined changes to CD4+ CD25+ Treg in patients with MS receiving maintenance anti-CD25 monoclonal antibody treatment to determine the effect of treatment on Treg and, consequently, on immunological tolerance.

Design: Peripheral blood and cerebrospinal fluid samples obtained from a before-and-after trial of anti-CD25 antibody monotherapy were examined to compare baseline and treatment differences in CD4+ CD25+ Treg.

Subjects: A total of 15 subjects with MS. One subject was withdrawn owing to an adverse effect.

Results: Sustained reduction of the frequency of CD4+ CD25+ Treg was observed during treatment. Anti-CD25 antibody treatment led to evidence of impaired in vivo Treg proliferation and impaired ex vivo Treg suppression. Inflammatory MS activity was substantially reduced with treatment despite reduction of circulating Treg, and there was no correlation between changes in the frequency of Treg and changes in brain inflammatory activity. However, new-onset inflammatory disease, notably dermatitis, was also observed in a number of subjects during treatment.

Conclusion: The reduction in Treg did not negatively affect maintenance of central nervous system tolerance during anti-CD25 antibody treatment. The incidence of new-onset inflammatory disease outside of the central nervous system in a subset of patients, however, warrants further studies to examine the possibility of compartmental differences in the capacity to maintain tolerance in the setting of reduced CD4+ CD25+ Treg.


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has been implicated in the pathogenesis of a growing number of disorders including systemic lupus erythematosus, psoriasis, aplastic anemia, and MS, suggesting a potentially broad relevance with respect to human autoimmune diseases.

The shared expression of CD25 on conventional activated T cells and CD4+CD25+ Treg suggest that both are potentially targeted by anti-CD25 antibody. Based on the knowledge that CD4+CD25+ Treg contribute to maintenance of tolerance, an inhibitory effect on Treg could potentially exacerbate existing inflammatory disease or unmask underlying predilection for new inflammatory disease. We therefore examined the changes to the CD4+CD25+ T cell subset in subjects with MS undergoing anti-CD25 antibody treatment. In particular, we asked what effect an antihuman CD25 antibody has on CD4+CD25+ Treg, whether changes to CD4+CD25+ Treg affected the immunomodulatory effect of treatment, and whether changes to CD4+CD25+ Treg affected maintenance of overall immunological tolerance.

METHODS

SAMPLES

Subjects with MS13 were enrolled in an open-label trial of anti-CD25 antibody (daclizumab). Subjects were free of immunomodulatory therapy for 24 weeks prior to enrollment and received intravenous infusion of daclizumab monotherapy (1 mg/kg) every 4 weeks for 54 weeks. Peripheral blood was obtained at baseline and during treatment. Cerebrospinal fluid was obtained just prior to administration of treatment samples were obtained just prior to administration of treatment (trough sample). Unless otherwise stated, baseline PBMC samples were compared with treated PBMC samples obtained at month 2.5 (trough sample following third dose). Brain magnetic resonance images were obtained monthly as previously described.12 Informed consent was obtained from each subject. The study was reviewed and approved by the National Institute of Neurological Disorders and Stroke institutional review board.

FLOW CYTOMETRY

The following antibodies were used according to manufacturer's instructions: CD3, CD4, CD8, CD25 (M-A251), CD56, CD127, IL-2, Ki67, and pSTAT5 (signal transduction and activator of transcription 5) antibodies were obtained from BD Biosciences (San Jose, California). The Foxp3 phycoerythrin (PE) or allophycocyanin antibodies were obtained from eBioscience (San Diego, California). The CD25 (Anti-Tac) fluorescein isothiocyanate isotype conjugate was from Immunotech (Westbrook, Maine). The CD25 (7G7) PE was from Ancell (Bayport, Minnesota). The FACS analysis of surface markers were performed on erythrocyte-lysed washed whole blood samples. The PBMC were used for all other analyses. Green Dead Stain (Invitrogen, Carlsbad, California) was used for live or dead cell discrimination with Foxp3 staining. Flow cytometric data was acquired on FACScalibur (BD Biosciences) and analyzed on FlowJo (TreeStar, Ashland, Oregon).

Treg SUPPRESSION ASSAY

To compare Treg suppression between patients with MS at baseline and healthy donors, CD4+ T cells were purified from PBMC by negative selection magnetic beads (Miltenyi, Auburn, California). Purity was more than 95%. The CD25-PE–labeled CD4+ T cells were then sorted (FACS diva; BD Biosciences) into CD4+CD25−/−Treg (highest, 3% CD25 expression) and CD4+CD25+ responder cells. The Treg coculture suppression assay was performed as previously described,4 with minor modifications. Soluble anti-CD3 (HT13a, 0.1µg/mL; BD Pharmingen, Franklin Lakes, New Jersey) was used for stimulation. Irradiated (3000 rad) T cell–depleted (MACS [magnetic activated cell sorting] cell separation/anti-CD3 microbeads; Miltenyi) autologous PBMC was used as accessory cells. Cells were plated in triplicate into 96-well plates, incubated at 37°C for 5 days, and pulsed with [3H] thymidine for the final 18 hours of incubation. The percentage of suppression was calculated as %suppression=[1−(cpm of coculture well/cpm of responder well)]×100, where cpm indicates counts per minute, and the coculture well ratio was 1:1 (responder, Treg). To compare the baseline and treatment Treg suppression, CD4+ T cells were purified from viably cryopreserved PBMC by negative selection magnetic beads. Purified CD4+ T cells were labeled with CD127-PE and CD25-PE/Cy5 antibodies. The CD4+CD25+ CD127low/−Treg (Treg) and CD4+CD25+ CD127+ (responder) cells were sorted by FACS and used for Treg coculture suppression assay as described above.

IMMUNOHISTOCHEMISTRY

Formalin-fixed paraffin-embedded tissue sections were prepared on poly-L-lysine coated slides. Immunohistochemistry for CD3 (Dako, Glostrup, Denmark) and Foxp3 (Abcam, Cambridge, Massachusetts) was carried out on consecutive tissue sections and developed with 3,3′-diaminobenzidine, tetrahydrochloride chromogen. A semiquantitative assessment of CD3 and Foxp3 expression was carried out by counting the number of positively stained lymphocytes under an objective microscopy lens at magnification ×40.

STATISTICAL ANALYSIS

Statistical significance was determined by unpaired t test to compare subjects with MS with healthy donors and by paired t test to compare baseline and treatment values. Where appropriate, comparisons were made using general linear model repeated measures analysis of variance. Pearson correlation coefficients were used to analyze relationships between parameters.

RESULTS

BASELINE CD4+CD25+ Treg CHARACTERISTICS IN SUBJECTS WITH MS

To establish pretreatment characteristics of CD4+CD25+ Treg in this cohort of subjects with MS, Treg suppression was measured by an in vitro Treg coculture assay.8 Figure 1A). Most subjects in this cohort demonstrated baseline Treg suppression within the range for healthy donors (P = .27) (Figure 1B). Likewise, the frequency of circulating CD4+Foxp3+ Treg in this cohort of subjects with MS did not differ significantly at baseline compared with that of age-, sex-, and race-matched healthy
Figure 1. Baseline regulatory T cell (Treg) characteristics in subjects with multiple sclerosis (MS). A, Representative Treg coculture assay [3H] thymidine incorporation data (mean counts per minute [standard deviation]) showing dose-dependent Treg suppression for a subject with MS (86% suppression). Up to 5 × 10⁵ fluorescence-activated cell sorter (FACS) sorted CD4⁺CD25⁺ Treg cells were titrated in coculture with 5 × 10⁵CD4⁺CD25⁻ (responder) cells for 5-day stimulation with anti-CD3 antibody. B, Baseline Treg suppression in subjects with MS (mean [SD], 69.7% [18%]) compared with healthy donors (HD) (mean [SD], 79.7% [13%]; P = .27). C, Frequency of peripheral blood CD4⁺Foxp3⁺ cells from subjects with MS (mean [SD], 2.7% [1.6%] of lymphocytes) compared with age-, race-, and sex-matched HD (mean [SD], 3.2% [1.0%] of lymphocytes; P = .37). D, Representative CD4⁺ gated FACS plots from a subject with MS showing negative CD69 and low or negative CD127 expression in forkhead box P3 (Foxp3⁺) cells (data representative of 6 subjects with MS and 3 HD), and attenuation of interleukin (IL)-2 production following 6-hour phorbol myristate acetate/ionomycin stimulation (data representative of 4 subjects with MS and 3 HD).

REDUCTION OF FOXP3⁺ REGULATORY T CELLS DURING ANTI-CD25 ANTIBODY TREATMENT

Antibody saturation was monitored during the course of anti-CD25 antibody treatment by flow cytometry using 2 fluorochrome-labeled antibodies (anti-Tac and 7G7) that bind noncompeting epitopes on CD25. Complete antibody saturation of CD25, demonstrated by the absence of fluorochrome-labeled anti-Tac binding, was maintained during the course of treatment (Figure 2A, anti-Tac), whereas reduction in the mean total CD25 expression on lymphocytes was less pronounced (13%) but nevertheless statistically significant (P < .001) (Figure 2A, 7G7). Examination of cerebrospinal fluid demonstrated complete antibody saturation of CD25 on cerebrospinal fluid lymphocytes and a 20% decline in mean total CD25 expression during treatment (P = .04) (Figure 2B).

Signaling of IL-2 was inhibited by anti-CD25 antibody treatment. STAT5 phosphorylation, which mediates downstream IL-2 signaling, was used as a marker of IL-2 signaling. Lymphocytes obtained during treatment demonstrated nearly complete absence of STAT5 phosphorylation in response to low-level (10 U/mL) IL-2. Significant reductions in STAT5 phosphorylation were also observed at higher levels (50 and 100 U/mL) of IL-2 (P = .005) (Figure 2C).

The effect of anti-CD25 antibody treatment on CD4⁺CD25⁺ Treg was analyzed by examining Foxp3 as a marker of Treg. Flow cytometric analysis (Figure 2D) demonstrated a reduction in mean fluorescence intensity of Foxp3 expression during treatment compared with baseline (P < .001) (Figure 2E). Reduction in Foxp3 expression at the single cell level during treatment is consistent with previous studies implicating STAT5 as a regulator of Foxp3 gene transcription. Furthermore, the frequency of total Foxp3-expressing CD4⁺ cells were reduced, with approximately 30% reduction in the mean frequency of CD4⁺Foxp3⁺ cells observed by month 2.5 and 44% reduction by month 7.9 (P < .001) (Figure 2F). Similar reductions were observed in the frequency of CD4⁺CD25⁺Foxp3⁺ cells (45% reduction; P < .001). Post-treatment samples available from a limited number of subjects demonstrated recovery of Treg frequencies to near baseline levels.
REDUCTION OF T<sub>reg</sub> PROLIFERATIVE CAPACITY AND IMPAIRED T<sub>reg</sub> SUPPRESSION BY ANTI-CD25 ANTIBODY TREATMENT

Based on the known role of IL-2 in promoting cell cycle progression in conventional T cells, we asked whether altered homeostatic proliferation of T<sub>reg</sub> could account for the reduction in frequency of T<sub>reg</sub> during treatment. The effect of anti-CD25 antibody treatment on T<sub>reg</sub> proliferation was examined using Ki67 expression to estimate the in vivo proliferating fraction, determined as the proportion of CD4<sup>+</sup>Foxp3<sup>+</sup> cells expressing Ki67. The PBMC from baseline and treatment were stained ex vivo for intracellular expression of Ki67. Consistent with a pre-
vious study demonstrating high in vivo proliferative kinetics of human Treg, CD4^+Foxp3^+ cells demonstrated high Ki67 expression at baseline compared with total CD4^+ cells (mean [SD], 11.7% [2.2%] vs 2.13% [0.8%], respectively). Analysis of treatment samples showed a reduction in the Ki67-expressing proliferating Treg fraction (P= .001) (Figure 3A), suggesting impaired homeostatic proliferation of Treg during anti-CD25 antibody treatment.

To assess Treg function, coculture suppression assays were performed to compare Treg suppression at baseline and during treatment. Because of altered CD25 expression during treatment, an additional surface marker, CD127, was used to sort Treg, which express low or no CD127 (CD127^low/neg). The FACS analysis demonstrated that most CD4^+Foxp3^+ cells were contained within the CD25^−CD127^low/neg subset at baseline and during treatment (Figure 3B). To determine the effect of anti-CD25 antibody treatment on Treg suppressive capacity, CD4^+CD25^−CD127^low/neg cells were sorted by FACS from PBMC obtained at baseline and during treatment and co-cultured with autologous CD4^+CD25^− (responder) cells. Treatment samples demonstrated im-

Figure 3. Regulatory T cell (Treg) in vivo proliferation and ex vivo suppression are impaired during anti-CD25 antibody treatment. A, Representative fluorescence-activated cell sorter (FACS) analysis comparing Ki67 expression in forkhead box P3 (Foxp3^+) cells at baseline and during anti-CD25 antibody treatment (the percentage of Ki67-expressing cells is in parentheses). The Bar graph compares the mean (SD) proportion of CD4^+Foxp3^+ cells expressing Ki67 at baseline and during treatment (P= .001; n=12). B, Representative CD4^+ gated FACS analysis showing that most CD4^+Foxp3^+ cells (blue) are CD25^−CD127^low/neg during treatment. The numbers indicate the frequency of cells that are Foxp3^+ within the polygonal gate and, in parentheses, the percentage of total lymphocytes that are CD4^+Foxp3^+ cells. C, Representative [3H] thymidine incorporation data (mean [standard deviation] counts per minute) shown for up to 5 x 10^3 FACS-sorted Treg (CD4^+CD25^−CD127^low/neg) titrated in coculture with 5 x 10^3 responder (CD4^+CD25^−CD127^−) cells. Closed circles represent cells from baseline and open circles represent cells obtained during anti-CD25 antibody treatment. D, Reduced suppressive capacity (mean [standard deviation] percentage of suppression) of circulating Treg during anti-CD25 antibody treatment (P=.03; n=4).
paired T<sub>reg</sub> suppression compared with baseline samples (P = .03) (Figure 3C and D), indicating a functional impairment of ex vivo T<sub>reg</sub> suppression during anti-CD25 antibody treatment.

**LACK OF CORRELATION BETWEEN REDUCTION OF T<sub>reg</sub> AND ACUTE CENTRAL NERVOUS SYSTEM INFLAMMATION**

In addition to changes in T<sub>reg</sub>, anti-CD25 antibody treatment led to significant alteration in conventional activated CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD56<sup>bright</sup> natural killer (NK) cells. The frequency of conventional activated T cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) was reduced during anti-CD25 antibody treatment (P < .001), which corresponded to the contraction of Ki67<sup>+</sup> proliferating fraction of conventional activated T cells. In contrast, the proportion of proliferating CD56<sup>bright</sup> NK cells was increased during anti-CD25 antibody treatment and corresponded to the expansion of CD56<sup>bright</sup>NK cells observed during the course of therapy (P < .001).

In the setting of simultaneous changes to activated conventional T cell and NK cell compartments, T<sub>reg</sub> were not necessarily the major determinants of acute central nervous system inflammation. Contrast (gadolinium dithlenetetramine pentaacetic acid [Gd-DTPA]) enhancement of MS lesions on brain magnetic resonance imaging, a marker of MS inflammatory activity, was assessed on a monthly basis. The number of MS lesions demonstrating Gd-DTPA enhancement (Gd-DTPA<sup>+</sup> lesions) was significantly reduced during treatment with anti-CD25 antibody (P < .001) (Figure 4A). To assess whether the reduction in T<sub>reg</sub> had any negative effect on the immunomodulatory effect of anti-CD25 antibody, we analyzed the relationship between changes in the frequency of T<sub>reg</sub> and brain inflammatory activity. No significant correlations were observed between changes in the frequency of T<sub>reg</sub> and changes in brain inflammatory activity measured as the total number of Gd-DTPA<sup>+</sup> lesions per month (r² = 0.0171; P = .69) (Figure 4B) or the number of new Gd-DTPA<sup>+</sup> lesions per month (r² = 0.0157; P = .71) when assessed at month 7.5. Analysis of earlier and later time points (months 2.5 and 12.5) yielded similar results (r² = 0.068 and r² = 0.0391, respectively, for correlation between change in total number of Gd-DTPA<sup>+</sup> cells and T<sub>reg</sub>).

**NEW-ONSET INFLAMMATORY DERMATITIS AS AN ADVERSE EVENT DURING ANTI-CD25 ANTIBODY TREATMENT**

Dermatitis occurred in 3 of 15 individuals who were taking anti-CD25 antibody (Table). The onset of dermatitis occurred during anti-CD25 antibody treatment in 2 subjects and at the end of treatment in one subject who nevertheless still demonstrated more than 80% saturation of CD25 at the onset of dermatitis. An additional subject with a family history of rheumatoid arthritis developed palpindromic rheumatism during treatment. Reductions in the frequencies of Foxp3<sup>+</sup> cells for subjects who developed dermatitis are shown in the Table. Histologic examination of lesional skin from 2 subjects who developed dermatitis during treatment showed spongiform to psoriasiform epidermal changes with perivascular lymphocytic inflammatory infiltrate in the subjacent superficial dermis (Figure 4C and D). In situ quantitative detection of Foxp3 in the lesional skin showed that approximately 13% of infiltrating CD3<sup>+</sup> cells were T<sub>reg</sub> (Figure 4E and F).

**COMMENT**

Here we demonstrate that long-term maintenance anti-CD25 antibody treatment led to sustained reduction of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> in subjects with MS. In contrast to the hypoproliferative nature of T<sub>reg</sub> in culture, we and others<sup>22</sup> find evidence that T<sub>reg</sub> exhibit high replicative capacity in vivo. Reduced proliferating T<sub>reg</sub> fraction corresponds to decline in T<sub>reg</sub> numbers in the setting of impaired IL-2 signaling and suggests that IL-2–supported T<sub>reg</sub> proliferation accounts for a substantial portion of the human circulating T<sub>reg</sub> pool. Clinical trials in patients with cancer demonstrated upregulation of Foxp3 and increased frequency of T<sub>reg</sub> following administration of IL-2.<sup>23,24</sup> Our data demonstrates, conversely, that negative perturbation of IL-2 signaling reduces Foxp3 expression and reduces the circulating T<sub>reg</sub> pool. Collectively, these studies indicate that IL-2 plays a major role in controlling the homeostatic set point for the size of the human circulating T<sub>reg</sub> pool.

We asked whether reduction in circulating T<sub>reg</sub> during anti-CD25 antibody treatment negatively affected MS inflammatory activity. Overall brain inflammatory activity was reduced during treatment, suggesting a shift toward tolerance. The lack of a correlation between changes in frequency of T<sub>reg</sub> and changes in brain inflammatory activity suggests that sustained reduction in circulating T<sub>reg</sub> did not negatively affect disease activity. One likely explanation is that simultaneous changes in other cell subsets during anti-CD25 antibody treatment countered any negative affect of reduced T<sub>reg</sub>. Anti-CD25 antibody treatment led to a contraction of the CD4<sup>+</sup>CD25<sup>+</sup> activated conventional T cell fraction and an expansion of CD56<sup>bright</sup>NK cells. A previous study demonstrated the capacity of CD56<sup>bright</sup>NK cells to suppress inflammation; expansion of CD56<sup>bright</sup>NK population is potentially a major determinant of acute brain inflammatory activity during anti-CD25 antibody treatment.<sup>25</sup> Alternatively, the lack of correlation between changes in circulating T<sub>reg</sub> and changes in brain inflammatory activity suggests the possibility that T<sub>reg</sub> are not a major determinant of acute inflammatory activity in MS. Data from experimental autoimmune encephalomyelitis, an animal model of MS, have not yet reconciled what role CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> play in modulating acute central nervous system inflammation. Loss of T<sub>reg</sub> appears to confer susceptibility to experimental autoimmune encephalomyelitis in an otherwise resistant strain of mice,<sup>26</sup> but central nervous system antigen-specific T<sub>reg</sub> failed to inhibit central nervous system effector T cells during the acute phase of experimental autoimmune encephalomyelitis, possibly owing to the in situ cytokine milieu, particularly IL-6, that renders effector T cells resistant to T<sub>reg</sub> suppression.<sup>27</sup>
Dermatitis occurred in 3 of the 15 subjects receiving treatment. An additional subject with a family history of rheumatoid arthritis developed migratory tenosynovitis during treatment, diagnosed as palindromic rheumatism. The incidence of new-onset inflammatory disease during anti-CD25 antibody treatment raised the possibility that there may be compartmental differences in the capacity to maintain tolerance in the setting of reduced circulating $T_{reg}$. A recent study showed that CD4+ CD25+ $T_{reg}$ contribute to routine immune surveillance and inflammatory response in the human skin. Furthermore, the availability of circulating $T_{reg}$ capable of mi-
grating into the skin was shown to be critical to the maintenance of skin-specific tolerance in an animal model, suggesting that the skin may be particularly vulnerable to reduction in circulating T<sub>reg</sub>.<sup>20</sup> Histologic findings from skin biopsies taken from our subjects were relatively nonspecific, but not inconsistent with what has been described in the Foxp3-deficiency syndrome immune dysregulation, polyendocrinopathy, enteropathy, X-linked (syndrome).<sup>30</sup> In situ quantitative detection of Foxp3 in the lesional skin showed that approximately 13% of infiltrating CD3<sup>+</sup> cells were T<sub>reg</sub>, which represents a lower frequency of T<sub>reg</sub> at the site of skin inflammation compared with historical controls.<sup>31</sup> Reduction in the frequency of T<sub>reg</sub> was relatively high (above the cohort mean/median) in subjects who developed new-onset inflammatory disease, but did not reach statistical significance compared with those who did not develop new inflammatory disease on treatment. The relationship between reduction in T<sub>reg</sub> and new-onset inflammatory disease during anti-CD25 antibody treatment, though suggestive, is inconclusive, and further work is required to determine whether there are compartmental differences in requirements for CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> to maintain organ-specific tolerance.

A functional defect of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells has been reported in subjects with MS.<sup>14,32</sup> We found no significant difference in mean T<sub>reg</sub> suppression between our cohort of subjects with MS and healthy donors, suggesting either that a functional defect of T<sub>reg</sub> may not be a uniform finding in all subjects with MS or that the differences are on a scale that requires a larger cohort to adequately power such comparisons. Studies comparing T<sub>reg</sub> suppression in subjects with MS and healthy volunteers suggest that the differences may be age-dependent<sup>33</sup> or disease stage-dependent.<sup>34</sup>

The collective clinical experience with anti-CD25 antibody treatment constitutes a large body of data that demonstrates its safety and efficacy as induction therapy in the prevention of allograft rejection<sup>35</sup> and suggest its utility in an array of human disorders.<sup>36</sup> The effect of short-term anti-CD25 antibody induction therapy on T<sub>reg</sub> is likely transient or modified by concomitant use of immunosuppressive agents.<sup>37</sup> We now demonstrate that a consequence of long-term maintenance monotherapy with anti-CD25 antibody is a sustained reduction of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>. Maintenance therapy with anti-CD25 antibody is likely to be a valuable therapeutic option in a number of immune-mediated inflammatory diseases.<sup>3,38</sup> Our findings underscore the need to clarify the organ-specific consequences of sustained reduction in human CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>.


