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Cutting Edge: Anti-CD25 Monoclonal Antibody Injection Results in the Functional Inactivation, Not Depletion, of CD4⁺CD25⁺ T Regulatory Cells

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CD4⁺CD25⁺ T regulatory (T₉) cells are an important regulatory component of the adaptive immune system that limit autoreactive T cell responses in various models of autoimmunity. This knowledge was generated by previous studies from our lab and others using T₉ cell supplementation and depletion. Contrary to dogma, we report here that injection of anti-CD25 mAb results in the functional inactivation, not depletion, of T₉ cells, resulting in exacerbated autoimmune disease. Supporting this, mice receiving anti-CD25 mAb treatment display significantly lower numbers of CD4⁺CD25⁺ T cells but no change in the number of CD4⁺FoxP3⁺ T₉ cells. In addition, anti-CD25 mAb treatment fails to both reduce the number of Thy1.1⁺ congenic CD4⁺CD25⁺ T₉ cells or alter levels of CD25 mRNA expression in treatment recipients. Taken together, these findings have far-reaching implications for the interpretation of all previous studies forming conclusions about CD4⁺CD25⁺ T₉ cell depletion in vivo. The Journal of Immunology, 2006, 176: 3301–3305.

C D4⁺CD25⁺ T regulatory (T₉) cells are a member of the growing family of regulatory cell populations that serve to limit the activation, trafficking, and/or effector function of both CD4⁺ and CD8⁺ T cells (1, 2). Although the exact mechanism by which T₉ cells serve to limit T cell functionality remains unknown, IL-10 production, surface CTLA-4 expression, IL-2 sequestration, costimulatory molecule blockade, and surface/secreted TGF-β expression are all proposed mechanisms by which T₉ cells may down-regulate effector CD4⁺ and CD8⁺ T cell responses. Regardless of the exact mechanism of action, T₉ cells are believed to contribute to the protective processes that govern susceptibility to, progression of, and remission from various autoimmune diseases. T₉ cells were described originally as CD4⁺ T cells that coexpress CD25 and high levels of CD62L in naive mice and are now more appropriately characterized as CD4⁺CD25⁺FoxP3⁺ cells (3).

Recent studies have revealed a functional role for CD25 expression on T₉ cells such that interruption of the IL-2R/IL-2 signaling pathway blocks T₉ effector function potentially via alterations in the expression of the glucocorticoid-induced TNFR-family gene (GITR or TNFRSF18) (4, 5). Accordingly, a number of groups have targeted CD25 as a mechanism of depleting T₉ cells and studying resultant effects on T cell activation, trafficking, and/or effector function. It is widely believed that injection of anti-CD25 mAb results in the rapid and efficient depletion of CD4⁺CD25⁺ T₉ cells as determined by secondary staining with a mAb directed against a different CD25 epitope (6–8).

In the current study, we report that in vivo injection of anti-CD25 mAb fails to physically deplete CD4⁺CD25⁺ T₉ cells but, alternatively, down-regulates and/or induces shedding of CD25 from the surface of T₉ cells, resulting in exacerbated acute clinical experimental autoimmune encephalomyelitis (EAE). This conclusion is supported by our findings that anti-CD25 mAb treatment decreases the number of CD4⁺CD25⁺, but not CD4⁺FoxP3⁺ T₉ cells. These findings were confirmed using Thy1.1⁺ CD4⁺CD25⁺ congenic T₉ cells adoptively transferred into naive Thy 1.2⁺ recipients before anti-CD25 mAb treatment, which decreased the number of CD25⁺, but not Thy1.1⁺, CD4⁺ T cells. In light of the functional dependence of T₉ cells on CD25 expression, our data suggest that injection of anti-CD25 mAb induces functional inactivation, but not physical depletion, of CD4⁺CD25⁺ T₉ cells.

Materials and Methods

Mice and materials

SJL/J female mice, 5–6 wk old, were purchased from Harlan Sprague Dawley and SJL-Thy1⁺ congenic mice were bred in the Northwestern University Center for Comparative Medicine (Chicago, IL). Induction and clinical evaluation of proteolipid protein (PLP)139–151-induced EAE

Six- to 7-wk-old female mice were immunized s.c. with 200 μl of an emulsion containing 800 μg of Mycobacterium tuberculosis H37Ra (Difco) and a suboptimal dose (25 μg) of PLP139–151 distributed over three spots on the flanks.

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3 Abbreviations used in this paper: T₉, CD4⁺CD25⁺ T regulatory; EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; LN, lymph node.
Individual animals were observed daily, and clinical scores were assessed in a blinded fashion on a 0–5 scale as follows: 0, no abnormality; 1, limp tail; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; and 5, moribund. The data are reported as the mean daily clinical score.

In vitro ELISPOT assays were performed as described previously (9).

Immunohistochemistry and immunofluorescence

CNS immunohistochemistry was performed as described previously (9). For the detection of CD4⁺CD25⁺FOXP3⁺ T cells, single-cell suspensions were washed and first incubated with Abs directed against CD4 (L3T4; BD Biosciences) and CD25 (7D4 or PC61; BD Biosciences) for 60 min before cell permeabilization and incubation with anti-FOXP3 (FJK-161; eBiosciences) for 30 min per the manufacturer’s specifications. Fluorescent staining was analyzed using a LSRII flow cytometer and CellQuest Pro Analysis software (BD Biosciences).

Statistical analysis

Comparisons of clinical scores between the various treatment groups were analyzed by unpaired Student’s t test. Values of p < 0.01 were considered significant.

Results and Discussion

The capacity of CD4⁺CD25⁺ T<sub>R</sub> cells to efficiently influence effector T cell function has stimulated a significant level of interest in their potential to regulate immune responses during infection, autoimmune disease, and cancer. Using various experimental models, multiple groups have reached the common conclusion that CD4⁺CD25⁺ T<sub>R</sub> cells suppress autoreactive T cell effector function and autoimmune disease progression (10). More specifically, we have shown previously that supplementation of the T<sub>R</sub> cell population in naive mice confers protection from subsequent development/induction of EAE (11, 12).

To address the contribution of endogenous T<sub>R</sub> cells in regulating EAE onset and progression, we first examined the phenotype and distribution of T<sub>R</sub> cell populations at various times throughout the clinical disease course of EAE. Histological analysis revealed an influx of FOXP3⁺ cells into the CNS at times corresponding to the onset of clinical disease symptoms (Fig. 1, A and B), and these cells appeared to be localized directly within disease lesions, as indicated by the paucity of PLP staining. This detection of T<sub>R</sub> cells within the CNS target organ suggested that endogenous T<sub>R</sub> cells may regulate the acute clinical disease phase. To test this, mice were depleted of CD4⁺CD25⁺ T<sub>R</sub> cells at various times either before or after disease induction and were followed for clinical disease progression. As seen in Fig. 1C, anti-CD25 mAb injection at times either before, corresponding with, or after suboptimal disease induction (25 μg of PLP<sub>139–151</sub>) resulted in significant exacerbation of clinical disease incidence and severity, compared with the minimal disease noted in untreated mice. Accordingly, anti-CD25 mAb injection also enhanced the effector function of PLP<sub>139–151</sub>-specific T cells, as measured both by the level of IFN-γ produced (data not shown) and the number of IFN-γ-producing cells following in vitro restimulation with PLP<sub>139–151</sub> (Fig. 1D). These findings suggest that endogenous T<sub>R</sub> cells regulate normal EAE disease onset/progression, potentially via their presence in the CNS.

The injection of anti-CD25 mAb treatment to physically “deplete” CD4⁺CD25⁺ T<sub>R</sub> cells in vivo is common practice. We have reported previously that injection of anti-CD25 mAb results in an apparent rapid, but short-lived, depletion of CD4⁺CD25⁺ T<sub>R</sub> cells, such that normal levels of CD4⁺CD25⁺ T<sub>R</sub> cells are observed within 10–14 days following treatment (13). However, the short-lived nature of this Ab-mediated depletion raised the question about the true mechanism of anti-CD25 mAb in vivo, because the de novo recovery of the CD4⁺CD25⁺ T<sub>R</sub> cell population should require >14 days to return to control levels. To address this, naive mice were injected with anti-CD25 mAb, followed by phenotypic analysis of the secondary lymphoid tissue to determine any effects of the treatment on the CD4⁺CD25⁺FOXP3⁺ T cell population. As expected, the number of CD4⁺CD25⁺ T cells was significantly decreased

**FIGURE 1.** Regulatory role of T<sub>R</sub> cells during EAE. A and B, CNS T<sub>R</sub> cell infiltration during EAE. Naive SJL/J mice were immunized with 50 μg of PLP<sub>139–151</sub>/CFA s.c. on day 0 and then sacrificed at the onset of clinical disease for CNS histology. FOXP3⁺ T cells (red) were visualized in cerebellar tissue isolated from either naive (A) or diseased (B) mice. Tissue sections also were stained with PLP (green) and 4′,6′-diamidino-2-phenylindole (blue) as counterstains. Note the presence of FOXP3⁺ T cells within non-PLP staining areas of demyelinating lesions in diseased mice. All data are representative of three separate experiments. C, Effect of anti-CD25 mAb treatment on suboptimal EAE. Groups of five naive SJL/J mice were immunized with a suboptimal dose (25 μg) of the immunodominant epitope of PLP (PLP<sub>139–151</sub>) on day 0 and followed for clinical disease. Mice also received two injections of anti-CD25 mAb (7D4; 500 μg/injection i.p.) on either days −4 and −2 (pretreatment), 0 and 2 (induction), or 8 and 10 (postinduction) following disease induction. Data are presented as the mean clinical disease score of mice in each treatment group, and disease incidence is indicated by the numbers in parentheses. D, Effect of anti-CD25 mAb treatment on autoreactive CD4⁺ T cell effector function. Spleen and LN samples were isolated from mice at day 17 following disease induction and analyzed ex vivo for the number of IFN-γ-producing cells. Data are presented as the number of IFN-γ-secreting cells in response to PLP<sub>139–151</sub>.
in vivo within 3 days of treatment but returned to control levels by day 10 following anti-CD25 mAb injection (Fig. 2A). This relatively quick recovery of the T<sub>R</sub> cell population is in direct agreement with our previous findings (13). However, injection of anti-CD25 mAb failed to influence the number of CD4<sup>+</sup>FOXP3<sup>+</sup> cells (Fig. 2, B–D). In light of the rapid recovery of the CD4<sup>+</sup>CD25<sup>+</sup> T cell population following anti-CD25 mAb treatment and the fact that the majority of CD4<sup>+</sup>CD25<sup>+</sup> T cells also express FOXP3, it appeared that anti-CD25 mAb treatment was not physically depleting CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells. To validate these findings, we adoptively transferred sort-purified Thy1.2<sup>+</sup> CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells into naive Thy1.1<sup>+</sup> recipient mice before treatment with anti-CD25 mAb. In agreement with the findings above, anti-CD25 mAb injection decreased the percentage of CD4<sup>+</sup>CD25<sup>+</sup> (Fig. 2B), but not CD4<sup>+</sup>Thy1.2<sup>+</sup> (Fig. 2F), T cells in treated recipients. Thus, contrary to dogma, these data indicate that anti-CD25 mAb treatment enhances effector T cell function via mechanisms distinct from physical depletion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>R</sub> cells in vivo.

Because the primary technique for detecting T<sub>R</sub> cells involves the colocalization of CD4 and CD25 surface protein expression, it is possible that anti-CD25 mAb-mediated alterations in the level of CD25 expressed on the surface of T<sub>R</sub> cells may give the false impression of physical depletion. This is supported by the observation that both clones of anti-CD25 (7D4 and PC61) decreased the level of cell surface CD25 protein expression, but not CD25 or FOXP3 mRNA expression in the spleen and lymph nodes (LN) of treated recipients (Figs. 3, A and B). In light of these findings, possible mechanisms to explain the decreased level of in vivo CD25 expression following anti-CD25 mAb treatment include the following: 1) exposure of T<sub>R</sub> cells to anti-CD25 mAb leads to internalization and/or shedding of the CD25 molecule from the cell surface in response to the anti-CD25 mAb treatment; or 2) the two clones of anti-CD25 mAb used for depletion and subsequent detection compete for the same epitope.

To address these possibilities, we initially performed in vitro studies examining the kinetics of CD25 down-regulation, which revealed a significant decrease in the level of CD25 surface expression as early as 30 min following injection of anti-CD25 mAb (data not shown). To determine whether the Ab treatment induced internalization of CD25, spleen and LN cells were analyzed for both extracellular and extracellular/intracellular (total) CD25 expression at varying times following injection of anti-CD25 mAb in vivo. Importantly, CD4<sup>+</sup>FOXP3<sup>+</sup> T<sub>R</sub> cells displayed significantly decreased levels of both surface (extracellular) and total (intracellular/extracellular) CD25 expression 24 h following anti-CD25 mAb injection (Fig. 4A). Surprisingly, the level of CD25 mRNA was decreased in the total cell population of the spleen and LN (Fig. 4B). Although FOXP3 mRNA expression was also decreased, it was not as pronounced as the decrease in CD25 mRNA (Fig. 4C).

![FIGURE 2. CD25 Expression on T<sub>R</sub> cells following anti-CD25 mAb treatment. A–D. Anti-CD25 mAb treatment exerts differential effects on the detection of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>FOXP3<sup>+</sup> cell populations. Naive mice were injected with anti-CD25 mAb (7D4; 500 μg/injection i.p.) on days −2 and 0. Spleen and LN samples were then isolated 3, 6, and 10 days following treatment and then analyzed by flow cytometry (PC61 clone used for CD25 visualization) for the percentage of CD4<sup>+</sup>CD25<sup>+</sup> (A) or CD4<sup>+</sup>FOXP3<sup>+</sup> (B) cells. Data are presented as mean percent positive for each group. Spleen samples also were isolated from either control (C) or anti-CD25 mAb-injected (D) animals on day 2 following treatment to visualize CD25 and FOXP3 expression by immunohistochemistry. Isotype controls revealed no background staining, and data are representative of two separate experiments. E and F. Anti-CD25 mAb treatment does not deplete CD4<sup>+</sup>CD25<sup>+</sup> Thy1.2<sup>+</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> T cells were sort-purified from naive SJL-Thy1<sup>+</sup> mice and adoptively transferred into SJL-Thy1<sup>+</sup> congenic recipients before anti-CD25 mAb treatment (clone 7D4) as described above. Data are presented as the percentage of CD25<sup>+</sup> (E) and Thy1.2<sup>+</sup> (F) CD4<sup>+</sup> T cells in treatment recipients and are representative of three separate experiments.](http://www.jimmunol.org/)

![FIGURE 3. Effect of anti-CD25 mAb treatment on CD25 and FOXP3 mRNA expression. Naive SJL/J mice were injected with anti-CD25 mAb (7D4 or PC61; 500 μg/injection i.p.) on days −2 and 0. Spleen and LN samples were then isolated 5 days following treatment and analyzed by real-time PCR for the level of CD25 (A) and FOXP3 (B) mRNA expression. Data are presented as the amount of CD25 or FOXP3 mRNA (ag). Data are representative of two separate experiments.](http://www.jimmunol.org/)
CD25 expression was lower in both intact and anti-CD25 treated mice when we measured the combined intracellular/extracellular expression of the receptor in comparison to extracellular expression only. However, this decreased expression was most likely due to fixative-induced interference with the CD25 staining protocol. Intracellular FOXP3 expression was not altered by either injection of anti-CD25 mAb or by the technique of measuring extracellular vs intracellular/extracellular CD25 expression (Fig. 4B). Intracellular/extracellular CD25 expression also was significantly decreased as early as 30 min following in vitro exposure to anti-CD25 mAb (data not shown), and importantly, the two clones of anti-CD25 mAb (PC61 and 7D4) used for depletion and subsequent detection were shown to not compete for a common binding epitope (data not shown). Finally, Western blot analysis of whole cellular protein lysates of CD4⁺CD25⁺ TR cells isolated 24 h following either injection of anti-CD25 mAb in vivo (Fig. 4C) or the addition of anti-CD25 mAb in vitro (data not shown) revealed a decrease in total cellular CD25 expression as early as 24 h or 30 min, respectively.

The findings that anti-CD25 mAb treatment decreases both the level of CD25 surface and total protein expression, in the absence of alterations in either CD25 mRNA expression or FOXP3 protein expression, supports the conclusion that injection of anti-CD25 mAb does not induce the physical depletion of CD4⁺CD25⁺ TR cells, but rather down-regulates CD25 cell surface expression. This observation appears to directly contradict earlier findings from our lab and others reporting that anti-CD25 mAb treatment results in both Tregs cell depletion and exacerbated immune responses (7). However, one important consideration is that IL-2 binding of CD25 is critical to the activation/function of Tregs cells (5). Thus, our current findings suggest that increased T cell effector function following anti-CD25 mAb treatment results from Ab-induced shedding of the CD25 molecule and the subsequent functional inactivation of CD4⁺CD25⁺ Tregs cells as opposed to their physical depletion.

One interesting observation is that anti-CD25 mAb treatment decreased the level of cell surface CD25 expression without altering either the level of intracellular CD25 protein or CD25 mRNA expression (Figs. 3 and 4). This finding suggests that CD25 may be shed from the surface of Tregs cells, rather than internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalization, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment. In agreement with this, it is well established that IL-2 binding results in internalized, following anti-CD25 mAb treatment.


