

Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis

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CD4⁺CD25⁺ regulatory T cells (T reg cells) play a key role in controlling autoimmunity and inflammation. Therefore, therapeutic agents that are capable of elevating numbers or increasing effector functions of this T cell subset are highly desirable. In a previous report we showed that a superagonistic monoclonal antibody specific for rat CD28 (JJ316) expands and activates T reg cells in vivo and upon short-term in vitro culture. Here we demonstrate that application of very low dosages of the CD28 superagonist into normal Lewis rats is sufficient to induce T reg cell expansion in vivo without the generalized lymphocytosis observed with high dosages of JJ316. Single i.v. administration of a low dose of the CD28 superagonist into Dark Agouti (DA) rats or Lewis rats that suffered from experimental autoimmune encephalomyelitis (EAE) proved to be highly and equally efficacious as high-dose treatment. Finally, we show that T reg cells that were isolated from CD28-treated animals displayed enhanced suppressive activity toward myelin basic protein-specific T cells in vitro, and, upon adoptive transfer, protected recipients from EAE. Our data indicate that this class of CD28-specific monoclonal antibodies targets CD4⁺CD25⁺ T reg cells and provides a novel means for the effective treatment of multiple sclerosis and other autoimmune diseases.

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Abbreviations used: AT, adoptive transfer EAE; CFSE, carboxy-fluorescein diacetate succinimidyl ester; CNS, central nervous system; CTLA-4, CTL-associated antigen 4; DA, Dark Agouti; EAE, experimental autoimmune encephalomyelitis; gpMBP, guinea pig MBP; LNC, lymph node cell; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; T reg cell, regulatory T cell; T_{conv} cell, conventional T cell.

Immunologic self-tolerance is maintained by several mechanisms, including negative selection of autoreactive T cells in the thymus and peripheral regulatory mechanisms, such as anergy induction, ignorance, or dominant suppression by regulatory T cells (T reg cells; reference 1). Among the different T reg cell populations investigated so far, CD4⁺CD25⁺ T reg cells—the so-called “naturally occurring” T reg cells—play a key role in the control of immune responses to self-antigens (2–5). Reduced frequencies or impaired function of this T reg cell population leads to a wide range of autoimmune disorders in animals (6) and putatively also in humans (7–10); this indicated that an imbalance between T reg cells and autoreactive T cells may contribute to the pathogenesis of such

diseases. Thus, reagents that are capable of expanding the pool of CD4⁺CD25⁺ T cells and enhancing their suppressive activity should prove to be very efficient therapeutic tools.

We previously described a novel class of superagonistic CD28-specific monoclonal antibodies that can induce the in vitro and in vivo expansion of T cells without a need for TCR engagement (11–13). The dramatic polyclonal T cell response after in vivo application of a CD28 superagonist (JJ316) is well tolerated and not accompanied by a toxic cytokine storm of proinflammatory mediators, a finding we have previously attributed to the induction of counter-regulatory anti-inflammatory cytokines, such as IL-10 (14). Moreover, we could demonstrate that superagonistic mAbs to CD28 are capable of expanding functional CD4⁺CD25⁺ T reg cells (15), suggesting that CD28 superagonist therapy may be highly effective in the treatment of inflammatory and autoimmune diseases.

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In the present report, we show that in vivo application of the superagonistic mAb JJ316 to Lewis rats causes preferential expansion of T reg cells over conventional T cells (T_{conv} cells), and enhances their suppressive activity. The administration of very low dosages of the CD28 superagonist in vivo allowed us to segregate T reg cell expansion and activation from the generalized lymphocytosis that was observed with high dosages. By making use of experimental autoimmune encephalomyelitis (EAE), an animal model for human multiple sclerosis (MS), we showed that superagonistic CD28-specific mAbs are highly efficacious in treating a prototypic autoimmune disease. Furthermore, in vitro suppression assays with encephalitogenic T cells, and adoptive transfer of T reg cells in vivo indicated that this therapeutic effect is mediated by the in vivo activation of $CD4^+CD25^+$ T reg cells.

RESULTS

In vivo expansion of T reg cells by CD28 superagonists

In previous reports, we showed that application of JJ316 to normal Lewis rats led to a dramatic, but transient, expansion of T_{conv} and T reg cells with a clear preference for T reg cell expansion (11, 15). This suggested that T reg cells may be more sensitive to superagonistic CD28 stimulation than conventional CD4 T cells. Accordingly, we analyzed the effects of different doses of CD28 superagonist on the expansion and function of T reg and T_{conv} cells in vivo. Normal Lewis rats were treated with control antibody MOPC-31C or with the CD28 superagonist JJ316 at doses ranging from 0.1 to 1 mg antibody per animal. 3 d later, spleen and lymph node cells (LNCs) were isolated from these animals. T cell subsets defined by CD25 expression were analyzed by flow cytometry (Fig. 1 A), and relative and absolute cell numbers for all doses of antibody injected

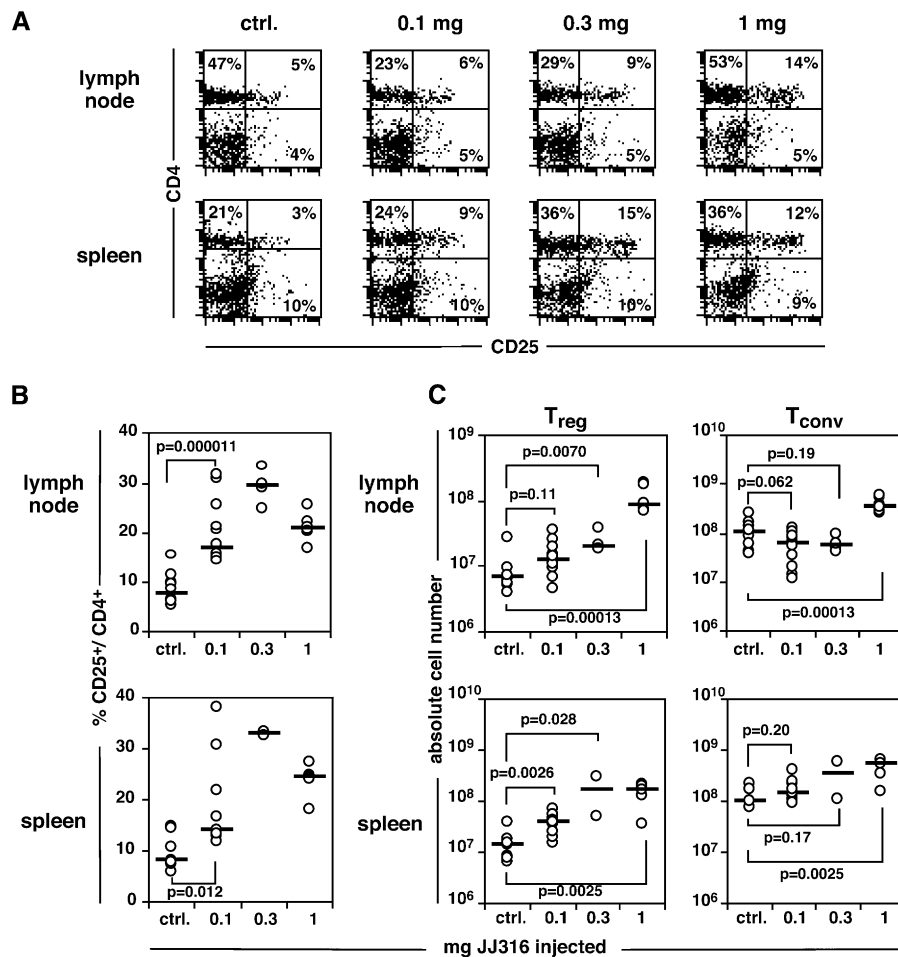


Figure 1. In vivo dose-response titration of mAb JJ316. Adult Lewis rats were injected i.v. with the three indicated amounts of mAb JJ316. 3 d after injection, peripheral LNCs and splenocytes were analyzed for the prevalence of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells by FACS analysis. (A) Representative dot plots of CD4 and CD25 expression in lymph nodes and spleens of control or JJ316-treated animals; the figure indicates the percentages of cells in the respective quadrant. (B) The proportion of

$CD25^+$ among $CD4^+$ cells is summarized for all animals analyzed. Absolute cell numbers were obtained by multiplying total cell numbers with the relative cell numbers obtained by FACS analysis (C). The plots in (B) and (C) show pooled data obtained on eight different occasions. Each circle represents one animal (i.e., 2 to 12 animals per group). Horizontal bars indicate medians. Mann-Whitney rank sum tests were performed between groups as indicated by the brackets, and the respective P values are given.

were calculated (Fig. 1, B and C). Fig. 1 B shows that as little as 0.1 mg of JJ316 per animal (0.5 mg/kg body weight) led to a significant increase in the percentage of CD25⁺ cells among CD4⁺ cells in peripheral lymph nodes and in the spleen. Absolute cell numbers of CD4⁺CD25⁺ T cells were increased significantly after injection of low amounts of the antibody (0.1 mg and 0.3 mg; Fig. 1 C), whereas CD4⁺CD25⁻ cells did not show a significant change in numbers in this dose range. Only upon injection of 1 mg (Fig. 1 C) or more (unpublished data) of mAb JJ316, a significant and very pronounced increase in CD4⁺CD25⁻ T cell numbers was observed; this is in accordance with our previous observations (11). These data indicate that low doses of CD28 superagonists drive T reg cell proliferation in vivo without expanding the pool of T_{conv} cells.

Direct monitoring of T reg cell proliferation in vivo

To track the in vivo proliferative response of T reg cells after application of the CD28 superagonist directly, we isolated

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, labeled them ex vivo with carboxyfluorescein diacetate succinimidyl ester (CFSE), and re-injected them i.v. into syngeneic normal Lewis rats. After 18 h the rats received a single i.v. injection of 0.1 mg of superagonistic CD28-specific mAb or control mAb MOPC-31C. When anti-CD28-treated rats were analyzed at day three after transfer, 40% of CFSE-labeled CD4⁺CD25⁻ T cells and 64% of transferred CD4⁺CD25⁺ T cells had undergone one or more cell divisions (Fig. 2 A). The marked CFSE dilution that was observed among transferred T reg cells provides direct evidence that JJ316 is capable of driving the proliferation of CD4⁺CD25⁺ T reg cells in vivo.

In the same experiment, we also determined CD25 expression on transferred T_{conv} cells and T reg cells (Fig. 2 B). CD25⁻CD4⁺ T cells failed to express CD25 upon CD28 treatment in vivo (Fig. 2 B), whereas transferred T reg cells further up-regulated CD25 expression on the cell surface (Fig. 2 B). Because 0.1 mg of JJ316 was not sufficient to induce very

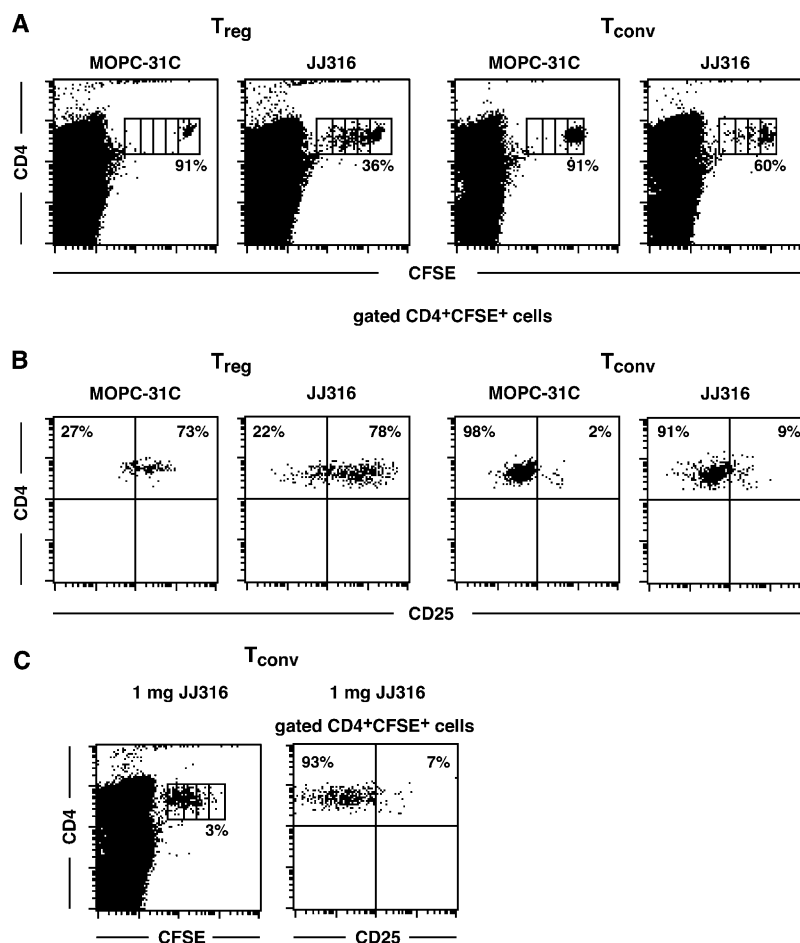


Figure 2. Direct monitoring of T reg and T_{conv} cell proliferation upon superagonistic anti-CD28 stimulation in vivo. T reg and T_{conv} cells were purified from pooled spleens and lymph nodes, labeled with CFSE, and adoptively transferred into syngeneic recipients. 0.1 mg of mAb JJ316 or control mAb MOPC-31C was injected on the following day. CFSE dilution was analyzed 2 d after antibody injection (A). Percentages repre-

sent the proportion of undivided cells among transferred cells. The result of one of at least three experiments is shown. (B) CD25 expression on transferred T reg and T_{conv} cells isolated from MOPC-31C-treated or JJ316-treated animals was determined by counter-staining with anti-CD25 mAb. (C) CFSE dye dilution among, and CD25 expression on, T_{conv} cells were assessed after stimulation with 1 mg of JJ316.

significant proliferation of T_{conv} cells, we also monitored CFSE dye dilution among, and CD25-expression, on T_{conv} cells after stimulation with 1 mg JJ316 in vivo (Fig. 2 C). With this dose, 97% of T_{conv} cells had undergone at least one cell division, but, again, without detectable induction of CD25 expression on these cells (Fig. 2 C; reference 15). Therefore, the increased number of $CD4^+CD25^+$ T cells after JJ316 stimulation in vivo clearly reflects the expansion of preexisting $CD4^+CD25^+$ cells.

Phenotype and function of in vivo expanded $CD4^+CD25^+$ T reg cells

To characterize the phenotype of conventional and T reg cell populations thoroughly, we stained lymph node and spleen cells from PBS- or JJ316-treated animals for CD4, CD25, CTL-associated antigen 4 (CTLA-4), and FoxP3 expression (Fig. 3). The forkhead transcription factor FoxP3 is the most specific marker for T reg cells (16–19). In lymph node and spleen of PBS- or CD28 superagonist-treated rats, FoxP3 was expressed almost exclusively by $CD4^+$ cells (i.e., 80–90% of FoxP3⁺ cells coexpress CD4; unpublished data). In peripheral lymph nodes and the spleen of JJ316-injected animals, the vast majority (80–90%) of $CD4^+CD25^+$ cells, and all $CD4^+CD25^{high}$ cells, continued to express FoxP3 (Fig. 3 A). We corroborated the flow cytometric data by visualizing FoxP3 expression by Western blot analysis. FoxP3 protein was detected readily in $CD4^+CD25^+$ T reg cells that were isolated from untreated or JJ316-treated animals. However, there was no detectable signal originating from $CD4^+CD25^-$ T cells regardless of whether JJ316 had been applied (Fig. 3 B). In addition to FoxP3, naturally occurring T reg cells constitutively express CTLA-4 (20, 21). Experiments with human T reg cell clones further showed that expression of CTLA-4 positively correlated with their suppressive capacity (22, 23). Therefore, we determined the expression of CTLA-4 by $CD4^+CD25^+FoxP3^+$ cells from PBS- or JJ316-treated animals. As compared with PBS-treated control rats, $CD4^+CD25^+FoxP3^+$ cells from animals that were injected with JJ316 revealed strongly enhanced expression of CTLA-4 (Fig. 3 C). The coexpression of FoxP3 and high amounts of CTLA-4 indicated that phenotypic and functional characteristics of T reg cells were maintained in JJ316-expanded $CD4^+CD25^+$ T cells.

To prove that the $CD25^+CD4^+$ cells that were recovered after superagonistic CD28 treatment were T reg cells, we tested their suppressive capabilities in ex vivo suppression assays. 3 d after injection of JJ316 (0.1 or 1 mg per animal) or PBS into normal Lewis rats, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from peripheral lymph nodes were assayed for their ability to inhibit the proliferation of CFSE-labeled $CD4^+$ indicator T cells induced by classical costimulation. Subsequent analysis of CFSE dye dilution revealed a pronounced proliferation of indicator cells in the absence of $CD4^+CD25^+$ T cells (Fig. 4 A; gray curves). Coculture of indicator cells with $CD4^+CD25^+$ T cells (black curves) that were isolated from the PBS-treated animal led to partial inhibition of indicator cell proliferation. The inhibitory activ-

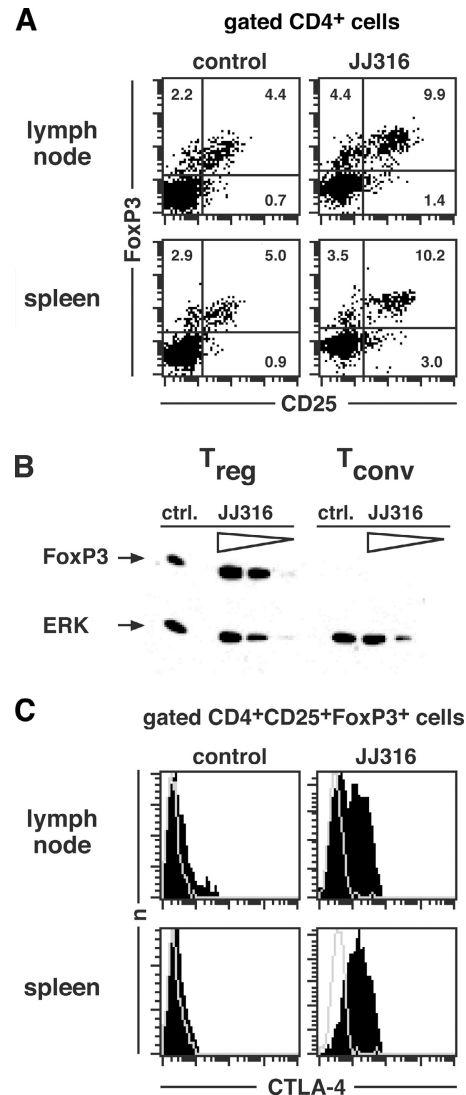


Figure 3. Phenotypic analysis of T reg and T_{conv} cells from treated animals. 3 d after administration of 1 mg JJ316, lymph node and spleen cells were stained at the cell surface with mAbs against CD4 and CD25, followed by intracellular staining for FoxP3 and CTLA-4. Control animals were injected with PBS only. (A) Representative expression of CD25 and FoxP3 on gated $CD4^+$ cells. The figure indicates the percentages of cells in the respective quadrant. (B) Detection of FoxP3 expression by Western blot analysis. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from untreated or JJ316-treated animals (1 mg), and protein lysates from whole cells were generated. FoxP3 expression was detected with a polyclonal rabbit-anti-mouse FoxP3 IgG. Lysates from cells purified after JJ316 treatment also were diluted serially and protein loading was assessed with polyclonal anti-rat ERK-2 Ig. The experiment was repeated with a similar result. (C) Representative anti-CTLA-4 staining profiles of $CD4^+CD25^+FoxP3^+$ cells are depicted (filled line graphs). Staining specificity was controlled by preincubation with unconjugated anti-CTLA-4 mAb (gray profile). A repeat experiment rendered similar results.

ity of $CD4^+CD25^+$ T cells that were derived from JJ316-treated animals was more pronounced after low-dose treatment, and was enhanced dramatically after high-dose treat-

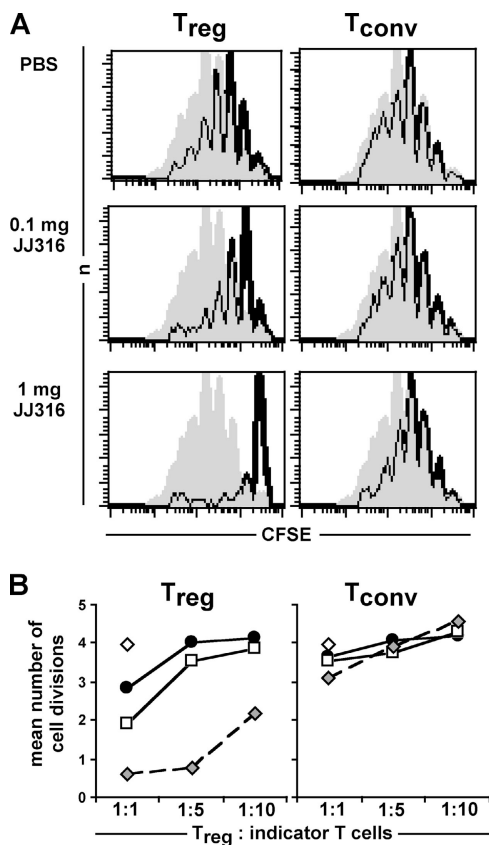


Figure 4. Functional analysis of T_{reg} and T_{conv} cells from JJ316-treated animals ex vivo. (A) Purified T_{reg} and T_{conv} cells from the lymph nodes of animals treated as indicated 3 d before isolation were cocultured with CFSE-labeled CD4⁺CD25⁻ T cells from the PBS-treated animal (indicator T cells), and stimulated with costimulatory beads for 5 d. Line graphs show proliferations of indicator T cells in the absence (gray shadow) or the presence of T_{reg} or T_{conv} cells at a 1:1 ratio (black line). (B) T_{reg} and T_{conv} cell/indicator T cell ratios of 1:1, 1:5, and 1:10 were determined by translating CFSE dilution profiles into the average number of cell divisions of the indicator T cells after 5 d of culture. The symbols indicate proliferation of indicator T cells in the absence of T_{reg}/T_{conv} cells (open diamonds) or in the presence of T_{reg}/T_{conv} cells from the animal treated with PBS (filled circles), 0.1 mg JJ316 (open squares), or 1 mg JJ316 (gray diamonds). The result is representative of two experiments performed.

ment (Fig. 4, A and B). In accordance with our phenotypic analysis, CD4⁺CD25⁻ T cells did not display immunoregulatory capacities after JJ316 treatment. Taken together, our results show that high- and low-dose superagonistic CD28 treatment increase the pool of naturally occurring T_{reg} cells, and enhance their suppressive effector functions.

Superagonistic CD28-specific mAbs ameliorate disease activity in EAE

The results outlined so far suggested that, as a first step, application of CD28 superagonists into models for human autoimmune diseases might have beneficial effects. Thus, we investigated the protective effect of JJ316 in different rat EAE models. First, we focused on preventive or therapeutic

application of JJ316 in the relapsing EAE model in Dark Agouti (DA) rats that were immunized with recombinant human myelin oligodendrocyte glycoprotein (MOG). Rats were treated i.v. on day 0 (disease induction), day 10 (disease onset), or on days 0 and 10 with 1 mg JJ316 (Fig. 5 A). Control animals received 1 mg of the isotype-matched mAb MOPC-31C at the same time points. Preventive administration of 1 mg JJ316 at the time point of active EAE induction (day 0) in DA rats led to almost complete suppression of the first disease episode of EAE. Only delayed and mild signs of disease were observed. Dual injection of JJ316 on days 0 and 10 protected DA rats even more effectively during the first disease stage. Therapeutic injection of JJ316 at the time of disease onset on day 10 immediately ameliorated disease activity, and the animals underwent remission significantly earlier compared with control animals. In contrast to the other regimens, therapeutic application at day 10 did not protect from severe signs of the disease during the subsequent relapse. Protection from clinical signs of EAE by the CD28 superagonist was accompanied by a decrease in T cell and macrophage infiltrates into the spinal cord and prevention of demyelination (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051060/DC1>).

Next, we wanted to determine the minimal effective dose of JJ316 and, therefore, treated DA rats after onset of MOG-EAE with 0.03 mg, 0.1 mg, or 0.3 mg per animal (Fig. 5 B). The maximal therapeutic effect of JJ316 was achieved at 0.3 mg; however, a dose as small as 0.03 mg was effective in reducing the severity of EAE ($P < 0.05$). Administration of 0.1 mg of the conventional CD28 mAb JJ319 had no effect on disease manifestation, with clinical scores comparable to those of MOPC-31C-treated controls.

To assess whether protection by CD28 superagonists may have a wider application beyond MOG-induced EAE in the DA rat, we also induced EAE in Lewis rats by immunizing them with guinea pig myelin basic protein (gpMBP) in CFA. 11 d later (i.e., after onset of disease), the rats received a single injection of JJ316 at different dosages (0.1, 0.3, and 1 mg/animal) or an isotype control antibody (MOPC-31C, 1 mg/animal). In analogy to standard treatment schemes applied in human autoimmune diseases, including MS (24), another group of rats received daily treatment with 2 mg of methylprednisolone from days 11 to 15 after immunization. We observed a clear reduction of disease activity (Fig. 5 C) in all rats that had received CD28 superagonist treatment, whereas the glucocorticosteroid had a comparatively weaker impact on the clinical course of the disease.

Our findings demonstrate the efficacy of superagonistic anti-CD28 in two independent models of EAE, and show that it is superior to standard glucocorticoid treatment. Moreover, protection by CD28 superagonist application also was observed in the adoptive transfer model of Lewis rat-EAE—a model that mimics a very aggressive type of autoimmune disease where there is no active induction of pathogenic T cells (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20051060/DC1>).

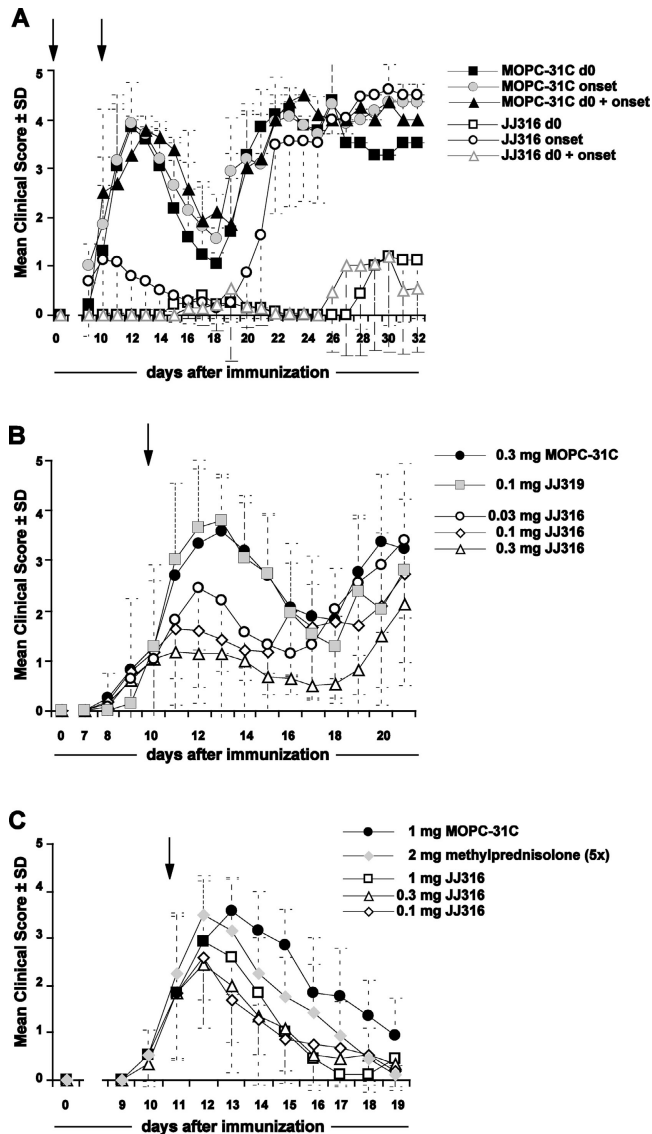


Figure 5. Therapeutic potential of superagonistic CD28-specific mAb JJ316 on active EAE of the Lewis and the DA rat. (A) To induce EAE in DA rats, animals were immunized with 100 μ g recombinant human MOG in CFA at day 0. mAb JJ316 (1 mg) was administered i.v. to groups of animals ($n = 8$) at day 0 (induction of EAE, open squares), day 10 (onset of EAE, open circles), or on days 0 and 10 (open triangles). Control animals ($n = 5$) were injected with 1 mg of MOPC-31C at the different time points as indicated. Animals were scored for clinical signs of EAE as described in Material and methods. (B) Therapeutic application of 0.3 mg, 0.1 mg, or 0.03 mg of JJ316 after onset of disease (day 10, arrow) protected DA rats ($n = 6$) immediately from severe EAE. Control animals were injected with 0.3 mg of MOPC-31C or 0.1 mg of the conventional anti-CD28 mAb JJ319. Mann-Whitney U-tests comparing JJ316- and sham-treated rats on days 13 and 14 after disease onset were significant ($P < 0.05$) for animals receiving 0.03 mg of JJ316 and highly significant ($P < 0.01$) for animals receiving 0.3 mg of JJ316. (C) Active EAE of the Lewis rat was induced by immunization with gpMBP in CFA in the hind footpad at day 0. On day 11 (arrow), animals received a single injection of 1 mg of an isotype control antibody (filled circles); a single injection of 0.1 mg, 0.3 mg, or 1 mg of mAb JJ316; or were administered 2 mg of methylprednisolone

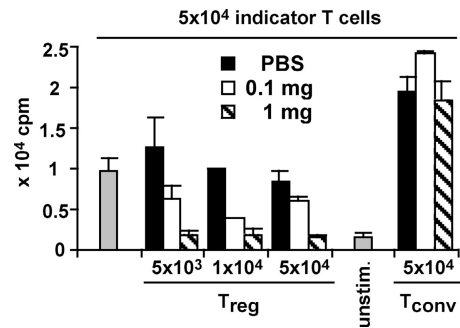


Figure 6. mAb JJ316-primed T reg cells suppress the proliferation of gpMBP-specific T cells in vitro. T reg and T_{conv} cells were isolated from the draining lymph nodes of animals that had been immunized with gpMBP in CFA 9 d before functional analysis. mAb JJ316 (0.1 mg or 1 mg per animal) or PBS was administered 3 d before the in vitro suppression assay. Conventional $CD4^+CD25^-$ T cells from the PBS-treated animal (gray bars) served as indicator T cells, and were cocultured with T reg or T_{conv} cells from the animal treated with PBS, 0.1 mg JJ316, or 1 mg of JJ316. T cells were stimulated with gpMBP in the presence of irradiated splenic APCs. A repeat experiment rendered similar results.

CD28 superagonist activated T reg cells suppress myelin basic protein (MBP)-directed T cell responses in vitro

Encephalitogenic (myelin basic protein specific) $CD4^+$ T cells play a pivotal role in initiating and maintaining EAE (25). Hence, one mechanism underlying the therapeutic effect of CD28 superagonists on EAE could be the successful suppression of these pathogenic T cells by activated and expanded $CD4^+CD25^+$ T reg cells. Therefore, we tested whether $CD4^+CD25^+$ T reg cells that are stimulated by the CD28 superagonist in vivo could suppress MBP-driven T cell proliferation in vitro. For in vivo priming, we immunized Lewis rats with gpMBP, and treated them 6 d later with PBS or JJ316 (0.1 or 1 mg per animal). After another 3 d we isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the PBS- or JJ316-treated animals, and cocultured the cells with $CD4^+CD25^-$ indicator cells (derived from the PBS-treated control animal) in the presence of irradiated splenic APCs and gpMBP. We did not observe any suppressive activity toward MBP-driven T cell proliferation of $CD4^+CD25^+$ T reg cells that were isolated from the PBS-treated animal (Fig. 6). In contrast, significant, or even very strong, suppression was detectable using $CD4^+CD25^+$ T reg cells that were derived from the animals that were treated with 0.1 mg or 1 mg of the CD28 superagonist, respectively (Fig. 6). $CD4^+CD25^-$ T cells that were isolated from either treatment group proliferated in response to gpMBP stimulation, and failed to suppress antigen-specific indicator T cells. These results show that $CD4^+CD25^+$ T reg cells activated by CD28 superagonists in vivo are potent suppressors of

on five consecutive days. P values of Mann-Whitney rank sum tests of clinical scores that compared the isotype-treated group with the JJ316-treated groups on days 13, 14, and 15 were all significant; most were highly significant.

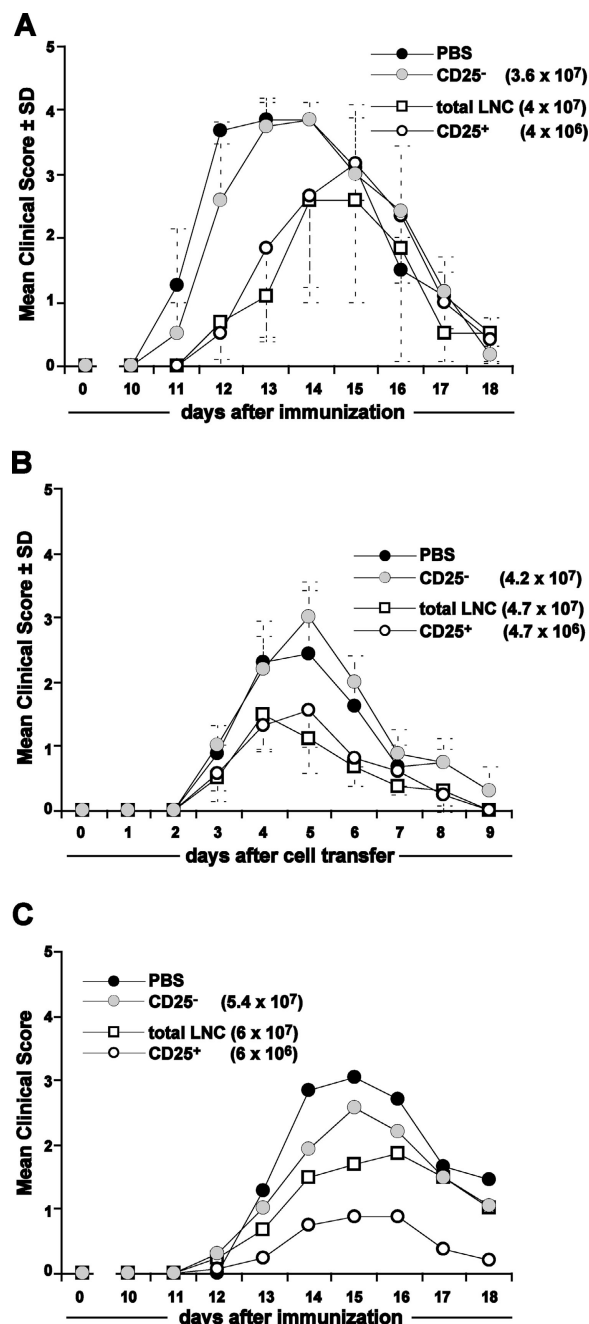


Figure 7. CD4⁺CD25⁺ T reg cells mediate reduced disease severity. Donor animals were immunized with gpMBP in CFA and received 1 mg of mAb JJ316 i.v. 3 d later, draining LNCs were prepared and separated, in part, into CD4⁺CD25⁻ and CD4⁺CD25⁺ cells. Recipient animals received PBS only, $4(4.7) \times 10^7$ total draining LNCs, $4(4.7) \times 10^6$ purified CD4⁺CD25⁺ T cells, or $3.6(4.2) \times 10^7$ CD4⁺CD25⁻ T cells; numbers in parentheses denote the amount of cells transferred in AT-EAE (see Fig. 4 B). EAE was induced in recipient animals by active immunization with gpMBP in CFA (A) or by adoptive transfer of encephalitogenic T cell line cells (B). These experiments were reproduced twice with similar results. Generation of protective CD4⁺CD25⁺ T reg cells is related to the application of superagonistic CD28-specific mAb and is antigen priming-independent (C). On the day of immunization with gpMBP in CFA, animals received 6×10^7 JJ316-primed total draining LNCs (open squares) or 6×10^6 separated

polyclonal T cell proliferation and mediate profound inhibition of antigen-specific T cell responses.

Transfer of protection from EAE by activated CD4⁺CD25⁺ T cells from CD28 superagonist-treated animals

To identify directly a potential contribution of JJ316-activated T reg cells to the therapeutic effects observed in vivo, we primed donor rats with 1 mg anti-CD28 superagonist, and immunized them together with gpMBP in CFA. 3 d after priming, recipient animals received $4(4.7) \times 10^7$ total LNCs (containing $\sim 10\%$ CD4⁺CD25⁺ T cells), $4(4.7) \times 10^6$ sorted CD4⁺CD25⁺ T cells, or $3.6(4.2) \times 10^7$ CD4⁺CD25⁻ T cells on the day of active EAE or adoptive transfer (AT)-EAE induction (Fig. 7, A and B); numbers in parentheses denote the amount of cells transferred in AT-EAE (Fig. 4 B). Compared with control rats, which were injected with PBS only, clinical signs of active EAE and AT-EAE in recipients of total LNCs or of the respective fraction of CD4⁺CD25⁺ JJ316-primed T cells were mitigated significantly (ANOVA: $P < 0.001$). In contrast, the adoptive transfer of CD4⁺CD25⁻-JJ316 primed T cells did not alter EAE severity, which suggested that CD4⁺CD25⁺ T reg cells constitute the protective cell population within whole LNCs.

To investigate the impact of antigen during the recruitment of T reg cells by the application of superagonistic anti-CD28 mAb in vivo, nonimmunized donors received 1 mg of JJ316 3 d before isolation of LNCs (Fig. 7 C). This time, 6×10^7 unsorted total LNCs, 6×10^6 CD4⁺CD25⁺ lymphocytes, or 5.4×10^7 CD4⁺CD25⁻ lymphocytes were transferred i.v. into Lewis rats that were immunized with gpMBP in CFA. Transfer of total JJ316-primed LNCs resulted in a significantly milder disease course (ANOVA: $P < 0.05$). Transfer of CD4⁺CD25⁺ T cells resulted in a highly significant amelioration of EAE signs (ANOVA: $P < 0.0001$). The transfer of CD4⁺CD25⁻ T cells did not lead to significant alteration of EAE as compared with controls. This showed that the superagonistic CD28-specific mAb has the potency to activate EAE-protective CD4⁺CD25⁺ T reg cells—even in the absence of exogenously provided antigen—presumably by activating “natural” T reg cells with a preselected self-specific repertoire (26).

DISCUSSION

In our previous work (15) we showed that superagonistic CD28-specific antibodies preferentially expanded pre-existing CD4⁺CD25⁺ T reg cells because conventional CD4⁺CD25⁻ T cells are not recruited into the pool of CD25⁺ cells upon JJ316 stimulation in vivo. We confirmed this result in the present study (Fig. 2), and bypassed the pitfalls that are involved in using only CD25 as a marker for T reg cells by determining FoxP3 expression at the single cell

CD4⁺CD25⁺ T cells (open circles). Control treatment consisted of the transfer of 5.4×10^7 CD28 superagonist-activated CD4⁺CD25⁻ T cells (gray circles) or in the administration of PBS only (black circles).

level. This allowed us to provide the first phenotypical analysis of rat CD4⁺CD25⁺FoxP3⁺ T reg cells (Fig. 3). The vast majority of CD4⁺CD25⁺ cells, and all CD4⁺CD25^{high} cells, expressed FoxP3 in the steady state and after CD28 superagonist stimulation *in vivo*; this unambiguously identified them as bona fide T reg cells (Fig. 3). Furthermore, we extended those previous studies using a standard dose of CD28 superagonist (11, 15) by titrating its amount *in vivo* to identify a therapeutic window with as little generalized lymphocytosis as possible.

Low-dose CD28 superagonist treatment was sufficient to increase the pool of CD4⁺CD25⁺ T reg cells, but did not induce an increase in T_{conv} cell numbers that is seen regularly with high dosages (Fig. 1). Therefore, low-dose application of superagonistic anti-CD28 mAbs provides a very attractive therapeutic strategy for the treatment of autoimmune diseases.

As to the reasons for the differential response of T reg and T_{conv} cells to low-dose superagonistic CD28 treatment, we hypothesize that T reg cells may be more susceptible to this form of stimulation because they might receive a “first” signal constitutively by way of interaction of their autoreactive T cell receptor (26) with self-peptide/MHC complexes. It can be postulated further that T reg cells that are stimulated by CD28 superagonists *in vivo* actively suppress the proliferation of T_{conv} cells. In line with this possibility, superagonistic CD28 stimulation *in vitro* was sufficient for freshly isolated T reg cells to suppress the proliferation of T_{conv} cells partially (unpublished data). Moreover, CD28-derived signals are known to be essential for the survival of T reg cells (27, 28), and it may be that interfering with their natural turnover by supplying optimized CD28 signals contributes to their numeric expansion. We recently showed that T cell activation by CD28 superagonists is superior to conventional costimulation in protecting from apoptotic cell death (29).

Upon injection of a low dose of mAb JJ316 (0.1 mg), absolute numbers of T reg cells were increased significantly in the spleen, but not in peripheral lymph nodes. Because this low dose of antibody was administered *i.v.*, it could be that only antibody concentrations in the spleen reached sufficient levels to cause an increase in absolute cell numbers. The observed differences also may be due to better Fc receptor-mediated cross-linking of the antibody by splenic APCs, which was shown to be essential for superagonistic CD28 stimulation to function (11). Alternatively, differential migratory properties of lymph node and splenic T cells upon JJ316 treatment could account for the observed differences. Low-dose treatment, however, was sufficient to increase the relative numbers of T reg cells among CD4⁺ cells to near maximum levels, (*i.e.*, two- to fourfold). As shown in animal models of allotransplantation (30) and type I diabetes (31), the ratio of T reg cells/T_{conv} cells in the microenvironment of the target organ seems to determine the outcome of the immune response.

Apart from absolute or relative numbers, suppressor potency at the single cell level is believed to be critical for the functional balance between T reg cells and their targets (8).

Superagonistic CD28 treatment induces CD25^{bright}CTLA-4^{bright}FoxP3⁺CD4⁺ cells with greatly enhanced suppressor functions, whereas CD25⁻CD4⁺ cells that were isolated from the same animals were not suppressive (Fig. 4).

The greatly increased expression of CTLA-4 on T reg cells after high-dose JJ316-treatment (Fig. 3) may explain their strong suppressive activity (21, 32). CTLA-4 was shown to be an effector molecule of T reg cells (21) involved in direct suppression of other T cells (33), and in rendering dendritic cells tolerogenic (34). Additionally, the immunosuppressive cytokine, IL-10, which is known to be an important mediator of T reg cell effector functions (35)—at least *in vivo*—is synthesized by JJ316-activated T reg cells *in vitro* (15), and is induced strongly upon CD28 superagonist treatment *in vivo* (reference 14 and unpublished data). Thus, the suppressive function of JJ316-activated T reg cells *in vivo* may be mediated, at least in part, by IL-10.

The increase in the suppressive potency of T reg cells in response to CD28 superagonist stimulation probably is caused indirectly by IL-2 or IL-4, which were shown to be produced by T_{conv} cells upon high-dose CD28 superagonist treatment *in vivo* (14). Both cytokines are capable of strongly enhancing the suppressive potency of T reg cells *in vitro* and *in vivo* (36, 37).

The promising data obtained with healthy animals prompted us to extend our experiments to models of acute or relapsing central nervous system (CNS) autoimmunity. In a previous report, we showed that high-dose superagonistic CD28 treatment had a beneficial prophylactic and therapeutic effect on the peripheral nervous system autoimmune disease, experimental autoimmune neuritis, of the Lewis rat (38). Because even very low dosages of the CD28 superagonist expanded and activated T reg cells of normal Lewis rats (Fig. 1), we focused on using different dosages of the CD28 superagonist for the treatment of EAE. In analogous models of EAE in the mouse, a protective role for T reg cells has been established (39–41). Superagonistic CD28 therapy strongly down-modulated disease activity over a wide range of dosages (Fig. 5), including the low doses that selectively induced T reg cell expansion. At face value, it is surprising that the lowest dose of CD28 superagonist used—0.1 mg per animal—was as efficient in interfering with Lewis rat EAE as was the 10-fold higher dose which induced a much more pronounced expansion of T reg cells and a more potent suppressive activity per cell as read out *in vitro*. Possibly, at 1 mg per animal, the increase in activated CD4⁺CD25^{low} FoxP3⁻ effector T cells (Fig. 3) counterbalances the protective effect that is mediated by a highly active and expanded T reg cell population to a certain degree.

CNS inflammation is driven by MBP-specific T cells in EAE of the Lewis rat (25). Therefore, we analyzed T reg cells from gpMBP-immunized animals that were treated with the CD28 superagonist or remained untreated with regard to their suppressive capacity toward MBP-specific T cells *in vitro*. The failure to detect suppression of gpMBP-specific T cells by T reg cells from gpMBP-immunized, but

otherwise untreated, animals correlates with the inability of endogenous suppressor cells to prevent disease development in this immunization scheme. However, T reg cells that were derived from animals after high- or low-dose CD28 superagonist treatment showed clear suppression of MBP-specific T_{conv} cells. Suppression by CD28 superagonist-activated T reg cells was not restricted to the surrogate in vitro assay. Upon adoptive transfer, JJ316/gpMBP-primed $CD4^+CD25^+$ T reg cells protected naive recipients from EAE (Fig. 7). These activated T reg cells did not have to be present during the induction phase of the effector T cells because they also suppressed adoptive transfer EAE (Fig. 7).

Similar to the in vitro suppression assay, transfer of 10 times more T_{conv} cells had no impact on the course of EAE (Fig. 7). Therefore, activation and expansion of T reg cells seems to be the mechanism that underlies the protective effect of JJ316 in EAE. Moreover, there is no evidence that this mechanism is complemented by activation-induced cell death of effector T cells because we did not observe increased numbers of apoptotic T cells in tissue sections of the CNS (Fig. S2), and we could detect gpMBP-responsive effector T cells in vitro after JJ316-therapy in vivo (Fig. 6 and not depicted).

Technical reasons allowed us to transfer only up to 6×10^6 purified T reg cells per animal; i.e., $\sim 5\%$ were added to the pool of T reg cells that is assumed to be present in the recipient. With these small numbers of transferred cells, only strongly activated T reg cells can be expected to exert an effect. Accordingly, we used T reg cells that were activated by 1 mg JJ316 in vivo, which we had found to possess very high regulatory activity per cell. These were able to ameliorate clinical signs of EAE, whereas T reg cells that were derived from low-dose JJ316-treated animals were not (Fig. 7 and not depicted). Inclusion of gpMBP in the priming protocol was not necessary for CD28 superagonist-activated T reg cells to suppress MBP-specific T_{conv} cells in vitro (not depicted), or to protect from EAE in vivo (Fig. 7). Although this suggests that the preexisting natural TCR repertoire of T reg cells contains a sufficient frequency of cells that is autoreactive to self-antigens in the CNS, it remains to be solved whether the therapeutic effect that was observed in vivo is mediated largely by T reg cells with specificity for the autoantigen MBP, or achieved mainly by way of bystander suppression by T reg cells recognizing other autoantigens (39, 40).

Immunohistochemical analyses corroborated our findings, and revealed a decrease in T cell and macrophage infiltration into the spinal cord of DA rats protected from the first episode of EAE by JJ316 treatment (Fig. S1 and Fig. 5 A). However, in this therapeutic regimen, protection was not observed during the subsequent relapse, which was of similar severity as in control rats based on clinical appearance and histology (Fig. S1 and Fig. 5 A). This suggests that at least in this model, CD28 superagonist-activated T reg cells suppress encephalitogenic T cells in peripheral lymphoid tissues, and thereby, reduce cellular infiltrates into the CNS. In gpMBP-induced EAE of the Lewis rat, however, JJ316 therapy did not reduce T cell infiltration into the spinal cord,

and even strongly increased the number of infiltrating macrophages (unpublished data). Here, suppression of the pathogenic T cells by T reg cells in situ is the likely mechanism that underlies regulation of the T cell response by JJ316. As far as macrophage infiltration into the spinal cord after CD28 superagonist treatment is concerned, we hypothesize that these macrophages could have undergone so-called “alternative activation.” The cytokines IL-4 and IL-13 were responsible for this alternative activation of macrophages that led to the production of antiinflammatory cytokines by the macrophage (42). Because IL-4-synthesis is induced upon superagonistic anti-CD28 stimulation in vivo (14), it is possible that the macrophages that infiltrate the CNS after JJ316 treatment contribute to the prevention of tissue damage, rather than augment it.

Finally, we believe that superagonistic CD28-specific mAbs are very promising tools for targeting and manipulating $CD4^+CD25^+$ T reg cells in vivo. Our findings provide the basis for new therapies to treat MS—and perhaps other human autoimmune diseases—successfully.

MATERIALS AND METHODS

Animals. Normal Lewis rats were bred at the animal facility of the Institute for Virology and Immunobiology, University of Würzburg, and were used for experiments between 6 and 12 wk of age. EAE was induced and monitored in female DA and Lewis rats that were 6–8 wk of age and with a body weight of 120–140 g, that were obtained from Charles River Wiga. They were taken at random from individual cages to receive either treatment. All experiments were performed according to the Bavarian state regulations for animal experimentation, and were approved by the responsible authorities.

In experiments with histologic analyses, rats were anesthetized with 200 mg/kg body weight pentobarbital (Narcoren; Rhone Merieux) that was perfused through the left cardiac ventricle with PBS. Spinal cords were removed, and the tissue was embedded in OCT compound (Sakura Finetek) for cryopreservation at -80°C .

Purification of $CD4^+CD25^+$ (T reg) and $CD4^+CD25^-$ T cells (T_{conv}). Routinely, single-cell suspensions were prepared from inguinal, axillary, cervical, mesenteric, and paraortic lymph nodes, and T cell subsets were purified using MACS (Miltenyi Biotec) separation columns according to the manufacturer's instructions. To remove $CD8^+$ T cells and B cells, lymph node cells were incubated with a biotinylated mAb against CD8 (clone OX8 or G28, BD Biosciences) and biotinylated polyclonal mouse-anti-rat Ig (Dianova) followed by streptavidin microbeads (Miltenyi Biotec). $CD8^-Ig^-$ lymph node cells ($CD4^+$ T cells) were stained consecutively with anti- $CD25$ -FITC mAb (clone OX39, Serotec) and anti-FITC microbeads (Miltenyi Biotec). Cell purities of regulatory $CD25^+$ T cells and conventional $CD25^-$ T cells were, on average, 85 and 95%, respectively.

In vitro suppression assays. Cultures of purified T cells were set up in 96-well flat bottom plates (Costar) with cell densities of 5×10^5 cells/ml in *x*-vivo 15 medium (Bio Whittaker) supplemented with 15% heat-inactivated FCS, 1 mM sodium pyruvate, nonessential amino acids, 100 U/ml penicillin and streptomycin, 30 μM mercaptoethanol, and 2 mM L-glutamine (all from GIBCO BRL). To test for suppressor function of T reg cells, conventional $CD4^+CD25^-$ T cells or whole $CD4^+$ T cells were used as indicator cells and cocultured with different numbers of T reg cells. The T cells were stimulated with anti-TCR (clone R73) plus anti-CD28 immobilized at a 2:1 ratio on Dynabeads (Dyna) used at a bead/cell ratio of 5:1. Alternatively, T cells were cultured in the presence of irradiated lymph node

or spleen cells as APCs (5×10^5 cells/ml) and Con A (2 $\mu\text{g}/\text{ml}$, Sigma-Aldrich). Proliferation was measured by flow cytometric determination of dye dilution in CFSE-labeled (5 μM ; Molecular Probes) T_{conv} cells or by measuring [^3H]thymidine (Amersham Biosciences) incorporation during the final 16 h of a 3-d culturing period. The DNA of [^3H]thymidine-pulsed cells was harvested onto fiberglass filters, and the radioactive content was quantitated using a β -scintillation counter.

To measure suppression of gpMBP-specific T_{conv} cells by T reg cells, animals were immunized with guinea gpMBP in CFA as for active EAE induction. 6 d after immunization, the animals were treated with PBS or 0.1 or 1 mg of mAb JJ316. After a further 3 d, T cells were purified from the draining lymph nodes and restimulated in vitro in the presence of 10 $\mu\text{g}/\text{ml}$ gpMBP and irradiated splenic APCs. Co-cultures consisted of 5×10^4 indicator T cells ($\text{CD4}^+\text{CD25}^-$ from the PBS animal) and 5×10^4 , 10^4 , or 5×10^3 T reg cells, or 5×10^4 $\text{CD4}^+\text{CD25}^-$ T cells from the respective animal.

In vivo monitoring of T reg cell proliferation. Lymph node cells and erythrocyte-depleted spleen cells of five normal Lewis rats were pooled and passed over nylon wool columns. $\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells were purified from the nylon wool nonadherent fraction using MACS columns. Purified cells were washed extensively with protein-free balanced salt solution and labeled with 5 μM CFSE for 5 min at room temperature in the dark. The cells were washed twice with fully supplemented x-vivo 15 medium containing 15% FCS. The cells were resuspended in sterile PBS and transferred i.v. ($\text{CD4}^+\text{CD25}^+$: 10^6 per animal; $\text{CD4}^+\text{CD25}^-$: 5×10^6 per animal). On the following day, 0.1 or 1 mg of mAb JJ316 or 1.0 mg of isotype-control mAb MOPC-31C was injected i.v. into the tail vein. Recipient animals were killed on day three after cell transfer, and nylon wool non-adherent cells were isolated from pooled spleen and lymph nodes before staining for CD4 and CD25 expression.

FACS analysis. The following monoclonal antibodies were used: anti-rat CD4-CyChrome (clone OX35, BD Biosciences); anti-rat CD25-FITC or -biotin (clone Ox39); anti-rat CTLA-4-biotin (clone WKH203, all Serotec), and anti-mouse FoxP3 (clone mFOXY, eBioscience).

For FACS analysis, up to 10^6 cells resuspended in 50 μl of PBS/0.1% BSA/0.02% NaN_3 were incubated with 20 $\mu\text{g}/\text{ml}$ of normal mouse Ig (Sigma-Aldrich) to block unspecific Fc-receptor binding (15 min, 4°C). After the blocking step, FITC-, PE-, and CyChrome-conjugated or biotinylated mAbs were added (15 min, 4°C). Bound biotinylated antibodies were detected by incubation with CyChrome- or allophycocyanin-conjugated streptavidin (BD Biosciences). The cells were analyzed after a final washing step on a FACScan or FACSCalibur flow cytometer using Cell Quest software (all Becton Dickinson). Dot plots and histograms are shown as \log_{10} fluorescence intensities on a 4-decade scale.

Intracellular staining for FoxP3 and CTLA-4 expression was performed by incubating impermeabilized cells with FITC- and APC-conjugated antibodies and washing them once with ice-cold PBS before fixing them for 30 min at room temperature with fixation buffer (eBioscience). Cells were permeabilized (permeabilization buffer, eBioscience) before incubation with anti-CTLA-4 mAb (WKH203-biotin) and anti-FoxP3 mAb (mFOXY-PE) for 30 min at room temperature. Specificity of anti-CTLA-4 staining was verified by blockade with 100 $\mu\text{g}/\text{ml}$ unconjugated mAb WKH203.

Induction of EAE. Lewis or DA rats were inoculated in one of the hind footpads with 50 μl of an emulsion containing equal volumes of 100 μg of gpMBP or recombinant human MOG (43) in saline and CFA with Mycobacterium tuberculosis (0.5 mg/ml; Difco). I.v. treatment with mAbs JJ316, MOPC-31C or JJ319 was as indicated for individual experiments. In the case of adoptive transfer of in vivo primed lymphocytes cells were also injected i.v. Methylprednisolone (Urbason, Aventis) was given to Lewis rats at a dose of 2 mg per animal and day from days 11 to 15 after immunization.

AT-EAE was induced by i.v. injection of 8×10^6 gpMBP-specific CD4^+ activated T cells into Lewis rats (44). Animals were weighed and inspected for disease severity on a daily basis. Clinical scoring was performed as

follows: 0 = normal; 1 = impaired righting/limp tail; 2 = atactic gait/abnormal position; 3 = moderate paraparesis; 4 = tetraplegia; and 5 = death.

Detection of FoxP3 by Western blot analysis. T reg and T_{conv} cells were purified from JJ316-treated or untreated animals (control) as described before. Whole cells were lysed using 2x Laemmli buffer. SDS-PAGE was used for protein separation followed by protein blotting onto a polyvinylidene difluoride membrane (Millipore). FoxP3 protein expression was detected with a polyclonal rabbit anti-mouse-FoxP3 IgG (gift of A.Y. Rudensky). Protein loading was assessed by detecting ERK-2 expression with a polyclonal rabbit/goat-anti-rat-ERK-2 Ig (Santa Cruz Biotechnology, Inc.).

Statistical analyses. Data were subjected to Mann-Whitney rank sum tests or ANOVA (Prism 3.0, GraphPad software) where indicated in the text. p-values of <0.05 were considered to be significant; p-values of <0.01 were considered to be highly significant.

Online supplemental material. Fig. S1 depicts immunohistochemical data showing reduced T cell and macrophage infiltration in the CNS and less demyelination in animals that were protected from EAE by JJ316. Fig. S2 provides data that JJ316 also protects Lewis rats from clinical signs of AT-EAE. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051060/DC1>.

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T. Hünig and T. Kerkau declare a commercial interest in TeGenero ImmunoTherapeutics AG. They are among the cofounders and shareholders of TeGenero, which develops CD28 superagonists for clinical use. T. Hünig and T. Kerkau are scientific advisors to TeGenero. The authors have no other potential conflicting financial interests.

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