T Cell Apoptosis and Induction of Foxp3+ Regulatory T Cells Underlie the Therapeutic Efficacy of CD4 Blockade in Experimental Autoimmune Encephalomyelitis


*J Immunol* 2012; 189:1680-1688; Prepublished online 16 July 2012;
doi: 10.4049/jimmunol.1201269

http://www.jimmunol.org/content/189/4/1680
T Cell Apoptosis and Induction of Foxp3+ Regulatory T Cells Underlie the Therapeutic Efficacy of CD4 Blockade in Experimental Autoimmune Encephalomyelitis

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The pathogenesis of multiple sclerosis requires the participation of effector neuroantigen-specific T cells. Thus, T cell targeting has been proposed as a promising therapeutic strategy. However, the mechanism underlying effective disease prevention following T cell targeting remains incompletely known. We found, using several TCR-transgenic strains, that CD4 blockade is effective in preventing experimental autoimmune encephalopathy and in treating mice after the disease onset. The mechanism does not rely on direct T cell depletion, but the anti-CD4 mAb prevents the proliferation of naive neuroantigen-specific T cells, as well as acquisition of effector Th1 and Th17 phenotypes. Simultaneously, the mAb favors peripheral conversion of Foxp3+ regulatory T cells. Pre-existing effector cells, or neuroantigen-specific cells that undergo cell division despite the presence of anti-CD4, are committed to apoptosis. Therefore, protection from experimental autoimmune encephalopathy relies on a combination of dominant mechanisms grounded on regulatory T cell induction and recessive mechanisms based on apoptosis of neuropathogenic cells. We anticipate that the same mechanisms may be implicated in other T cell-mediated autoimmune diseases that can be treated or prevented with Abs targeting T cell molecules, such as CD4 or CD3. The Journal of Immunology, 2012, 189: 1680–1688.
effects due to T cell depletion (48). The mechanisms underlying the putative tolerogenic effect of anti-CD4 treatment in neuroinflammation were never fully characterized.

In this study, we evaluate the mechanisms underlying the protective effect of CD4 blockade in EAE. Using models of spontaneous and active EAE, we show that a nondepleting isotype of anti-CD4 mAb can prevent and treat EAE without leading to CD4 T cell depletion. Treated mice remained immunocompetent against viral infection.

We found that the underlying mechanism of CD4 blockade is distinct in naive and preactivated cells. Whereas CD4 blockade on activated cells favored their commitment to apoptosis, CD4 blockade during the priming of naive cells halted proliferation and effector differentiation and promoted the emergence of Foxp3+ Tregs.

It is a combination of halting the effector function of preactivated cells together with the recruitment of naive cells into the regulatory pool that allows CD4 blockade to be effective in TCR-transgenic mice, even after the onset of the disease. As such, our data provide insight into the cellular mechanisms that are deployed to achieve EAE protection with putative tolerogenic mAbs.

Materials and Methods

Animals
C57BL/6 (B6; H-2b), MBP-specific TCR-transgenic Rag1−/− (TR−) mice (H-2b) (49), and 2D2 MOG-specific TCR-transgenic mice (H-2b) (50) were bred and maintained under specific pathogen-free conditions and used sex matched and 8 to 10 wk age. All experimental protocols were approved by the Local Ethics Committee and are in compliance with European Union guidelines.

EAE induction
For active EAE, B6 mice were s.c. immunized with 100 μg MOG35–55 peptide (MEVGYWRRSPFSRVHLYRNGK) (Biopolymers Laboratory, Harvard Medical School) emulsified in CFA solution (4 mg/ml mycobacteria in IFA). On the day of immunization and 2 d after, mice received 200 ng pertussis toxin (List Biological Laboratories) in 100 μl PBS i.v. For 2D2 Th1 transfer, purified CD4+ T cells from 2D2 mice at day 6, viable cells were restimulated with APCs and 20

In vitro Th17 polarization
MBP-specific CD4+ T cells were sorted by magnetic separation with CD4 (L3T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from healthy 30-d-old TR− mice. Cell purities were between 92 and 96%. The T cells were cultured for 5 d with bone marrow-derived dendritic cells (52) and 10 μg/ml MBP peptide (MedProbe, Oslo, Norway) in IMDM/5% FBS with 1 ng/ml TGF-β (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 10 ng/ml IL-1β (ebioscience), and 10 μg/ml anti-INF-γ (RA642) with or without 10 μg/ml anti-CD4 (YTS177). At the end of the culture, cells were harvested and processed for flow cytometry.

Cytokine production assays
Splenocytes (1 × 10^6) were plated in 96-well flat-bottom plates and cultured in complete medium (RPMI 1640 with GlutaMAX, supplemented with 10% FCS, 1% HEPES, 1% sodium pyruvate, 0.1% 2-ME; Invitrogen) with 20 μg/ml peptide (MBP or MOG35–55), supernatants were recovered after 72 h and cytokines were quantified by sandwich ELISA for IL-10, INF-γ (PreproTech, London, U.K.), or IL-17 (R&D Systems).

Adoptive transfer of naive T cells
CD4+CD26LTCD25+ T cells were purified from CD45.1 congenic 2D2 mice in two steps using microbeads (MACS; Miltenyi Biotec): first by negative selection using mAbs to CD8 (YTS169), B220 (RA3-2B2), Mac1 (CI:A3-1), and CD25 (7D4); followed by positive selection with anti-CD26LT-coated beads, providing 80% CD4+ T cells, of which 95% were CD62L−CD25+ expressing the transgenic 2D2 TCR. Cells (1 × 10^6) were adoptively transferred into syngeneic B6 mice the day before immunization.

Viral plaque assay
Mice were infected with murid herpesvirus (MuHV)-4 by intranasal inoculation of 10^6 PFU in 20 μl PBS under halothane anesthesia. At days 7 (peak of lytic infection) and 14 (resolution of lytic infection), lungs were recovered and frozen at −80°C. On the day of the assay, lungs were homogenized in glutamine-free MEM complete media and submitted to 10-fold serial dilutions. To each dilution 5 × 10^5 BHK-21 cells were added. Viruses were left to adsorb for 1 h in six-well plates, at 37°C, followed by 4 d incubation after addition of 3 ml media. At day 4 of culture, the cell monolayer was fixed with 4% formaldehyde and stained with 0.1% toluidine blue. Viral plaques were counted with a Stemi SV6 magnifying glass (Zeiss). Virus titers were determined from the average of the number of counted viral plaques in duplicates.

Histology
Animals were perfused postmortem with 2% paraformaldehyde in PBS. Tissues were embedded in paraffin, and tissue sections were stained with H&E.

Statistical analysis
Statistical significance was determined using the two-tailed nonparametric Mann–Whitney U test, and p values of <0.05 were deemed significant (⁎p < 0.05, **p < 0.01, ***p < 0.001).
Results
Nondepleting anti-CD4 mAb prevents EAE

Previous studies primarily focused on the clinical amelioration afforded by CD4 mAb treatment on EAE in Biozzi AB/H (45), PL/J mice (44), and Lewis rats (32). Little is known regarding the underlying mechanisms. To study the impact of nondepleting CD4 mAb on the encephalitogenic T cell response, we chose the most widely used mouse model of EAE that is induced in C57BL/6 mice after immunization with MOG35-55. Control mAb-treated mice (Fig. 1A) developed EAE on average 12 d after immunization causing hind limb paralysis to most animals (maximum disease score, 3 ± 0.4; Supplemental Table I). This paralytic disease was fully prevented when mice were treated with CD4 mAb on days 3 and 2 prior to immunization (Fig. 1, Supplemental Table I). Histological analysis indicated that disease prevention correlated with a reduced accumulation of immune cells in the CNS (Fig. 1B), including CD4+ T cells (Fig. 1C). In vitro Ag recall experiments indicate that treatment with CD4 mAb reduces the intensity of the MOG35-55-specific T cell response. Fourteen days after immunization, splenocytes from control mAb-treated mice produced significantly higher concentrations of IL-17 and IFN-γ in response to MOG restimulation in vitro as compared with immunized mice pretreated with CD4 mAb (Fig. 1D).

To test whether the efficacy of the treatment would persist after disease onset we treated mice with a single injection of 1 mg anti-CD4 mAb on the day of disease onset. As shown in Fig. 1E, the isotype control mAb-treated mice progressed from an initial tail atony to full paralysis of the hind limbs. Anti-CD4 mAb treatment halted disease progression, providing full disease remission 1 wk after treatment (Fig. 1E, Supplemental Table I).

Mice treated with nondepleting anti-CD4 mAb remain immunocompetent

To evaluate whether CD4 mAb-treated mice remain immunocompetent after treatment withdrawal, we evaluated the capacity of successfully treated mice to mount an antiviral immune response against a γ-herpesvirus infection. MuHV-4 causes transient pneumonia in B6 mice. This acute viral infection is efficiently cleared from the lungs after ~14 d by a cell-mediated immune response comprising both CD8+ and CD4+ T cells (53). We validated the implication of the adaptive immune response and the CD4+ T cell response by infecting RAG2−/− mice, respectively (Fig. 2). The RAG2−/− mice succumbed to uninhibited viral infection, causing increased viral titers 7 d postinoculation that proved lethal to all mice before day 14 (Fig. 2A). The role of CD4+ T cells was revealed in MHC class II−/− mice where the absence of CD4+ T cells increased the dissemination of MuHV as...
we transferred a traceable population of naive MOG35–55-specific CD4+ T cells into CD45.2 B6 mice prior to EAE induction. These CD62L+CD25+ CD4+ T cells were purified from CD45.1 congenic, 2D2 transgenic mice expressing an I-Ab–restricted TCR (Vα3.2–Vβ11) specific for MOG35–55 (50). As previously published, the transferred MOG-specific T cells differentiate into pathogenic Th1 and Th17 cells and expand 3700-fold by day 9 after immunization with MOG35–55 (54). As shown in Fig. 3A, the recipient mice develop severe EAE that renders mice quadriplegic (maximum disease score, 4.3 ± 0.33) when injected with isotype control mAb at days 0, 2, and 4 after immunization with MOG35–55. A similar treatment with nondepleting CD4 mAb fully protected the recipient mice from active EAE, as none of the animals developed any clinical signs of EAE (Fig. 3A). This is not because of depletion of the CD4 T cells, as the size of the CD4 T cell compartment was unaltered between isotype control- and nondepleting CD4 mAb-treated mice (Fig. 3B). However, when selectively enumerating the MOG35–55-specific T cells a statistically significant reduction in the accumulation of 2D2 T cells was observed 9 d after immunization in mice treated with the CD4 mAb (Fig. 3C). This reduction was not associated with increased apoptosis, as the percentage of 2D2 T cells binding annexin V was similar in the isotype control- and CD4 mAb-treated groups 3 d after immunization with MOG35–55 (Fig. 3D).

CD4 blockade inhibited T cell priming of the encephalitogenic T cell response as measured by CFSE dilution of the congenic MOG-specific 2D2 T cells. In the isotype control-treated mice most 2D2 T cells had undergone at least five cell divisions (51%) by day 3. In the CD4 mAb-treated mice this represented only 8% of the 2D2 T cells (Fig. 3E, 3F, Supplemental Fig. 2). The origin of this difference is likely to be bimodal, with less naive 2D2 T cells being activated after CD4 blockade, and second, that 2D2 T cells activated in the presence of CD4 blockade abort their expansion, as suggested by the limited number of cell divisions (Fig. 3E). Abolitive T cell activation is characterized by T cell proliferation in the absence of effector differentiation. Consequently, we assessed whether CD4 blockade would prevent the commitment of naive 2D2 T cells to the Th1 and Th17 effector subsets 9 d after immunization. In vitro MOG35–55 recall demonstrated a sizeable release of IL-17 and IFN-γ in lymph node cultures from isotype control-treated mice (Fig. 3G, Supplemental Fig. 2C, 2D). In CD4 mAb-treated mice the Ag recall response was downregulated, as indicated by the significant reduction in the release of effector cytokines (Fig. 3G). This was associated with a similar reduced frequency of 2D2 T cells expressing IFN-γ or IL-17 (Fig. 3H, Supplemental Fig. 2C, 2D), indicating that CD4 mAb treatment blocks the differentiation of naive 2D2 T cells into Th1 or Th17 effector cells. All data presented (Fig. 3B–H) were obtained from the draining inguinal lymph nodes.

Lastly, we addressed the contribution of regulatory CD4+ T cells to the immunomodulatory impact of nondepleting CD4 mAb treatment. We assessed whether CD4 blockade during active immunization could favor the differentiation of naive T cells into induced Foxp3+ Tregs. On day 9 after immunization with MOG35–55 in CFA, we found an increased frequency of Foxp3+ 2D2 T cells (p < 0.05), whereas the frequency of Foxp3+ Tregs within polyclonal T cells remained unchanged (Fig. 3I).

**Effectors T cells are committed to apoptosis under anti-CD4 treatment**

To study the mechanism by which nondepleting anti-CD4 mAbs treat established disease, as shown in Fig. 1E, we investigated the in vivo impact of anti-CD4 on terminally differentiated effector T cells. To this end, we passively induced EAE in C57BL/6 mice by adoptively transferring 14 × 10^6 MOG-specific 2D2 cells that were primed in vitro under Th1 polarization conditions (Fig. 4A) (55). Treating the recipient mice on days 0, 2, and 4 after Th1 transfer with anti-CD4 mAbs prevented the onset of disease in all treated mice (Fig. 4A). To study the fate of the transferred congeneric 2D2 cells, we assessed their accumulation in the CNS and cervical lymph nodes 9 d after transfer, just prior to death of control mice (mortality on days 11–13). In the diseased isotype control-treated
mice an average of $4 \times 10^5$ pathogenic 2D2 T cells could be recovered per CNS, whereas in the CD4 mAb-treated group the number of CNS infiltrating 2D2 T cells was significantly reduced (10-fold) (Fig. 4B). The transferred 2D2 T cells were also detectable in the cervical lymph nodes, but the cell numbers did not significantly differ from the control-treated group (Fig. 4C). However, in the CD4 mAb-treated group the fate of the pathogenic 2D2 T cells was significantly altered. As shown in Fig. 4D and 4E, up to 40% of transferred encephalitogenic T cells were undergoing apoptosis as assessed by annexin V binding within 48 h (Fig. 4D). Splenocyte cultures were restimulated with 100 μg/ml MOG35–55 (filled bars) or cultured in the absence of Ag (open bars), and IL-17 release (left panel) and IFN-γ (right panel) were measured after 72 h by sandwich ELISA. (H) Ex vivo analysis of CD4+CD45.1+ 2D2 T cells for intracellular IL-17 (left) and IFN-γ (right) expressed as absolute number of cytokine-positive 2D2 T cells. (I) Histograms representing the frequency of Foxp3+ cells within the polyclonal CD4+CD45.1+ T cells (left) and within MOG-specific 2D2 T cells (CD4+CD45.1+, right). The histograms represent cells from mice treated with anti-CD4 (dotted line) or an isotype control (thick line, frequency values in italics). The data represent the mean ± SEM of two independent experiments. *p < 0.05.

**Anti-CD4 mAb treatment prevents fatal EAE in TCR-transgenic mice**

MBP-specific TCR-transgenic mice that are Rag1-deficient (TR-/-) spontaneously develop EAE between days 40 and 60 of life (49). All of the TR-/- mice develop a disabling disease that progresses rapidly, leading to death within 5 d upon the initial clinical manifestations. This model permitted us to assess the efficiency of non-depleting CD4 mAb treatment during spontaneous neuro-inflammation that develops without the need of adjuvant immunization. Treating the TR-/- mice with two shots of 1 mg anti-CD4 mAb between days 30 and 35 of age protected all mice from EAE (incidence 0%; Supplemental Table I) when compared with isotype control-treated TR-/- mice, all of which developed fatal EAE (incidence 100%; Fig. 5A). The efficacy of the non-depleting CD4 mAb treatment is such that even delaying treatment after...
out of two. (control mAb (histograms). *) Absence of anti-CD4 (top) or subjected to CD4-blockade (bottom), showing higher frequency of cells binding annexin V in mice treated with anti-CD4 (histograms). *p < 0.05.

Discussion

The availability of different TCR-transgenic animal models of inflammatory demyelinating disease allows the study of mechanisms underlying putative tolerance-inducing regimens that influence different components of the immune system (57). In the last decade several studies have supported a role for T cell-directed therapies in the treatment of MS, where alemtuzumab is perhaps the most prominent example (58). Additionally, depleting (27, 28) and nonmitogenic (26, 59) anti-CD3 mAbs have been shown effective in inducing long-term protection from EAE. We used a nondepleting anti-CD4 mAb and found that CD4 blockade can lead to long-term protection from EAE while triggering different outcomes in naive or preactivated effector T cells.

We found that CD4 blockade was able to prevent the onset of EAE in MOG-CFA–immunized mice, as well as to impair disease progression when anti-CD4 was administered following the onset of clinical manifestations of the disease. Importantly, anti-CD4–treated mice remained fully competent to respond to unrelated Ags (Supplemental Fig. 1) and to mount protective immune responses toward a γ-herpesvirus (MuHV-4). However, at the time of anti-CD4 infusion there is a period where exposure to foreign Ags leads to tolerance rather than to protective immunity. Therefore, and depending on the pathogen characteristics, active infection when anti-CD4 is administered may interfere with effective pathogen clearance. In fact, in the early 1990s there were some promising studies with anti-CD4–induced EAE protection (32, 44–46) that led to some of the earliest clinical trials with therapeutic mAbs. However, the clinical development of this anti-CD4 therapy for MS was soon abandoned because of adverse effects related to CD4 T cell depletion (48). Our data show that, similar to what was previously shown in transplantation (60), autoimmune arthritis (61), and allergic diseases (62, 63), the therapeutic effect mediated by anti-CD4 mAb does not require direct T cell depletion.

To follow the fate of pathogenic T cells in vivo we adoptively transferred MOG-specific cells from 2D2 mice. It is well described that in EAE, the CNS suffers a massive infiltration by activated lymphocytes, namely CD4 effector T cells, which break the blood–brain barrier and recruit other proinflammatory cells to the site of inflammation, leading to an increased severity of the disease (64). We found that anti-CD4–treated mice did not show any significant infiltrates in the CNS while presenting a reduction of MOG-specific TCR-transgenic cells in the cervical lymph node.
FIGURE 5. Anti-CD4 treatment prevents fatal EAE in TR<sup>−</sup> mice. (A) TR<sup>−</sup> mice develop fatal spontaneous EAE between days 40 and 60 of life. TR<sup>−</sup> mice were treated with 2 × 1 mg anti-CD4 between days 30 and 35 of life. Treated mice were protected from the onset of EAE, whereas isotype-treated controls developed fatal disease (n = 8; ***p < 0.001). These data are representative of three independent experiments. (B) To investigate the impact of anti-CD4 treatment in established disease, TR<sup>−</sup> mice were treated with anti-CD4 or an isotype control upon the first manifestations of the disease. CD4-treated mice were protected from severe EAE whereas control animals died within days (n = 5; ***p < 0.001). (C) Number of CD4<sup>+</sup> T cells in cervical lymph nodes of anti-CD4-treated and control mice (NS). (D) Frequency of apoptotic cells, identified by binding of annexin V and 7-aminoactinomycin D (7AAD), in anti-CD4–treated and control mice (NS). (E) Number of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in control and anti-CD4–treated mice, as well as representative dot plots. Mice treated with anti-CD4 showed significantly increased levels of Tregs (n = 4; **p < 0.01). (F and G) In vitro cultures of TR<sup>+</sup> T cells stimulated with MBP, under Th17 polarization conditions. (F) Representative dot plots and (G) quantification of Foxp3<sup>+</sup> (left) and IL-17<sup>+</sup> (right) T cells. Addition of anti-CD4 mAb led to a decrease in Th17 commitment and an increase in the frequency of Foxp3<sup>+</sup> T cells expression in a dose-dependent way (n = 4; *p < 0.05).

The number of nontransgenic CD4 cells did not change, confirming the nondepleting nature of the mAb used. We also found that CD4 blockade acts by preventing T cell proliferation, as well as preventing the differentiation of effector cell producing pro-inflammatory cytokines. Our data show that the few encephalitogenic cells that proliferate in mice treated with anti-CD4 are committed to apoptosis, whereas in the control group they acquire IL-17– and IFN-γ–producing phenotypes (Supplemental Fig. 2). The molecular triggers that lead to apoptosis in the proliferating cells exposed to anti-CD4 mAb remain to be established. Additionally, we also found that among the transferred MOG-specific cells there is an expansion of Foxp3<sup>+</sup> T cells. These presumably induced Tregs can be especially important for the long-term maintenance of the tolerance state.

We then studied the effect of CD4 blockade on preactivated MOG-specific T cells, generally considered difficult to regulate. In fact, it was previously reported that MOG-specific Foxp3<sup>+</sup> Tregs are able to control naive neurotropic T cells but not activated effector T cells (65). We found that preactivated 2D2 cells do not cause pathology in the presence of CD4 blockade. Our data show that preactivated 2D2 cells under the control of anti-CD4 fail to accumulate in the CNS, while being committed to apoptosis.

To further investigate the regulation of the balance between Tregs and effector T cells we used Rag1-deficient mice bearing a T cell repertoire exclusively composed of MBP-specific T cells and devoid of Foxp3<sup>+</sup> Tregs (TR<sup>−</sup>) (49, 51). Despite the number of potentially aggressive cells these animals were protected from the development of EAE following a short course of nondepleting anti-CD4. We found protection from EAE correlated with the emergence of Foxp3<sup>+</sup> Tregs in the periphery. This is in agreement with several studies that support a role for Tregs in the control of CNS inflammation. In fact, some studies have shown that TR<sup>−</sup> mice can be protected from EAE following adoptive transfer of Tregs from anti-MBP TCR-transgenic Rag1<sup>+/−</sup> mice (51, 66, 67).

Our in vitro studies have confirmed that blocking CD4 during the activation of TCR-transgenic T cells under Th17 polarizing conditions results in a decrease in IL-17–producing cells concomitantly with a greater frequency of Foxp3<sup>+</sup> cells in an anti-CD4 mAb dose-dependent way. It was indeed previously reported that strategies leading to a reduction of stimulatory signals received by the T cell (namely by reducing the concentration of agonist peptides or using CD4 blockade) can favor Foxp3 upregulation (52, 68). In all RAG<sup>−/−</sup> TCR-transgenic strains tested, we observed that the T cells that had acquired Foxp3 expression maintained normal levels of transgenic TCR expression.

Taken together, our data show that T cells under distinct stages of functional maturation respond differently to tolerance-inducing protocols. Therefore, it is likely that some reagents able to induce tolerance in naive T cells may be inadequate to effectively control preactivated T cells. Similarly, strategies that suppress terminally differentiated effector cells may be insufficient to induce long-term protection from the disease by promoting the peripheral induction of Tregs. In a similar way to what we have previously reported in an animal model of autoimmune arthritis (61), it appears that the key to long-term tolerance in EAE is the resetting of the abnormal effector Th1-Th17/Treg ratio. Importantly, naive and effector cells appear to be regulated differently: CD4 blockade prevents the proliferation and effector polarization of naive
T cells, while favoring Treg conversion; however, activated T cells are predominantly regulated by favoring their commitment to apoptosis.

Overall, our data show that effective tolerance-inducing strategies in autoimmunity will have to induce naive T cells to acquire regulatory function important for the long-term maintenance of tolerance, while simultaneously disarming the preactivated effector T cells that may resist Treg-mediated regulation.

Acknowledgments

We are grateful to Juan J. Lafaille, Joecelyn Demengeot, and Catarina Martins for helpful advice and for providing TR− mice, Miguel Soares and Andreia Cunha for advice regarding CNS-related techniques, Marta Martins for helpful advice and for providing TR− mice, and Herman Waldmann for nondepleting anti-CD4 hybridomas.

Disclosures

The authors have no financial conflicts of interest.

References


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**Supplemental Table 1.** *Clinical severity of EAE in mice from the different experiments represented in the figures.* In some of the experiments the day of onset is not represented because none of the mice developed clinical manifestations of the disease (Figures 1A, 3A, 4A, and 5A), or because the experiment was initiated after the onset of the disease (Figure 1E and 5B). In these cases it is represented as N.A., not applicable.
Supplemental Figure 1. Non-depleting anti-CD4 MAb induces immune tolerance to foreign antigens that are present at the time of treatment. (A) C57Bl/6 mice initially treated with non-depleting anti-CD4 (left column) were compared with mice that received PBS instead of anti-CD4 treatment (middle column). Both groups of mice were immunized with ovalmumin on day 50 following anti-CD4 (or PBS) treatment. Serum concentration of OVA-specific IgG1 was quantified 20 days following OVA immunization. The concentration of OVA-specific IgG1 was similar in both groups of animals irrespective of prior treatment with non-depleting anti-CD4, and significantly higher than in control group ($P<0.01$). (B) Tolerance can be induced to OVA if administered at the time of anti-CD4 treatment. C57Bl/6 mice were treated with PBS or OVA-alum + anti-CD4. After 50 days both groups of mice were challenged with OVA-alum. The production of OVA-specific IgG1 was impaired in animals previously exposed to OVA together with anti-CD4 ($P<0.01$).
Supplemental Figure 2. Treatment with anti-CD4 prevents proliferation and acquisition of terminal effector function by MOG-specific T cells. CFSE-labeled naïve 2D2 T cells (1x10^6) were adoptively transferred into B6 mice prior to MOG_{35-55} immunization. Flow cytometry analysis was performed on day 3 in the draining lymph nodes of mice treated with anti-CD4 MAb (n=4, white bars) and control MAb (n=4, black bars) on days 0, 2, and 4. Representative dot-plots are shown in Figure 3F. (A) The number of cell divisions that each MOG-specific 2D2 T cell had undergone can be inferred from the CFSE dilution. The graph represents the frequency of 2D2 cells that have divided the indicated number of times. (B) Proliferating cells from anti-CD4 treated mice are committed to apoptosis. Frequency of Annexin V^+ 2D2 T cells by number of cell divisions. (C) Number of IFNγ^+ 2D2 T cells and (D) IL-17^+ 2D2 T cells within cells that undergone different number of divisions.