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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 not 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 between 8 and 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 (MEVGWYRSPFSRVVHLYRNGK) (Biopolymers Laboratory, Oxford, U.K.) 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 0.5 × 10⁶ cells/ml were stimulated in vitro with 20 μg/ml NF-M-35 (RRVTEFRSSRFVGGPGSF) peptide in the presence of IL-12 (20 ng/ml), IL-2 (1 ng/ml), and irradiated syngeneic spleenocytes (5 × 10⁶ × 10⁵). On day 6, viable cells were restimulated with APCs and 20 μg/ml NF-M-35 in the presence of IL-12 (20 ng/ml) and IL-2 (1 ng/ml). On day 9, 14 × 10⁶ Th1 cells were injected into lightly irradiated (300 rads) syngeneic recipients. Disease severity was scored daily on a five-point scale: 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, quadriplegia; 5, moribund.

**Abs**

Nondepleting anti-CD4 (YTS177) and isotype control (YKIX302) mAbs were produced in our laboratory using Integra CL1000 flasks (IBS Integra Biosciences, Chur, Switzerland), purified by 50% ammonium sulfate pre-precipitation, dialyzed against PBS, and purity was checked by native and SDS gel electrophoresis. Hybridomas were generously provided by Prof. Herman Waldmann (Oxford, U.K.).

**Preparation of CNS mononuclear cells**

Mice were perfused through the left cardiac ventricle with cold PBS. The brain and spinal cord were dissected. CNS tissue was cut into pieces and digested with collagenase type VIII (0.2 mg/ml; Sigma-Aldrich) in HBSS at 37°C for 30 min. Mononuclear cells were isolated by passing the tissue through a 70-μm cell strainer, followed by a 30% Percoll (Sigma-Aldrich) gradient and 20 min centrifugation at 2500 rpm. Mononuclear cells were recovered from the pellet, resuspended, and used for further analysis.

**Flow cytometry**

Single-cell suspensions were stained with CD25-Alexa Fluor 488 and PE-Cy7 (PC06.1), CD4-PE and PerCP-Cy5.5 (RM4-5), CD8-allophycocyanin-Cy7 (53-67), Th1.2-allophycocyanin-Cy5 (53-2.1), anti-MBP TCR clonotype biotin (3H12) (51), TCR Vβ11 (RR3-15), TCR Vα3.2 FITC (RR3-16), CD45.1 A700 and biotin (A20; all from ebioscience), and CD3-PerCP-Cy5.5 (145-2C11; BioLegend). Apoptotic cells were identified with 7-aminoactinomycin D and annexin V-allophycocyanin labeled in annexin V binding buffer (eBioscience). To prevent unspecific Ab capture by the Fc receptors, cells were incubated with anti-CD16/32 (clone 2.4G2) prior to staining. Samples were acquired on a FACS Canto or LSRII and analyzed with FlowJo.

**CFSE labeling**

Cells were resuspended at 1 × 10⁶ cells/ml incubated at 37°C for 10 min in the presence of 5 μM CFSE. To stop the staining, cold complete medium was added.

**Intracellular stainings**

For intracellular cytokine staining, cells were isolated as described and stimulated for 4 h in complete culture medium containing 50 ng/ml PMA, 500 ng/ml ionomycin, and 10 μg/ml brefeldin A (Sigma-Aldrich). After staining of surface markers, cells were treated with a fixation and permeabilization kit (eBioscience) and stained with IFN-γ (XMG1.2), IL-17A (ebio 17B7), and Foxp3 (FJK165) Abs from BD Biosciences and eBioScience according to the manufacturers’ recommendations.

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 purity was 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 (R46A2) 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 × 10⁶) were plated in 96-well flat-bottom plates and cultured in complete medium (RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPS, 1% penicillin/streptomycin, 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 (PrepTech, London, U.K.), or IL-17 (R&D Systems).

**Adaptive transfer of naive T cells**

CD4 CD62L−CD25− 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 (RA3-6B2), Mac1 (Cl-A3-1), and CD25 (7D4); followed by positive selection with anti-CD62L−coated beads, providing 80% CD4+ T cells, of which 95% were CD62L−CD244+ expressing the transgenic 2D2 TCR. Cells (1 × 10⁶) 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 10⁶ 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× serial dilutions. To each dilution 5 × 10⁵ 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−/− and MHC class II−/− 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

![FIGURE 1.](http://www.jimmunol.org/)

CD4 blockade treats active EAE in C57BL/6 mice. (A) CD4 mAb treatment prevents active EAE. Average clinical score of MOG35-55-immunized mice treated with 1 mg nondepleting anti-CD4 mAb (n = 5; ▲) or isotype control mAb (n = 5; ■) at 3 and 2 d prior to immunization. The mean clinical score ± SEM is presented for one representative experiment out of three. (B) Parenchymal infiltration of inflammatory cells is prevented following CD4 blockade. H&E staining of transversal sections of the spinal cord 20 d after MOG35-55 immunization (original magnification ∼400). Control mAb (n = 3; middle column) and nondepleting CD4 mAb (n = 3; right column) were injected 3 and 2 d prior to immunization. Aged-matched nonimmunized mice (n = 3; left column) were used as control. (C) CD4 mAb treatment prevents immune invasion of the CNS. Fifteen days after MOG35-55 immunization, CNS mononuclear cells were obtained by Percoll gradient from mice treated with an isotype control (black bar; n = 5) and anti-CD4 mAb (white bar; n = 5) 3 and 2 d before immunization. Age-matched nonimmunized mice (gray bar; n = 5) were used as control. CD4+ TCRβ+ CD8+ T cells were quantified by flow cytometry. The data represent the mean number of CD4+ T cells per CNS ± SEM and two representative dot plots. (D) Anti-CD4 mAb treatment reduces the magnitude of the MOG35-55-specific T cell response. Ag recall experiments were performed on splenocyte cultures 14 d after MOG35-55 immunization. The concentration of IFN-γ and IL-17 was measured 72 h after in vitro stimulation with 20 μg/ml MOG35-55 (filled bars) or medium alone (open bar). Mice were treated with control mAb (n = 5; middle) and anti-CD4 mAb (n = 5; right) 3 and 2 d prior to immunization. Age-matched nonimmunized mice were used as controls. Data represent mean cytokine concentration ± SEM. ND, not detected. (E) CD4 mAb therapy ameliorates established EAE. Average clinical score of MOG35-55-immunized mice treated with a single injection of 1 mg nondepleting anti-CD4 mAb (n = 5; ▲) or isotype control mAb (n = 5; ■) on the day of disease onset. The data present the mean clinical score ± SEM after disease onset (day 0) and correspond to one representative experiment out of two. *p < 0.05, ***p < 0.001.
To study the fate of encephalitogenic T cells at the single-cell level, polarization of MOG-specific T cells and CD4 blockade inhibits proliferation and functional IgG1 following subsequent OVA recall (Supplemental Fig. 1B). Ag, which manifested as impaired production of OVA-specific Fig. 1A). In contrast, administration of OVA at the time of anti-CD4 mAb treatment with nondepleting anti-CD4 mAb prior to OVA immunization did not hamper subsequent immune responses to OVA (Supplemental Fig. 2A). This model of viral clearance was used to test the immunocompetence of mice previously treated with anti-CD4 or control mAb 2 and 3 d prior to MOG35–55 immunization. Thirty days after immunization mice were intranasally infected with MuHV-4 and the lungs were analyzed for virus content 7 and 14 d postinfection. Control B6 mice were infected with MuHV-4 in the absence of prior MOG-CFA immunization or CD4 blockade. As shown in Fig. 2B, mice that were initially treated with CD4 mAB were able to resolve the MuHV-4 virus infection detected at day 7 (Fig. 2B, top) by day 14 (bottom) with an efficiency equivalent to nonmanipulated control mice.

To further illustrate that nondepleting CD4 mAB treatment tolerizes only to Ags present at the time of treatment, we differentially applied CD4 mAB treatment to mice immunized with OVA and alum (Supplemental Fig. 1). We found that treatment with nondepleting anti-CD4 mAB prior to OVA immunization did not hamper subsequent immune responses to OVA (Supplemental Fig. 1A). In contrast, administration of OVA at the time of anti-CD4 treatment led to the induction of tolerance to this foreign Ag, which manifested as impaired production of OVA-specific IgG1 following subsequent OVA recall (Supplemental Fig. 1B).

CD4 blockade inhibits proliferation and functional polarization of MOG-specific T cells
To study the fate of encephalitogenic T cells at the single-cell level, 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-A2-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 congeneric 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). Abortive 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).

Effector 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 × 106 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 congenic 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).

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 nondepleting 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 100%: Fig. 5A). The efficacy of the non-depleting CD4 mAb treatment is such that even delaying treatment after
out of two. (control mAb (*))

FIGURE 4. CD4 blockade prevents Th1-induced EAE through induction of apoptosis. (A–D) MOG-specific 2D2 T cells were polarized toward Th1 phenotype in vitro and transferred (14 × 10^7/mouse) into syngeneic B6 mice. (A) Average clinical score of mice treated with anti-CD4 mAb (n = 4; ■) or control mAb (n = 4; ▲) at the day of Th1 transfer and on days 2 and 4. The mean clinical score ± SEM is presented for one representative experiment out of two. (B–E) Nine days after 2D2 Th1 transfer anti-CD4 mAb (n = 4; open bars) or control mAb (n = 4; filled bars) treated mice were sacrificed and the number of CD4^+CD45.1^+ TCR Vα3.2^+ T cells was determined by flow cytometry among CNS-infiltrating mononuclear cells (B) and in the cervical lymph nodes (C). (D) Proportion of cells binding annexin V among CD45.1^+TCR Vα3.2^+CD4^+ 2D2 T cells in the cervical lymph nodes. The data represent the mean ± SEM of two experiments. (E) Representative flow cytometry data from cervical lymph nodes of mice transferred with Th1-polarized cells in the 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.

onset (clinical score 1) still permitted the progression of disease to be halted (Fig. 5B). In contrast, the isotype control treatment failed to delay the lethal disease progression, resulting in the death of all mice (Fig. 5B).

Similar to the active EAE setting, the overall CD4^+ T cell number is maintained following anti-CD4 treatment, confirming the nondepleting nature of the mAb (Fig. 5C). Unlike active EAE, we did not find evidence for increased apoptosis on autoantigen-specific T cells from anti-CD4–treated mice (Fig. 5D). Because TR^− mice are devoid of natural Tregs, we could conveniently evaluate the induction of Treg differentiation in anti-CD4–treated mice (Fig. 5E) (49). Two injections of 1 mg CD4 mAb in 30- to 35-d-old mice permitted the de novo generation of CD4^+Foxp3^+ T cells in cervical lymph nodes (Fig. 5E). The observed low percentage of induced Foxp3^+ Tregs is consistent with previous reports of Treg-mediated tolerance induced in TCR-transgenic Rag-deficient mice (52, 56).

To study the underlying mechanism, we evaluated whether in vitro stimulation of CD4^+ MBP-specific TR^− cells with MBP-loaded dendritic cells in the presence of anti-CD4 would alter the balance between the protective and proinflammatory Treg and Th17 populations. Addition of anti-CD4 to T cell cultures in Th17 polarizing conditions (in the presence of TGF-β and IL-6) led to a decrease in Th17 cells with a simultaneous increase of Treg frequency in a dose-dependent manner (Fig. 5F, 5G). This suggests that, in addition to recessive tolerance, nondepleting CD4 mAbs are able to convey dominant tolerance in animal models of CNS autoimmunity.

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− mice. (A) TR− mice develop fatal spontaneous EAE between days 40 and 60 of life. TR− 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− 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+ T cells in cervical lymph nodes of anti-CD4-treated and control mice (NS). (D) Number of CD4+Foxp3+ 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− T cells stimulated with MBP under Th17 polarization conditions. (F) Representative dot plots and (G) quantification of Foxp3+ (left) and IL-17+ (right) T cells. Addition of anti-CD4 mAb led to a decrease in Th17 commitment and an increase in the frequency of Foxp3+ T cell 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 cells producing proinflammatory 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+ 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+ Tregs are able to control naïve 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 cover 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+ Tregs (TR−) (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+ 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− mice can be protected from EAE following adoptive transfer of Tregs from anti-MBP TCR-transgenic Rag1−/− 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+ 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−/− 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 naïve 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, naïve 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 dismissing the preactivated effector T cells that may resist Treg-mediated regulation.

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Disclosures

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References


