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Treatment with Nonmitogenic Anti-CD3 Monoclonal Antibody Induces CD4⁺ T Cell Unresponsiveness and Functional Reversal of Established Experimental Autoimmune Encephalomyelitis

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In vivo administration of anti-CD3 Ab induces both immune tolerance and undesirable side-effects resulting from nonspecific proinflammatory cytokine production. In the current study, we investigated the therapeutic potential of two structurally altered forms of the anti-CD3 Ab in ameliorating established experimental autoimmunne encephalomyelitis. Administration of either a chimeric (NM-IgG3) or digestion product (NM-F(ab’)₂ form of the anti-CD3 Ab during established experimental autoimmune encephalomyelitis conferred significant protection from clinical disease progression and was associated with decreased Ag-specific T cell proliferation, cytokine production, and CNS inflammation. Interestingly, while this protection correlated with an increase in the frequency of CD4⁺CD25⁺ regulatory T cells, neither prior depletion of regulatory T cells nor anti-CD4-Ig treatment abrogated the treatment’s efficacy. Importantly, both treatments induced normal levels of intracellular Ca²⁺-flux, but significantly diminished levels of TCR signaling. Consequently, this decreased level of TCR-mediated signaling were alterations in the level of apoptosis and CD4⁺ T cell trafficking resulting in a profound lymphopenia. Collectively, these results indicate that nonmitogenic anti-CD3 directly induces a state of immune unresponsiveness in primed pathogenic autoreactive effector cells via mechanisms that may involve the induction of T cell tolerance, apoptosis, and/or alterations in cell trafficking. The Journal of Immunology, 2005, 174: 4525–4534.

CD4⁺ T cells are key contributors to the underlying pathogenic mechanisms responsible for the onset and progression of most autoimmune diseases. However, these cells are also critical to the induction of adaptive immunity, thus creating a functional dichotomy that underscores the necessity for active regulatory mechanisms that both promote immunity against foreign Ags, while protecting against self-directed responses. One endogenous regulatory mechanism is the phenomenon of central tolerance which protects against the generation of autoimmune responses by negative selection against autoreactive CD4⁺ T cells. However, T cells with autoreactive potential can escape negative selection to persist in normal individuals with the capacity of mediating various autoimmune diseases (1).

CD4⁺ T cells recognize both foreign and self-Ags (e.g., myelin proteins) via their Ag-specific TCR during cognate interactions with APCs displaying peptide/MHC class II complexes. Typically, endogenous ligation of the TCR alone produces a signal of insufficient strength to activate a T cell. Therefore, TCR stimulation in the absence of costimulatory signals leads to CD4⁺ T cell anergy, tolerance, or depletion and this may be one therapeutic strategy to down-regulate pathogenic CD4⁺ T cell activity during autoimmune disease. In light of this, various therapeutic approaches have been used to block autoreactive CD4⁺ T cell function during experimental autoimmune encephalomyelitis (EAE) and other autoimmune disease models, including the administration of Abs directed against a variety of epitopes including CD3, CD4, CD28, CD40, CD80, CD86, CD154, ICOS, OX40, and 4-1BB, as well as CTLA4-Ig (2, 3). However, these treatment strategies often result in either nonspecific immune suppression or other undesirable side-effects.

For example, the hamster 145-2C11 Ab (anti-CD3) binds and cross-links the CD3ε chains of the mouse TCR (4) resulting in costimulation-dependent CD4⁺ T cell activation in vitro. Because in vivo administration anti-CD3 Ab induces T cell down-regulation, T cell tolerance, and CD4⁺ T cell depletion (5), previous studies have used this Ab as an immunosuppressive agent to treat various autoimmune disorders and transplant rejection (6–13). However, cross-linking of the TCR by anti-CD3 Ab in vivo can deliver a signal of sufficient strength to overcome the need for costimulation resulting in release of proinflammatory cytokines and significant side-effects that is often referred to as “first dose”...
syndrome (5, 14–16). Contributing to the complications of anti-CD3 Ab treatment is the generation of anti-CD3 Ab-specific humoral immune responses that neutralize the effectiveness of the treatment (17). Thus, it would be advantageous to retain the immunosuppressive properties of anti-CD3 Ab treatment in the absence of nonspecific T cell activation and the generation of humoral responses directed against the treatment. Because the Fc region determines the potential of an Ab molecule to bind FcR (18), and thus the ability of anti-CD3 Ab to cross-link the TCR (19), we investigated the therapeutic potential of two structurally modified (nonmitogenic) forms of anti-CD3 that exhibit a significantly reduced FcR-binding capacity for the treatment of established EAE: a F(ab\(^\prime\))\(_2\) (NM-F(ab\(^\prime\))\(_2\)) that lacks the FcR-binding region, and a chimeric Ab (NM-IgG3) in which the native Fc region with a high affinity for FcRs (IgG) has been replaced with an Fc region with very low FcR affinity (IgG3) (20). Nonmitogenic anti-CD3 mAb has been reported to successfully treat NOD mice with overt diabetes (8–10, 13) and has had some success in phase I/II clinical trials for delaying onset of type I diabetes and treating psoriatic arthritis (12, 21). Importantly, anti-CD3 immunotherapy has been reported to lead to the induction of tolerance to β cell Ags via a mechanism perhaps related to the activation of CD4^+CD25^+ regulatory T (T\(_{reg}\)) cells (8, 9, 22).

In the current studies, we used the proteolipid peptide (PLP) peptide (PLP\(_{139–151}\))-induced model of relapsing EAE (R-EAE) in SJL mice to determine whether treatment with nonmitogenic forms of anti-CD3 mAb could ameliorate the progression of established disease by blocking clinical relapses and associated T cell responses. PLP\(_{139–151}\)-induced R-EAE is a well-characterized CD4^+ T cell-mediated model of relapsing-remitting multiple sclerosis characterized by the de novo activation of T cell responses to endogenous myelin epitopes, via the process of epitope spreading, which play a major role in the pathogenesis of disease relapses (23, 24). We show that both nonmitogenic forms of anti-CD3 mAb confer significant protection from the onset and progression of R-EAE in the absence of nonspecific T cell activation, proliferation, and cytokine production. Most notably, the treatments led to induced unresponsiveness in PLP\(_{139–151}\)-specific T cells potentially mediated by the direct activation of inhibitory signaling mechanisms within the CD4^+ effector cells, as well as alterations in CD4^+ T cell trafficking.

**Materials and Methods**

**Mice**

Wild-type SJL/J female mice, 5–6 wk old, were purchased from Harlan Sprague Dawley. 5B6 PLP\(_{139–151}\) TCR transgenic mice were generously provided by Dr. V. Kuchroo (Harvard University, Boston, MA). Mice were maintained on standard laboratory food and water ad libitum. Paralyzed animals were afforded easier access to food and water.

**Induction and clinical evaluation of PLP\(_{139–151}\)-induced EAE**

Six- to 7-wk-old female SJL mice were immunized s.c. with 100 µl of an emulsion containing 200 µg of Mycobacterium tuberculosis H37Ra (BD Biosciences) and 50 µg of PLP\(_{139–151}\) distributed over three spots on the flank. Mice received five daily i.v. injections of either 50 µg of mouse IgG3, hamster F(ab\(^\prime\))\(_2\), NM-F(ab\(^\prime\))\(_2\), or NM-IgG3 on days 0–4, 6–10, or 13–17 postimmunization. For CD4^+CD25^+ Tc cell depletion experiments, mice were injected with 0.5 mg of anti-CD25 mAb (clone 7D4; American Type Culture Collection) i.p. on days −6 and −3 with respect to PLP\(_{139–151}\)/CFA immunization. Individual animals were observed daily and clinical scores assessed in a blinded fashion on a 0–5 scale as follows: 0, no abnormality; 1, limp tail; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; and 5, moribund. The data are reported as the mean daily clinical score.

**In vitro assays**

Draining lymph node or spleen cells were isolated from either naive mice or mice immunized at indicated times following disease induction. Single-cell suspensions (3 × 10^6 cells per milliliter in HL-1; Fisher Scientific) were stimulated with increasing concentrations of either PLP\(_{139–151}\) (GeneMed Synthesis), anti-CD3 (eBioscience), NM-IgG3 (20), and/or NM-F(ab\(^\prime\))\(_2\) (Bio Express). In some experiments, Ab was immobilized via covalent linkage to 1 µM polylysine beads (Interfacial Dynamics) by incubating varying concentrations of Ab with the beads for 60 min on ice followed by repeated washes. Cultures were incubated at 37°C for 24–96 h and 1 µCi/well [H]H37Ra was added to each well for the final 24 h of culture. [H]H37Ra uptake was detected using a Topcount Microplate Scintillation Counter and results are expressed as the mean cpm of triplicate cultures ± SEM. For cytokine bead array analysis, supernatants were collected between 72 and 96 h and cytokines detected using the Mouse Cyto-10 Plex system and Luminex Liquidchip analyzer (Qiagen). All apoptosis assays were performed using the Guava PCA-96 MultiCaspase Kit and Analyzer (Guava Technologies).

**Elicitation of Ag-specific delayed-type hypersensitivity (DTH)**

DTH responses were quantitated using an ear swelling assay as previously described (25). Prechallenge ear thickness in metofane-anesthetized mice was measured with a Mitotoy dial thickness gauge. A total of 10 µg of PLP\(_{139–151}\) (in 10 µl of PBS) was injected intradermally into the dorsal surface of the ear using a 100-µl Hamilton syringe fitted with a 30-gauge needle, and ear swelling (in units of 10^-4 m) was measured 24 h later.

**Immunohistochemistry and immunofluorescence**

CNS immunohistochemistry for the detection of CD4^+, CD8^+, and F4/80^+ cells within the CNS of perfused animals was performed as previously described (26). For immunofluorescence experiments, single-cell suspensions were washed and incubated with fluorescently tagged Abs specific for a panel of cell surface markers including CD4, CD25 (PC61), CD54, CD62L, and CD69 (eBioscience). In some experiments, spleen, lymph node, or PLP\(_{139–151}\) TCR transgenic CD4^+ T cells were stimulated with either PLP\(_{139–151}\), isotype control Ab, anti-CD3, NM-IgG3, and/or NM-F(ab\(^\prime\))\(_2\) for 24 h before cell staining and analysis. Fluorescent staining was analyzed using a LSRII Flow Cytometer and CellQuest Pro software (BD Biosciences).

**Intracellular calcium-flux measurement**

Lymph node cells were harvested from naive mice and labeled with anti-CD4 Ab and 1 µM Indo-1 (Molecular Probes) for 15 min at 37°C followed by repeated washes and continued incubation at 37°C for an additional 2 h. In some experiments, cells were also labeled with anti-CD25 mAb (PC61) for the distinction of CD4^+CD25^+ and CD4^+CD25^- cell populations. Cell samples were analyzed by flow cytometry and data was expressed as the ratio of 398 nm (Indo-1 bound to Ca^{2+})/482 nm (unbound Ca^{2+}) in CD4^+ T cells over a period of 7 min.

**Statistical analyses**

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

**Results**

**Structural alterations to anti-CD3 block induction of T cell activation and effector function**

Because the mitogenic effect of anti-CD3 Ab treatment (19) is dependent upon FcR-mediated stabilization of the molecule before TCR cross-linking (18), and in light of previous reports that anti-CD3 mAb treatment induces nonspecific cytokine production and subsequent cytokine-induced sickness (5, 14–16), we investigated the mitogenic capacity of two structurally altered forms of anti-CD3 that were designed to significantly decrease their FcR-binding capacity (Fig. 1A). As expected, naive SJL lymph node T cells did not proliferate (Fig. 1B), produce IFN-γ (Fig. 1C), or up-regulate surface activation markers (data not shown) in response to either NM-F(ab\(^\prime\))\(_2\) or NM-IgG3, in contrast to exposure to the.
may lead to APC activation/costimulatory molecule up-regulation, thus providing a source of concurrent costimulatory signals that may reverse the nonmitogenic phenotype of the Ab treatments. Importantly, the concurrent stimulation of CD28 failed to reverse the nonmitogenic properties of both NM-F(ab')2 and NM-IgG3 (Fig. 1E), but enhanced the level of proliferation induced by the native form of the anti-CD3 mAb as expected, suggesting that the nonmitogenic phenotype of the Ab treatments persists in the presence of exogenous or nonspecific costimulatory signals.

Nonmitogenic anti-CD3 ameliorates progression of established EAE in SJL mice

We next investigated the therapeutic efficacy of nonmitogenic anti-CD3 mAb treatment on the clinical progression of R-EAE. We and others have previously reported the therapeutic potential of NM-IgG3 in the prevention of other autoimmune and transplant rejection responses (8–10, 12, 13, 21). However, because only a few studies have investigated the efficacy of nonmitogenic anti-CD3 mAb treatment administered at time after disease induction/onset (9, 11, 33), it was critical that the efficacy of the NM-IgG3 and CD3-F(ab')2 treatments administered at times following disease initiation be determined in our model system. To accomplish this, SJL mice were first immunized with the immunodominant epitope PLP139–151 in CFA followed by treatment with NM-IgG3 or NM-F(ab')2 on days 6–10 postimmunization. Importantly, administration of nonmitogenic anti-CD3 mAb efficiently prevented the onset of clinical disease in the previously immunized mice (Fig. 2A), decreased in vivo PLP139–151-specific Th1 effector function as determined by DTH (Fig. 2B) and reduced the degree of CNS lymphocyte infiltration (Fig. 2C), which correlates with both the level of macrophage infiltration/activation and disease severity (34). Taken together, these findings suggest that nonmitogenic anti-CD3 mAb therapy induced anergy or tolerance in PLP139–151-specific T cells resident in the splenic compartment or altered trafficking of PLP139–151-specific T cells to the CNS.

We next investigated the effect of altering the timing of nonmitogenic anti-CD3 mAb treatment during EAE. SJL mice immunized with PLP139–151 in CFA received five daily treatments of NM-F(ab')2 either on days 0–4, 6–10, or 13–17 postimmunization, times corresponding with the initiation, preacute, and acute phases of disease, respectively. Interestingly, NM-F(ab')2 treatment on days 0–4 had no effect on either the day of onset or the level of disease severity (Fig. 3A). In contrast, NM-F(ab')2 treatment either during days 6–10 or 13–17 postimmunization (Fig. 3, B and C) significantly reduced the primary acute or subsequent relapse phases of disease, respectively. To further test the efficacy of the nonmitogenic treatments, we also used an adoptive transfer model of EAE in which previously activated PLP139–151-specific T cells were transferred into naive recipients. Mice receiving PLP139–151-specific T cells resident in the splenic compartment or altered trafficking of PLP139–151-specific T cells to the CNS.
production. In contrast, both PLP139–151-specific Th1 and Th2 cytokine production was significantly suppressed in mice treated from days 6–10 indicating immune deviation does not play a major role in disease protection.

Contribution of CD4+CD25+T<sub>R</sub> cells to nonmitogenic anti-CD3-induced protection

In light of induction of unresponsiveness by nonmitogenic anti-CD3 without alterations in the Th1/Th2 balance, we asked whether Ab treatment expanded or activated the endogenous CD4+CD25+CD62L<sup>high</sup> population that normally displays the CD4+CD25+CD62L<sup>high</sup> phenotype (35) similar to what has been reported in Ab-treated NOD mice (8, 9, 22). Interestingly, nonmitogenic Ab treatment appeared to double the percentage of lymph node CD4+ T cells that also expressed high levels of CD25 and CD62L (Fig. 4A).

**FIGURE 2.** Treatment of PLP<sub>139–151</sub>-primed SJL mice on days 6–10 with nonmitogenic anti-CD3 mAb blocks onset of clinical EAE and accompanying autoreactive T cell responses. A, NM-F(ab′)<sub>2</sub> and NM-IgG3 both alleviate clinical disease onset in active EAE. Mice were immunized s.c. on day 0 with PLP<sub>139–151</sub> (50 μg) in CFA and received five daily i.v. injections (50 μg/injection in PBS) of either NM-F(ab′)<sub>2</sub>, NM-IgG3, or a hamster F(ab′)<sub>2</sub> isotype control on days 6–10 postimmunization (as indicated by arrows). Data are presented as the mean clinical score of five mice per group and are representative of three separate experiments. B, Effect on in vivo T cell effector function. Mice were challenged intradermally in the pinnae of the ear with PLP<sub>139–151</sub> (1 μM) on day 14 postimmunization and DTH response determined by the increase in ear swelling 24 h thereafter. Data are presented as the mean of 24 h of ear swelling (in units of 10<sup>−4</sup> in) ± SEM and are representative of two separate experiments. C, Nonmitogenic anti-CD3 mAb treatment blocks CNS inflammation during active EAE. Spinal cord tissues from mice sacrificed days 15–20 postpriming were examined for the presence of CD4<sup>+</sup>, CD8<sup>+</sup>, and F4/80<sup>+</sup> (APC) cells (red) (<100 magnification). Tissues were also counterstained with 4′,6-diamidino-2-phenylindole (blue). No positive staining was observed in isotype-matched controls, and only a few B220<sup>+</sup> B cells were observed (data not shown). Data presented is representative of samples analyzed from five mice per group.

**FIGURE 3.** The efficacy of nonmitogenic anti-CD3 mAb therapy in EAE is dependent on treatment timing. A–C, Relationship between treatment timing and efficacy. Mice were immunized s.c. on day 0 with PLP<sub>139–151</sub> (50 μg) in CFA and followed for clinical disease. Mice also received five daily i.v. injections (50 μg/injection in PBS) of either NM-F(ab′)<sub>2</sub> or hamster F(ab′)<sub>2</sub> isotype control Ab either on days 0–4 (A), 6–10 (B), or 13–17 (C) postimmunization (as indicated by arrows) corresponding with the induction, preacute, and postacute disease phases, respectively. Data are presented as the mean clinical score of five mice per group and are representative of three separate experiments. D, Treatment of active (adoptive transfer) disease. On day 0, naive mice received 3 × 10<sup>6</sup> activated PLP<sub>139–151</sub>-specific T cells followed by five daily i.v. injections (50 μg/injection in PBS) of NM-F(ab′)<sub>2</sub> on days 0–4 (Early) or days 6–10 (Late) following disease induction. Data are presented as the mean clinical score of five mice per group and are representative of three separate experiments. E, Effect of nonmitogenic anti-CD3 mAb therapy on PLP<sub>139–151</sub>-specific cytokine production. Mice were primed with PLP<sub>139–151</sub> in CFA on day 0 and received i.v. injections of NM-F(ab′)<sub>2</sub> or NM-IgG3 on either days 0–4 or 6–10 postimmunization. Spleen cells were harvested on day 18 and restimulated with PLP<sub>139–151</sub> (10 μM) for 96 h in vitro before cytokine bead array analysis. Data are presented as the mean cytokine concentration (picograms per milliliter) and are representative of two separate experiments. *, Significant difference between cytokine levels in control and Ab-treated mice (p < 0.01).
Although TCR-mediated activation is known to increase the level of CD25 expression on nonregulatory CD4+ T cells, the observed increases in the CD25+ population were most likely the result of a distinct mechanism, because the levels of CD25 expression were unchanged in untreated PLP139-151-primed animals, and the CD25high cells also expressed high levels of CD62L. In contrast, nonmitogenic Ab treatment did not influence the percentage of CD4+CD25+ T cells in the spleens of treated animals, which was surprising in light of our findings that NM-F(ab')2 treatment suppresses Ag-specific proliferation and cytokine production in the spleens, but not lymph nodes, of treated animals (data not shown).

To determine the functional relevance of the nonmitogenic anti-CD3 treatment-induced increase in T<sub>R</sub> cell frequency, SJL mice were depleted of CD4+CD25+ T<sub>R</sub> cells before PLP139-151 immunization and subsequent Ab treatment. As shown in Fig. 4B, the percentage of CD4+ T cells in SJL mice that express the CD4+CD25+ T<sub>R</sub> cell phenotype is significantly smaller (2.78%) than most inbred mouse strains; however, the anti-CD25 treatment protocol resulted in nearly complete depletion of the T<sub>R</sub> cell population at a time corresponding with PLP<sub>139-151</sub>/CFA immunization. Interestingly, T<sub>R</sub> cell depletion failed to reverse the total protective effect of NM-F(ab')2 treatment on EAE induction in mice treated on days 6–10 postimmunization (Fig. 4C). This suggests that the activity of CD4+CD25+ T<sub>R</sub> cells is not required for the potent protective effect of nonmitogenic anti-CD3 in regulating established EAE. However, T<sub>R</sub> cells were detected in the lymph nodes and spleens of CD25-depleted mice at levels comparable to control mice 10 days following immunization (data not shown), a time corresponding to the last NM-F(ab')2 treatment.

Although the exact mechanism by which Tr cells exert their suppressive influence is still unknown, one proposed mechanism for down-regulation of effector CD4+ and CD8+ T cell is via surface/secreted TGF-β (35), and previous studies in the NOD model system suggest that the protective effects of NM-F(ab')2 treatment may be reversed by the blockade of TGF-β (9). However, concurrent administration of anti-TGF-β mAb at either 200 μg/injection (data not shown) or 1 mg/injection (Fig. 4D) with each nonmitogenic anti-CD3 treatment failed to reverse the protective influence of the therapy in our model system. Thus, the relative contribution provided by CD4+CD25+ T<sub>R</sub> cells to the protective influence of the nonmitogenic Ab treatments remains unclear, as both T<sub>R</sub> cell depletion and TGF-β blockade fail to block the protective effects of the nonmitogenic therapy during established EAE.

**Nonmitogenic anti-CD3 blocks Ag-specific activation of naive T cells in vitro, but induces intracellular Ca<sup>2+</sup> flux**

If the protective effect of the nonmitogenic anti-CD3 was indeed due to mechanisms distinct from shifts in cytokine profile and T<sub>R</sub> cell induction/activation, the question remained as to whether the treatment could directly initiate a signaling cascade in the target cells leading to the induction of anergy or tolerance. To initially test this hypothesis, PLP139-151-specific T cells were isolated from 5B6 TCR transgenic mice and activated with an increasing concentration of PLP<sub>139-151</sub> in combination with increasing concentrations of NM-F(ab')2 in vitro. Twenty-four hours following activation, the level of T cell activation was assessed by measuring cell surface expression levels of CD54, CD62L, and CD69. NM-F(ab')2 treatment blocked the Ag-induced up-regulation of CD54 and CD69, and down-regulation of CD62L in a concentration-dependent manner (Fig. 5A). In contrast, cotreatment with the native form of anti-CD3 Ab produced the expected increase in T cell activation. In agreement with T cell activation marker analysis, NM-F(ab')2 cotreatment also blocked PLP<sub>139-151</sub>-specific proliferation of the naive T cells (Fig. 5B). However, the efficacy of the NM-F(ab')2-mediated blockade of activation on naive T cells in vitro could be overcome in cultures stimulated with high doses of PLP<sub>139-151</sub> suggesting that either the NM-F(ab')2 simply functioned as a nonsignaling TCR antagonist or that the efficacy of NM-F(ab')2 treatment was inversely related to the level of Ag-induced TCR signaling.

To determine whether nonmitogenic Ab treatment directly initiated an intracellular signaling cascade, we measured the capacity of each molecule to induce intracellular Ca<sup>2+</sup>-flux in CD4+ T cells, because this is an early event in TCR/CD3-mediated signaling. As expected, the native anti-CD3 Ab and ionomycin induced a significant level of Ca<sup>2+</sup>-flux in naive CD4+ T cells (Fig. 5C) and previously activated CD4+ T cells (data not shown) within 2 min of ligand addition, which is in agreement with previous findings (20). Of importance is the observation that both nonmitogenic Ab treatments also induced significant levels of Ca<sup>2+</sup>-flux in both CD4+CD25+ effector and CD4+CD25+ T<sub>R</sub> cells, suggesting that
Nonmitogenic anti-CD3 alters lymphocyte trafficking in vivo

Our observations that nonmitogenic anti-CD3 prevented CD4+ T cell infiltration to the CNS during EAE (Fig. 2C) suggested that the treatment may exert its protective effect via mechanisms that involve either population deletion or alterations in lymphocyte trafficking. Although neither nonmitogenic form of the anti-CD3 Ab should induce complement-mediated cell lysis, we confirmed this in vitro and in vivo. As expected, treatment with either form of nonmitogenic Ab failed to induce complement-mediated cell lysis in vitro or bulk CD4+ T cell depletion in vivo (data not shown). However, in light of the data indicating that the treatment preferentially affects previously activated CD4+ T cells (Fig. 3, A–D), it was possible that the attenuated TCR signal resulting from nonmitogenic anti-CD3 mAb treatment initiated cell death/apoptosis. This possibility was supported by the fact that NM-F(ab’)2 and NM-IgG3 treatment increased the percentage of total CD4+ T cells undergoing either mid- or late-apoptosis in the spleens of treated mice (Fig. 6A). However, even though nonmitogenic anti-CD3 induced slightly elevated levels of apoptosis, we observed no decrease in the number of PLP139-151-specific CD4+ T cells in either the spleen or lymph nodes of treated recipients (Fig. 6B), and, in fact, nonmitogenic anti-CD3 treatment significantly increased the percentage of PLP139-151-specific CD4+ T cells in both organs. As neither NM-F(ab’)2 nor NM-IgG3 induce T cell proliferation (Fig. 1), the increased numbers of PLP139-151-specific CD4+ T cells in the lymphoid organs of treated animals are likely due to alterations in lymphocyte trafficking.

We next directly assessed the effects of nonmitogenic anti-CD3 therapy on trafficking of PLP139-151-specific CD4+ T cells. We observed a rapid and dramatic decrease in number of PLP139-151-specific CD4+ T cells in the circulation of mice receiving mitogenic anti-CD3 or either form of the nonmitogenic anti-CD3 mAb treatment. This decrease was observed as early as 1 h following treatment (Fig. 7B) and persisted until at least 2 days following the last treatment (Fig. 7, C and D). Taken together, these findings suggest that nonmitogenic anti-CD3 mAb treatment rapidly decreases the number of circulating PLP139-151-specific CD4+ T cells leading to their sequestration in the primary lymphoid tissues which may be an important mechanism by which this therapeutic intervention confers protection from established autoimmune disease.

Discussion

Guided by the problems of past studies, we and others have previously reported encouraging findings concerning the therapeutic potential of nonmitogenic forms of anti-CD3 Abs for the treatment of a number of diseases/conditions such as allograft rejection, arthritis, and diabetes (8–10, 12, 13, 21). In the current study, we show that treatment with either of two forms of nonmitogenic anti-CD3 (NM-F(ab’)2 and NM-IgG3) significantly prevented the onset/progression of clinical EAE in the absence of nonspecific T cell
activation, proliferation, or cytokine production both in vitro and in vivo. Importantly, this lack of nonspecific T cell activation appeared to be the direct result of the structural alterations to the Abs that decreased their FcR binding ability, because covalent linkage of either nonmitogenic anti-CD3 Ab to polystyrene beads converted them into potent T cell activators. However, while NM-CD3 treatment eliminates a number of concerns associated with immunotherapy strategies using the native form and the anti-CD3 Ab, future studies must focus on potential mechanisms for targeting this therapy to Ag-specific populations of T cells, in contrast to all activated CD4+ T cells, because desired immune responses against opportunistic infections during treatment may be negatively affected as a secondary outcome of the immunotherapy.

The role of the cytokines in altering the EAE disease course is widely accepted with cells producing Th1-like cytokines such as IFN-γ, IL-2, IL-23, and TNF-α mediating disease progression (36–39) and cells producing Th2/3-like cytokines such as IL-4, IL-10, and TGF-β exerting protective influences (40, 41). We have also shown that CNS expression Th1 cytokine mRNA is associated with periods of disease progression while expression of Th2 cytokine mRNA with periods disease remission in SJL R-EAE (42). In addition, because lower levels of TCR signaling favor T cell differentiation along the Th2 lineage, it was possible that the nonmitogenic anti-CD3 mAb treatments, which cannot be stabilized by FcR binding, ameliorate clinical disease symptoms by switching the Ag-specific responses from a Th1-like to a Th2-like phenotype. However, we failed to detect a shift from Th1-like to Th2-like cytokine production by PLP139–151-specific T cells from mice protected from disease onset by treatment with NM-F(ab')2, in that both Th1 and Th2 cytokine production was inhibited, whereas others report that nonmitogenic anti-CD3 Ab treatment may preferentially influence Th1 cells (19, 33). Both forms of nonmitogenic anti-CD3 mAb treatment induced a general state of unresponsiveness or tolerance in PLP139–151-specific T cells as measured by proliferation, cytokine production, and DTH. The therapies also prevented the ensuing CNS inflammation that precedes onset of clinical disease during EAE. Thus, these findings are consistent with the possibility that NM-F(ab')2 and NM-IgG3 conferred protection against EAE progression via the induction of tolerance.

Importantly, the timing of nonmitogenic therapy in relation to EAE induction appears to be a critical determinant of the treatment’s potential efficacy and suggests that the therapy primarily targets previously activated T cells. Interestingly, nonmitogenic anti-CD3 mAb administration at the time of PLP139–151/CFA priming failed to influence the clinical disease course, whereas treatment at times following disease initiation corresponding to disease onset or peak clinical disease conferred significant protection from disease progression. Presumably, treatment administration at the time of immune challenge failed to confer protection against disease initiation due to the presence of costimulatory and other innate immune signals generated by Ag and adjuvant administration which is supported by previous findings that the level of costimulatory signals present at the time of nonmitogenic mAb treatment may influence the treatment efficacy (43). It is important

FIGURE 6. Nonmitogenic anti-CD3 mAb treatment increases PLP139–151-specific T cell retention in lymphoid organs. A, Nonmitogenic mAb treatment increases the level of apoptosis in CD4+ T cells. Naive SJL mice were primed with 50 μg of PLP139–151 on day 0 followed by either control, NM-F(ab')2, or NM-IgG3 treatment on days 6–10. Spleen and lymph node cells were harvested 2 days following the last treatment (day 12) to measure the levels of apoptosis as determined by caspase expression. Data are presented as the percentage of CD4+ T cells expressing the caspase profile of live, mid-apoptotic, late-apoptotic, and dead cells. B, Nonmitogenic anti-CD3 treatment increases the number of Ag-specific CD4+ T cells in the spleen and lymph nodes. Naive SJL mice received 5 × 106 PLP139–151-specific Thy 1.1+ CD4+ T cells 2 days before EAE induction by PLP139–151/CFA immunization and were then treated on days 6–10 with either isotype control, NM-F(ab')2, or NM-IgG3 mAb. Two days following the last treatment, spleens and lymph nodes were harvested for analysis of Thy1.1+CD4+ T cells. Data are representative of three separate experiments.
to note that mice receiving NM-F(ab')2 on days 6–10 postimmunization develop clinical disease symptoms ~40 days following disease initiation (data not shown); however, this is most likely due to the short half-life of the treatment, the emergence of new CD4+ T cell populations, and/or the Ag bolus that remains in the animals. Supporting this, no significant clinical disease is observed in treated animals when disease is initiated by the transfer of activated PLP139–151-specific CD4+ T cells regardless of whether NM-CD3 treatment is administered during either days 0–4 or 6–10 following disease initiation (Fig. 3D). In addition, NM-CD3 treatment of adoptive transfer-induced disease also resulted in lymphopenia, an accumulation of Ag-specific cells in the spleen and lymph nodes, and decreased levels of cytokine production.

These findings support the hypothesis that the structurally altered forms of anti-CD3 mAb used in this study provided a subthreshold activation signal to previously activated T cells in the absence of costimulatory signals, thus inducing a functional state of immune tolerance in both active and adoptive transfer models of EAE. However, our in vitro findings showing that providing exogenous costimulatory signals (anti-CD28 mAb) at the time of nonmitogenic Ab-induced activation failed to reverse the nonmitogenic phenotype of the treatments and that increasing concentrations of NM-F(ab')2 could prevent the Ag-induced activation and proliferation of naive PLP139–151-specific TCR transgenic T cells. Thus, the mechanism(s) by which the nonmitogenic Ab treatments confer protection from established autoimmune disease may not be simply the provision of “Signal 2” in the absence of “Signal 1”. Equally important was the observation that increasing the concentration of the cognate PLP139–151 peptide served to increase the minimal effective concentration of NM-F(ab')2, suggesting that the structurally altered Ab may simply act as a TCR complex antagonist interfering with the TCR:peptide-MHC II interaction and not acting to initiate intracellular signaling leading to an active functional state of immune tolerance. However, both NM-F(ab')2 and NM-IgG3 efficiently induced intracellular Ca2+ mobilization in CD4+ T cells with kinetics and at levels comparable to the native form of the anti-CD3 mAb which is in agreement with similar studies using the nonmitogenic HuOKT3γδ (Ala-Ala) form of anti-CD3 mAb (44). These findings suggest that the nonmitogenic anti-CD3 may indeed actively influence CD4+ T cell effector function by inducing inhibitory intracellular signaling pathways that cannot be reversed by concurrent costimulatory signals. Thus, it currently remains unclear as to whether apparent competition between Ag-induced TCR activation and nonmitogenic Ab blockade of T cell activation is the result of physical and/or biochemical competition for cellular resources.

An alternative mechanism for nonmitogenic anti-CD3-induced tolerance is the induction/activation of CD4+ Treg cells which display a mixed phenotype of naive and activated cell surface markers, e.g., CD4+CD25+CD62L+hhigh (35), and inhibit T cell proliferation in a TCR-dependent manner. We and others have previously reported the inhibitory capacity of Treg cells in various models of autoimmune disease (45–50) and have described a potential role for the functional suppressive activity of Treg cells in nonmitogenic anti-CD3-mediated disease protection (9, 10, 51). In the current study, we observed an approximate doubling of the CD4+CD25+CD62L+hhigh population in the lymph nodes of treated SJL recipients, suggesting a treatment-induced expansion and/or activation of the Treg cell population. In addition, Ca2+ mobilization experiments suggested that the nonmitogenic anti-CD3 mAb may activate Treg cells. Paradoxically, we found that both the depletion of Treg cells by in vivo anti-CD25 Ab treatment before disease induction and the administration of anti-TGF-β failed to reverse the potent ability of nonmitogenic anti-CD3 mAb to inhibit onset of clinical EAE in recipients treated from days 6–10 after PLP139–151 immunization. However, we found that Treg cells had recovered to levels normally seen in untreated SJL mice by day 10 post-EAE induction, the day of the final NM-F(ab')2 treatment. Thus, the question remains as to exact activation state and functional contribution of Treg cells to regulation of the effector function of myelin-specific T cells in SJL mice. It is possible that either the anti-CD25 Ab simply down-regulates the CD25 complex without depleting the Treg cell population or that self-Ag immunization followed by nonmitogenic anti-CD3 Ab therapy leads to a rapid functional recovery of this Treg cell population. In either case, we continue to investigate the mechanism(s) underlying nonmitogenic anti-CD3-induced protection from clinical EAE to determine whether immune tolerance is induced in the pathogenic effector T cells either via a direct and/or indirect mechanism.
The 2C11 anti-CD3 mAb (4) efficiently facilitates CD4+ T cell deletion via complement-mediated cell lysis contributing to the rationale for its early use to treat autoimmune disease (6–13). Conversely, NM-IgG3 and NM-F(ab’)2 both fail to fix complement and mediate cell lysis (data not shown), and nonmitogenic anti-CD3 mAb treatment increased both the level of CD4+ T cell apoptosis (Fig. 6A) and the number of Ag-specific CD4+ T cells (Fig. 6B) in the spleen and lymph nodes. This finding suggests the mechanism by which nonmitogenic anti-CD3 mAb treatment confers protection from established autoimmune disease may be due to the retention of activated CD4+ T cells within the lymphoid organs. In concert with this, PLP139–151-specific T cells rapidly transmigrate via complement-mediated cell lysis contributing to the pathogenesis of relapsing-remitting experimental autoimmune encephalomyelitis (EAE) in SJL mice correlates with inhibition of neuroantigen-specific cell-mediated immune responses. J. Neuroimmunol. 164:670.

Collectively, these studies indicate that nonmitogenic anti-CD3 is an effective therapy for ameliorating established R-EAE. In addition, the data indicates that the protective effect can be mediated in the apparent functional absence of CD4+CD25+ Treg cells and likely involves direct induction of T cell tolerance/ergy secondary to suboptimal TCR signaling (Fig. 5D) and/or alterations in trafficking of encephalitogenic T cells (Figs. 6 and 7).

Disclosures

The authors have no financial conflict of interest.

References


