Naive T Cells Are Resistant to Anergy Induction by Anti-CD3 Antibodies

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Naive T Cells Are Resistant to Anergy Induction by Anti-CD3 Antibodies

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Anti-CD3 mAbs are potent immunosuppressive agents used in clinical transplantation. It has been generally assumed that one of the anti-CD3 mAb-mediated tolerance mechanisms is through the induction of anti-CD3 mAb-mediated tolerance mechanisms in naive T cells. We demonstrate in this study that naive T cells stimulated by anti-CD3 mAbs in vivo and in vitro do not respond to the superantigen staphylococcal enterotoxin B or to soluble forms of anti-CD3 mAbs and APCs, but express increased reactivity to plastic-coated forms of the same anti-CD3 mAbs and to their nominal Ag/class II MHC, a finding that is difficult to rationalize with the concept of anergy. Phenotypic and detailed kinetic studies further suggest that a strong signal 1 delivered by anti-CD3 mAbs in the absence of costimulatory molecules does not lead to anergy, but rather induces naive T cells to change their mitogen responsiveness and acquire features of memory T cells. In marked contrast, Ag-experienced T cells are sensitive to anergy induction under the same experimental settings. Collectively, these studies demonstrate that exposure of naive T cells in vivo and in vitro to a strong TCR stimulus does not induce Ag unresponsiveness, indicating that sensitivity to negative signaling through TCR/CD3 triggering is developmentally regulated in CD4\(^+\) T cells.


Optimal T cell stimulation requires Ag recognition by the TCR (signal 1) and signals provided by the APC (collectively referred to as signal 2 or costimulatory signals) (1, 2). An important concept that emerged from studies performed mostly on cloned murine and human CD4\(^+\) cells is that provision of signal 1 in the absence of costimulation does not represent a neutral event, but rather is perceived by T cells as a negative stimulus, leading to a state of cell-autonomous unresponsiveness referred to as anergy (3, 4). As a consequence, negative signaling by Ag recognition in the absence of adequate accessory cell functions is thought to represent a powerful mechanism for inducing peripheral cell tolerance (5, 6). Central to this proposed mechanism of peripheral tolerance is the concept that naive CD4\(^+\) T cells (thymic emigrant cells that have not encountered Ag) are sensitive to anergy induction. Although work performed on clonal cell populations has clearly demonstrated the signaling dichotomy of the TCR (activation vs anergy induction) (7–9), the extent to which TCR signals induce T cell anergy in naive populations has been a matter of debate. Naive T cells were found to be resistant to anergy induction following exposure to peptide-MHC complexes in the absence of costimulation (10–12). These reports contrast with studies performed in both human and murine models in which T cell anergy was induced in vitro following triggering of naive T cells with anti-CD3 mAbs (13–15).

Anti-CD3 mAb is a potent immunosuppressive drug altering T cell functions in vivo that is widely used for the prevention of allograft rejection or the treatment of autoimmune diseases (16). The precise mechanism of T cell unresponsiveness induced by anti-CD3 mAbs remains poorly defined. Among others, it has been proposed that anti-CD3 mAbs induced T cell anergy in vivo by delivering a partial T cell activation signal (16–18).

Because the ability to induce T cell anergy in naive lymphocytes is of both theoretical and clinical importance, we wished to investigate the consequences of anti-CD3-mediated signaling on resting naive T cells, both in vivo and in vitro. We demonstrate in this work that naive T cells are not sensitive to anergy induction by anti-CD3 mAbs. Anti-CD3-treated cells do not respond to conventional in vitro polyclonal mitogens such as soluble anti-CD3 mAbs or bacterial superantigens, but respond to nominal Ag/MHC complexes. Thus, lack of response to noncognate classical T cell mitogens does not result from anergy induction, but rather reveals a change in mitogen responsiveness that is characteristic of memory-type cells. Collectively, these observations demonstrate that in contrast to Ag-experienced T cells, Ab-mediated TCR ligation in the absence of costimulation does not induce anergy in naive CD4\(^+\) T cells.

Materials and Methods

Medium and reagents

The medium in all experiments was RPMI 1640 supplemented with 5% FCS, penicillin, streptomycin, glutamine, nonessential amino acids, and 5 × 10\(^{-5}\) M 2-ME. The chicken OVA peptide (OVA\(_{223-250}\)) was synthesized by NeoSystem (Strasbourg, France). Superantigen staphylococcal enterotoxin B (SEB)\(^4\) was purchased from Toxin Technology (Sarasota, FL). Ionomycin and PMA were obtained from Sigma-Aldrich (St. Louis, MO). The hamster mAb 37.51 to murine CD28 (19) was kindly provided by J. Allison (University of California, Berkeley, CA).

\(^4\) Abbreviations used in this paper: SEB, superantigen staphylococcal enterotoxin B; SSC, side light scatter.
Cell lines and mice
Six- to 8-wk-old BALB/c mice (Harlan, Horst, The Netherlands) and DO11.10 mice (20) were maintained in a pathogen-free environment. The DO11.10 T-cell line was derived following three rounds of biweekly stimulations of T cells from the DO11.10 transgenic mouse by the OVA\textsubscript{223-239} peptide and APC, as described (21).

Cell purifications and in vitro treatments
CD4\textsuperscript{+} T cells were purified from naive animals by magnetic depletion of B cells, macrophages, dendritic cells, NK cells, granulocytes, and CD8\textsuperscript{+} T cells (clone ALJ-1, 10/\textmu m/l cultured in 24-well plates coated with anti-CD8 mAbs (clone H11003, 10/\textmu m/l) for 30 min at 4°C before centrifugation and separation over an autoMACS column (Miltenyi Biotec) using the DepleS program, according to the manufacturer’s recommendations. The percentage of CD4\textsuperscript{+} T cells obtained ranged between 95 and 98%, as estimated by flow cytometry in all experiments.

Feeder cells were obtained following complement-mediated T cell depletion using an anti-Thy-1 mAb (clone HO13.4). Thy-1\textsuperscript{−} cells (containing B cells, dendritic cells, and macrophages) were irradiated at 1200 rad (from a cobalt source) before use.

Purified CD4\textsuperscript{+} cells (1.5 × 10\textsuperscript{5} cells) were incubated for 2 days in complete medium in 24-well plates coated with anti-CD3 mAbs (clone 145-2C11, 10/\textmu m/l) and then rested for 2 days in medium alone. Viable cells were recovered by centrifugation on Lympholyte M solution (Cedarlane Laboratories, Hornby, Canada) and restimulated (0.5–2 × 10\textsuperscript{5} in 200 \mu l of 96-well plates) in the presence of serial dilutions of plate-bound anti-CD3 mAbs or in the presence of freshly isolated syngenic feeder cells and graded doses of anti-CD3 mAbs, SEB, or OVA\textsubscript{223-239} (22) and then rested for 2 days in medium alone. Viable cells were recovered by centrifugation on Lympholyte M solution (Cedarlane Laboratories, Hornby, Canada) and restimulated (0.5–2 × 10\textsuperscript{5} in 200 \mu l of 96-well plates) in the presence of serial dilutions of plate-bound anti-CD3 mAbs or in the presence of freshly isolated syngenic feeder cells and graded doses of anti-CD3 mAbs, SEB, or OVA\textsubscript{223-239}.

In vivo treatments
Naive animals were injected i.v. with 10 \mu g of anti-CD3 mAbs asite or control hamster mAbs (clone PARSI-19, generated in our laboratory) ascite. Both asite solutions were endotoxin free, as judged by the Limulus amoebocyte lysate assay. CD4\textsuperscript{+} T cells were purified and restimulated in vitro 72 h later, as described in the previous section.

Flow cytometry
Specific cell surface staining was performed using standard procedures and analyzed with a FACSort cytometer (BD Biosciences). Cells were labeled with CFSE, as described (26). Briefly, T cells were resuspended at 10\textsuperscript{6} cells per ml in serum-free medium in the presence of 10 mM CFSE and incubated for 20 min at 37°C. The reaction was stopped by adding 5 vol of ice-cold medium, and, after two washes, CFSE-labeled cells were used for in vitro cultures and FACScan analysis.

Calcium mobilization assays
T cells were loaded with the Fluo-3 calcium-sensitive dye, as described (27). Briefly, cells were washed three times in calcium/magnesium-free HBSS (Invitrogen Life Technologies, Carlsbad, CA) and incubated at 10\textsuperscript{6} cells per ml with F-127 pluronic acid (100 \mu g/ml) and 5 \mu M Fluo-3 for 30 min at 37°C. Cells were then washed twice in complete medium and stored (2 × 10\textsuperscript{6} cells) at 4°C in the dark before use.

For stimulation with soluble anti-CD3 mAbs, Fluo-3-loaded T cells (400 \mu l) were incubated with anti-CD3 mAbs (clone 7D6, 0.5 \mu g/ml) for 2 min at 37°C, cross-linked by secondary rabbit anti-mouse Abs, and analyzed for Fluo-3 emission at 525 nm with the CellQuest software (BD Biosciences), as previously described (25).

For stimulations with plastic-coated anti-CD3 mAbs, we devised a method for simultaneous measurement of cytoplasmic calcium for free cells and cells conjugated with anti-CD3-coated plastic beads based on a report of Hashemi et al. (28). Polystyrene 6.0-\mu m beads (Polysciences, Warrington, PA) were incubated overnight with 10 \mu g/ml anti-CD3 mAbs or PBS (4 × 10\textsuperscript{6} beads/ml) on a rotator at 4°C. Beads were then stored at 4°C in the presence of the Ab or PBS for extended periods of time, extensively washed to eliminate unbound Ab, and resuspended at 2 × 10\textsuperscript{7} beads/ml before being used in the experimental test.

Aliquots of Fluo-3-loaded T cells were warmed to 37°C 5 min before each experiment. Typically, 400 \mu l of cells (8 × 10\textsuperscript{5} cells) were mixed with 40 \mu l of beads (to yield 1:1 T cell-bead ratio), centrifuged at 1000 rpm for 30 s, then gently resuspended and incubated for 30 s at 37°C before being analyzed by flow cytometry. We used a forward light scatter/side light scatter (FSC/SSC) gating procedure to separate the three populations of free cells, free beads, and bead-cell conjugates (as shown in Fig. 8). The free beads were smaller than T cells and displayed a strong SSC signal, due to their opacity. When anti-CD3-coated beads were added to T cells, a third population appeared in the sample mixture that had the size of the T lymphocytes and the SSC characteristic of the beads. Our condition of mixing cells and beads typically yielded a maximum of ~20% of the total number of cells in the form of cell-bead conjugates. We chose to keep this number low to minimize the occurrence of the more complex situation of multiple cell-bead aggregates that are difficult to interpret and also clog the cytometer.

Fluo-3 emission was measured on a linear scale in the free-cell and cell-bead doublet gates. The fluorescent signal for Fluo-3 was calibrated by saturating Ca\textsuperscript{2+} with 150 \mu g/ml ionomycin to obtain the maximum signal (Fmax) and then adding Mn\textsuperscript{2+} (2 mM MnCl\textsubscript{2}) to obtain the minimum signal (Fmin). The intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) can be calculated from the Fluo-3 fluorescence intensity using the formula derived by Tsien et al. (29): [Ca\textsuperscript{2+}]\textsubscript{i} = K\textsubscript{d}/(F – Fmin)/Fmax – F), where K\textsubscript{d} = 400 nM for the Fluo-3 intracellular dye.

Results
Anti-CD3 mAbs do not induce naive T cell anergy in vivo
To evaluate the effect of in vivo anti-CD3 mAb therapy on T cell immunocompetence, equal numbers of purified CD4\textsuperscript{+} T cells isolated from control (isotype-matched, Ab-treated) and anti-CD3-treated mice were stimulated by graded doses of soluble anti-CD3 mAbs in the presence of syngenic, FcγR-bearing APC or by graded doses of plastic-absorbed anti-CD3 mAbs in the absence of accessory cells. As previously demonstrated by us and others (17, 25), anti-CD3 mAb administration in mice led to T cell unresponsiveness to a subsequent in vitro stimulation by soluble anti-CD3 and accessory cells (Fig. 1A). The same CD4\textsuperscript{+} T cell population retained, however, the ability to respond to plastic-coated anti-CD3 mAbs (Fig. 1B), indicating that these cells were fully capable of transmitting activation signals, leading to cell proliferation through the TCR/CD3 receptor. Both cell populations responded to pharmacological agents bypassing early receptor signaling (a combination of ionomycin and PMA), further illustrating their functional proliferative competence (Fig. 1C). To evaluate the ability of anti-CD3 mAbs to induce unresponsiveness to nominal Ag, OVA-specific, TCR transgenic DO11.10 mice were inoculated with control and anti-CD3 mAbs, as above. Purified CD4\textsuperscript{+} T cells from both experimental groups were subjected to in vitro restimulation in the presence of anti-CD3 mAbs or Ag/MHC complexes. As previously shown, CD4\textsuperscript{+} T cells isolated from anti-CD3-treated mice responded poorly to soluble anti-CD3 mAbs and APC (Fig. 1D). However, anti-CD3-treated T cells proliferated in response to the cognate OVA peptide Ag (Fig. 1E), indicating that anti-CD3 therapy in vivo did not down-regulate T cell responsiveness to Ag.

Repeated anti-CD3 mAb injections in mice have been shown to favor the development of Th2-like cells, a cell population displaying reduced sensitivity to classical anergy induction (30). However, in vitro restimulation of anti-CD3-treated CD4\textsuperscript{+} T cells by plastic-coated anti-CD3 mAbs or OVA peptide/MHC complexes led to sustained or increased production of both IFN-\gamma and IL-4 (Fig. 1, F and G), suggesting that the anti-CD3 Ab in vivo treatment did not induce a shift toward the Th2 phenotype.
Anti-CD3 mAbs induce Ag-experienced T cell depletion and anergy in vivo

To evaluate the effects of the anti-CD3 injection on Ag-experienced T cell responsiveness, we inoculated DO11.10 transgenic mice with the cognate OVA peptide Ag in adjuvant before treatment with control or anti-CD3 mAbs. As shown in Fig. 2A, immunization of transgenic mice with the OVA peptide Ag in adjuvant induced Ag-specific effector/memory T cell differentiation in vivo, as judged by reduced expression of the CD62L marker on a large fraction of the transgene-bearing T cells specifically (Fig. 2A, compare left and right quadrants). When anti-CD3 mAbs were injected in these mice, we reproducibly observed a loss of the CD62L<sup>low</sup> transgenic (KJ1.26<sup>+</sup>/H11001<sup>+</sup>) CD4<sup>+</sup> T cells, suggesting that anti-CD3 mAb treatment selectively depleted activated/memory T cells (Fig. 2, compare lower right quadrants). Typically, anti-CD3 treatment led to the loss of 50–90% of memory OVA-specific T cells, indicating that Ag-experienced T cells are extremely sensitive (compared with naive transgene-bearing T lymphocytes) to activation-induced cell death in vivo. This conclusion was further supported by transfer experiments in which BALB/c mice were reconstituted with naive or in vitro activated DO11.10 T cells before anti-CD3 treatment. Anti-CD3 mAb injection induced clonal expansion of transferred naive transgenic cells, while the same treatment caused massive depletion of the Ag-experienced cells (data not shown).

The responsiveness status of the remaining Ag-activated anti-CD3-treated splenocytes was assessed following Ag restimulation in vitro. In the experiment shown in Fig. 3, naive and OVA-immunized transgenic mice were treated with anti-CD3 mAbs or isotype control mAbs, and CD4<sup>+</sup> splenic T cells were tested for OVA peptide responsiveness. As shown above, naive T cells treated in primary treatment: none

![Response of in vivo anti-CD3 mAb-treated CD4<sup>+</sup> T lymphocytes](image1)

FIGURE 1. Responsiveness of in vivo anti-CD3 mAb-treated CD4<sup>+</sup> T lymphocytes. A–E, Proliferative response of CD4<sup>+</sup> T cells purified from BALB/c (A–C) or DO11.10 (D and E) mice inoculated 3 days before with control or anti-CD3 mAbs and restimulated in vitro (10<sup>5</sup> cells/well) with graded doses of: A and D, soluble anti-CD3 and T-depleted syngenic APC (2 x 10<sup>5</sup> cells/well); B, plastic-coated anti-CD3 mAbs; C, calcium ionophore and PMA (10 ng/ml); and E, OVA<sub>223-239</sub> and APC. Proliferation was evaluated after a 48-h culture, and results are expressed as cpm of [<sup>3</sup>H]thymidine incorporation. F and G, Cytokine secretion induced following restimulation of CD4<sup>+</sup> T cells from control and anti-CD3-treated DO11.10 mice with coated anti-CD3 (10 μg/ml) or OVA<sub>223-239</sub> (1 μg/ml) and APC. IFN-γ (F) and IL-4 (G) culture supernatant titers are estimated by ELISA. Results are representative of 3 (F and G) to at least 10 (A–C) individual experiments.

![Response of in vivo anti-CD3 mAb-treated CD4<sup>+</sup> T lymphocytes](image2)

FIGURE 2. Selective depletion of Ag-experienced T cells following anti-CD3 injection. DO11.10 transgenic mice were inoculated with OVA peptide and adjuvant. Five days later, one group of mice received control isotype (A), while the other was injected i.v. with anti-CD3 mAbs (B). Splenocytes were recovered on day 4 and triple stained with anti-CD4 FITC, KJ1.26 PE, and anti-CD62L-biotin/avidin-CyChrome Abs. Panels show CD62L vs KJ1.26<sup>+</sup> expression on CD4<sup>+</sup> gated splenocytes. Percentage of gated cells in each quadrant is indicated.

![Response of in vivo anti-CD3 mAb-treated CD4<sup>+</sup> T lymphocytes](image3)

FIGURE 3. Anergy induction in Ag-experienced T cells following anti-CD3 inoculation. DO11.10 mice were injected with saline (A) or OVA peptide (B). Five days later, one-half of the mice in each group were inoculated i.v. with anti-CD3 mAb or the control isotype mAb. Splenocytes were tested on day 4 after Ab injection for their responsiveness to the OVA peptide Ag in vitro. Results are expressed as mean ± SD of [<sup>3</sup>H]thymidine incorporation (two mice/group).
vivo with anti-CD3 mAbs displayed a vigorous response to OVA peptide in vitro (Fig. 3A). In contrast, anti-CD3 mAb treatment led to a reduced response to the same Ag in immunized animals (Fig. 3B), suggesting that sensitivity to anergy induction in this experimental model could be a feature of Ag-experienced CD4$^+$ T cells.

**Anti-CD3 mAbs do not induce naive T cell anergy in vitro**

To further determine whether TCR triggering in the absence of costimulation was able to induce T cell anergy in naive T cells, purified CD4$^+$ T cells from naive BALB/c or DO11.10 mice were cultured on anti-CD3 mAb-coated dishes, according to a protocol originally developed by Jenkins et al. (31). Naive CD4$^+$ T cells were exposed to anti-CD3 mAbs for 2 days, allowed to rest for an additional period of 2 days in fresh medium, and assayed for immunocompetence during secondary restimulations with a panel of mitogenic stimuli. Control cells were either cultured for 4 days in the presence of medium alone or freshly purified from naive syngenic mice, as detailed in *Materials and Methods*. This anergy protocol was first validated using a DO11.10 T cell line derived in vitro following repetitive rounds of antigenic stimulation. Preincubation of the DO11.10 line with plate-bound anti-CD3 mAbs led to a global hyporesponsiveness characterized by a defective proliferation to both anti-CD3 mAb triggering (in soluble and coated forms; Fig. 4, A and B) and antigenic stimulation (Fig. 4C). In marked contrast, the same protocol failed to induce classical T cell anergy in naive T cells of identical genetic background. In keeping with the in vivo model previously described, in vitro anti-CD3-treated CD4$^+$ T cells displayed a reduced response to soluble anti-CD3 mAbs (Fig. 5, A and B) and to the V$\beta$8-specific SEB superantigen (Fig. 5E). These cells, however, proliferated vigorously to plastic-bound anti-CD3 mAbs (note the shift response of pretreated vs control cells toward lower activation threshold in Fig. 5, C and D) and in response to the cognate OVA/MHC complexes (Fig. 5F). Moreover, restimulation of preactivated cells in the presence of OVA peptide or insolubilized anti-CD3 led to increased secretion of both Th1 (IL-2, IFN-γ) and Th2 (IL-4) cytokines (Fig. 5G–I).

**Anti-CD3-treated CD4$^+$ T cells display phenotypic and functional characteristics of memory T cells**

The distinct ability of anti-CD3-treated cells to respond to different forms of anti-CD3 mAbs was reminiscent of early work performed on memory T cells. Memory T cells differ from naive T cells by the functional characteristics of memory T cells...
expression of a different set of surface markers (32, 33), a rapid production of polarized cytokines, and a rapid initiation of expansion after antigenic restimulation (34). Of relevance to this study, mouse memory CD4⁺ T cells have also been shown to be hyporesponsive to several noncognate TCR stimuli, including bacterial superantigens (35) and APC-directed anti-CD3 mAbs (36), a pattern of responsiveness very similar to the anti-CD3 mAb-treated cells described in the present study. Based on these considerations, we hypothesized that anti-CD3 treatment led to the differentiation of naive T cells into memory-like cells. The phenotypical analysis shown in Fig. 6 is in agreement with this hypothesis. Indeed, when compared with naive control lymphocytes, anti-CD3-treated CD4⁺ T cells expressed equivalent levels of the CD69 early activation markers and reduced levels of CD45RB and CD62L, a phenotype often associated with Ag-experienced cells. As memory T cells respond rapidly to Ag stimulation, a kinetic analysis of the proliferative response to distinct stimuli was performed. As a positive control, a population of T cells previously exposed to a combination of anti-CD3 and anti-CD28 mAbs was included in this analysis. The response of naive T cells to all stimuli tested peaked at ~days 2–4 (Fig. 7, A–C). In agreement with published evidence, anti-CD3/CD28 mAb-treated cells displayed a typical effector cell profile, characterized by a fast response (peak responses between days 1 and 2, depending on the stimulus) and a higher efficacy (note the increased thymidine uptake when compared with naive T cells). Anti-CD3 mAb-treated cells presented a unique pattern of responsiveness. These cells proliferated with a typical secondary-type response (faster kinetics and increased thymidine uptake) to both the OVA subsites and the plate-bound anti-CD3 mAbs (Fig. 7, C and B, respectively). Surprisingly, the response of anti-CD3-treated cells to soluble anti-CD3 peaked early, but faded rapidly when compared with the response of both control and anti-CD3/CD28-stimulated cells (Fig. 7A). This early and transient [³H]thymidine incorporation suggested that anti-CD3-treated cells underwent an abortive cell proliferation. This observation was supported by the FACS analysis of cell divisions with the CFSE fluorescent dye, showing that anti-CD3-treated CD4⁺ T cells did not undergo extensive cellular division in response to stimulation with APC-bound anti-CD3 mAbs, when compared with naive T cells (Fig. 7D).

**Anti-CD3 treatment affects early TCR-signaling capacities**

The previous observations indicated that rather than inducing anergy, exposure of naive T cells to anti-CD3 mAbs led to a change in their TCR activation requirement. To further support this contention at the level of early TCR signaling, control and anti-CD3-treated cells were loaded with the Fluo-3 calcium detector dye, and their response to distinct forms of anti-CD3 mAbs was analyzed by flow cytometry (Fig. 8 and Table I). In particular, control and anti-CD3-treated cells were incubated with a plastic-coated form of anti-CD3 mAbs suitable for flow cytometry analysis (anti-CD3-coupled beads; see Materials and Methods for details). Preliminary studies showed that anti-CD3-treated cells proliferate strongly in response to anti-CD3 mAbs coated on the plastic beads (data not shown). A significant proportion of the CD4⁺ T cells associated in doublets with the anti-CD3-coated beads as shown in the forward light scatter/SSC dot plot in Fig. 8, A and B. There was no significant difference in the proportion of cell-bead doublets obtained in the two T cell populations (ranging between 15 and 24% in three different experiments), and no doublet formation was observed with uncoated beads (data not shown). The mean fluorescence emitted by the Fluo-3 indicator dye was then compared in the single cell and cell-bead doublet gates of both T cell populations (Fig. 8, C and D). Calcium mobilization only occurred in T cells bound to Ab-coated beads. In agreement with the previous observations, anti-CD3-treated cells displayed an enhanced calcium response to anti-CD3-coated beads. As a control, calcium influx

![Figure 6](image-url)  
**FIGURE 6.** Surface phenotype of naive and anti-CD3-treated CD4⁺ T cells. Control (upper panels) and in vitro anti-CD3-treated cells (lower panels) were stained with Abs to CD45RB, CD62L, CD69 (solid lines), or control isotypes (dotted lines).

![Figure 7](image-url)  
**FIGURE 7.** Kinetic responses of control and anti-CD3 mAb-treated CD4⁺ T cells. A–C, Control, plastic-coated anti-CD3- or plastic-coated anti-CD3 + anti-CD28-treated naive T cells were restimulated with: A, soluble anti-CD3 mAbs (30 ng/ml) and syngenic APC; B, plastic-coated anti-CD3 mAbs (3 μg/ml); or C, OVA 223–239 (300 ng/ml) + APC. Proliferation was measured at the indicated times by adding [³H]thymidine during the last 6 h of the culture. D and E, Control and anti-CD3-treated cells were loaded with CFSE and stimulated (2 × 10⁵ cells, in 96-well plate) for 4 days in the presence of APC and soluble anti-CD3 mAbs (30 ng/ml) (solid lines) or medium (dotted lines) and analyzed for their fluorescence content. Similar results were obtained in three independent experiments.
upon addition of ionomycin was equivalent in both cell populations (data not shown). We next compared the ability of naive and anti-CD3-treated cells to increase intracellular calcium levels in response to distinct forms of anti-CD3 mAbs. Cells were stimulated by anti-CD3-coated beads or by soluble anti-CD3 mAbs cross-linked by a secondary anti-Ig reagent. As shown in Table I, naive and anti-CD3-treated cells displayed distinct activation requirement. Naive T cells preferentially responded to soluble Ab forms, while anti-CD3-treated cells displayed enhanced responses to coated forms of the Ab. Thus, in agreement with the in vitro cell-based studies (cell proliferation and cytokine production), anti-CD3-treated cells are not unresponsive to TCR complex triggering, but rather display an altered activation profile when compared with naive cells.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Calcium response of control and anti-CD3-treated naive T cells to plastic bead-insolubilized anti-CD3 mAbs. A and B, Illustration of flow cytometric gating for cell population analysis. Lower box gates, single cell gates; upper box gates, cell/anti-CD3-coated bead doublet gates. C and D, CD4<sup>+</sup> T cells were incubated for 1 min with beads coated with anti-CD3 mAbs, as described in Materials and Methods. Dot plots represent the fluorescence emission of the Fluo-3-loaded T cells in the single cell and cell-bead doublet gates. Results are representative of three different experiments.

### Table 1. Calcium influx in control and anti-CD3-treated cells upon restimulation with soluble or coated forms of anti-CD3 mAbs

<table>
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<sup>a</sup>Fluo-3-loaded CD4<sup>+</sup> T cells were incubated with anti-CD3 mAbs for 2 min at 37°C, cross-linked by secondary rabbit anti-Ig Abs. Calcium concentration was calculated from the mean fluorescence at the peak level.

<sup>b</sup>Fluo-3-loaded CD4<sup>+</sup> T cells were incubated with anti-CD3-coated beads, and calcium concentration was calculated from the mean fluorescence of the cell-bead doublets.

<sup>c</sup>Results are expressed as mean calcium concentration (see Materials and Methods).

<sup>d</sup>nd, Not done.

### Discussion

The ability of the TCR to deliver negative signals is thought to represent an important mechanism for self/nonself discrimination in the periphery (2). Although it has been clearly established that signals delivered by the APC are required for optimal stimulation of naive T cells, the extent to which these cells are sensitive to anergy induction by TCR engagement in the absence of costimulation is still a matter of debate. A series of in vitro observations indicate that engagement of the TCR of a naive T cell by an Ag/MHC complex in the absence of costimulation represents a neutral event (11, 12). In particular, naive T cells are resistant to anergy induction by Ag pulsed on 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-fixed APC, a classical protocol leading to unresponsiveness of T cell clones (37). Similarly, exposure of naive T cells to ionomycin and nonmitogenic anti-CD3 mAbs failed to induce T cell unresponsiveness (38, 39), supporting the notion that these cells are insensitive to multiple forms of anergy induction. These observations are in marked contrast with studies performed with mitogenic anti-CD3 mAbs, demonstrating the sensitivity of naive T cells to this form of anergy induction (14, 15). These observations could be rationalized by assuming that naive T cells display a higher threshold for anergy induction, requiring a strong TCR signal in the absence of costimulation (such as provided by mitogenic anti-CD3 mAbs) to enter the anergic state.

These considerations led us to re-evaluate the functional consequences of TCR complex ligation by anti-CD3 mAbs in naive CD4<sup>+</sup> T cells both in vivo and in vitro. Using this approach, we demonstrate in this study that naive T cells are not sensitive to anergy induction by mitogenic anti-CD3 mAbs. The major observation from our study is that although anti-CD3-treated cells failed to respond to soluble anti-CD3 mAbs in the presence of costimulatory-bearing APCs, they retained the ability to proliferate and produce cytokines in response to nominal Ag (Figs. 1, 5, and 7). These data call for caution in extrapolation from the common perception that failure to respond to soluble anti-CD3 mAbs reflects T cell unresponsiveness. Several reports have indeed demonstrated that while naive T cells are optimally stimulated by soluble anti-CD3 mAbs, memory cells preferentially respond to insoluble forms of the same Ab (36, 40). Similarly, other widely used T cell mitogens (including bacterial superantigens or lectins) have been shown to preferentially stimulate naive T cells, further illustrating the differences in the integration of TCR signals between T cell subsets (35, 41). These observations suggest that responsiveness to anti-CD3 mAbs reflects cell differentiation rather than immunocompetence, and suggest that conventional mitogens (including lectins, bacterial superantigens, and anti-TCR complex Abs) are not suited for the evaluation of T cell competence in anergy-related studies. These observations suggest that some of the conflicting results previously reported on naive T cell anergy (see Refs. 14 and 15) may derive from inadequate methodology.

In contrast to studies performed with nominal Ag presented by costimulatory-deficient APCs (37), stimulation of naive T cells by anti-CD3 mAbs cannot be considered as a neutral event. In vivo or in vitro anti-CD3 triggering induced T cell blastogenesis (as shown by cell size increase) and proliferation (as judged by thymidine uptake and CFSE-labeling studies; data not shown). Moreover, both in vivo and in vitro anti-CD3-treated cells expressed reduced levels of CD45RB and CD62L molecules (Fig. 6, and data not shown), a phenotype characteristic of Ag-experienced cells. CD4<sup>+</sup> experienced T cells have been shown to represent a heterogeneous pool of lymphocytes composed of effector (recently activated) and long-lived memory T cells (42). Ahmadzadeh et al. (40) recently...
developed an activation profile assay, using anti-CD3 and antigenic stimuli, that clearly identified two subsets of Ag-experienced CD4⁺ T cells. Compared with naive T cells, both effector (CD62L⁻/low) and resting memory (CD62L⁺/high) T cells responded vigorously to their nominal Ag/MHC, but differed in their responsiveness to anti-CD3-mediated triggering. Effector cells proliferated vigorously to both plastic-coated and APC-bearing anti-CD3 mAbs, while resting memory T cells exhibited hypo-responsiveness to anti-CD3 mAbs insulobilized on FcR-bearing APC. According to this view, anti-CD3/CD28-treated cells presented a typical effector cell responsiveness (a higher efficacy with a faster kinetic to all TCR stimuli tested; Fig. 7), while T cells treated with plate-bound anti-CD3 mAbs alone displayed activation requirement often associated with a resting memory T cell phenotype (Figs. 5 and 7).

Although the mechanism responsible for the selective unresponsiveness of anti-CD3-treated cells to soluble forms of the same Ab was beyond the scope of this study, we demonstrate in this study that this change in activation requirement is probably due to a modification in the signaling machinery, as anti-CD3-treated cells were preferentially stimulated by coated forms of the Ab at an early signaling step, as emphasized by studies of intracellular calcium influx described in Table I. These studies are in keeping with observations indicating that memory and naive cells differ in their TCR-associated signaling responses (43). Nonresponsiveness of memory CD4⁺ T cells to soluble anti-CD3 mAb has been previously linked to inhibitory signals delivered by CD4 upon MHC class II interaction (36, 44, 45). This hypothesis is hard to reconcile with the observations reported in this study, as: 1) anti-CD3-treated cells displayed a reduced response to soluble anti-CD3 mAbs even when presented by APCs from MHC class II knockout mice (our unpublished observations), and 2) cells unresponsive to soluble anti-CD3 mAbs or SEB responded to Ag presented in the context of MHC class II molecules (Figs. 1, 5, and 7).

The biochemical mechanism responsible for the selective unresponsiveness of anti-CD3-treated T cells to bacterial superantigens has not been identified to date. In particular, based on the notion that superantigens and peptide agonists interact with distinct regions of both the TCR and the MHC molecules, it is tempting to speculate that the composition of the macromolecular complex recruited by these ligands at the immune synapse may be slightly different, a hypothesis that is presently under investigation.

Finally, cell-mixing studies failed to provide any support for a regulatory influence of anti-CD3-treated cells upon naive T cells, indicating that the altered responsiveness to anti-CD3 mAbs reflected a cell-autonomous event (data not shown).

Collectively, our observations indicate that anti-CD3 stimulation in the absence of costimulation does not induce Ag unresponsiveness in naive T cells, but favors the differentiation of naive T cells toward a memory-like phenotype.

The conclusion that naive T cells are resistant to several anergizing protocols in vitro is at variance with numerous observations suggesting that T cells can be rendered unresponsive in vivo. A recent proposal by Schwartz (4) may help resolve this apparent paradox. Accordingly, T cell unresponsiveness can be classified into two broad categories. T cell clonal anergy can be defined as a growth arrest state that develops following acute exposure of T cells to an inadequate stimulus (such as a TCR ligand in the absence of costimulatory signals). In most situations, this anergic state is long lasting and persists in the absence of further TCR engagement. Adaptive tolerance has been proposed to unfold in vivo following chronic exposure of naive T cells to a persisting Ag in the absence of inflammation. In this setting, naive T cells respond vigorously to the Ag, but gradually down-regulate their proliferative response and cytokine production capacity. This form of unresponsiveness apparently develops following a productive response (proliferation and cytokine secretion) and is readily reversible upon removal of the antigenic stimulus. The observation that the biochemical block in signal transduction appears to be different between these two forms of unresponsiveness (see Table I in Ref. 4) concurs with the concept that these two phenomenon are biologically distinct. This conclusion may have important functional consequences in clinical studies. Indeed, anergizing protocols based on acute exposure of naive T cells to a costimulatory-deficient TCR stimulus (such as anti-CD3 mAbs or Ag-loaded, costimulatory-deficient APCs) may prove inefficient in inducing long-lasting unresponsiveness in vivo. Rather, induction of adaptive tolerance may require previous activation of T cell in vivo (possibly in a costimulatory-dependent fashion), followed by continuous exposure to Ag. However, the functional status of these adapted cells in vivo is still unclear, as despite defective proliferative responses, these cells acquire and retain cytolytic potential (see Bandeira et al. (46) and Stamou et al. (47)), calling for caution in translating these protocols in a clinical setting.

In conclusion, the present study extends previous observations and strongly suggests that naive T cells (with some possible exceptions; see Ref. 48) are not sensitive to T cell clonal anergy, as defined above. Thus, the ultimate impact of the cell-autonomous, antiproliferative states (clonal anergy and/or adaptive tolerance) on peripheral tolerance is still unclear, and needs to be integrated with the emerging role of regulatory cells in containing the immune response to self constituents.

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References


