Title
Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell receptor signal and induce clonal anergy.

Permalink
https://escholarship.org/uc/item/7c06520q

Journal
The Journal of experimental medicine, 185(8)

ISSN
0022-1007

Authors
Smith, JA
Tso, JY
Clark, MR
et al.

Publication Date
1997-04-01

DOI
10.1084/jem.185.8.1413

Peer reviewed
Nonmitogenic Anti-CD3 Monoclonal Antibodies Deliver a Partial T Cell Receptor Signal and Induce Clonal Anergy

By Judith A. Smith, J. Yun Tso, Marcus R. Clark, Michael S. Cole, and Jeffrey A. Bluestone

From the Ben May Institute for Cancer Research, Committee on Immunology, Department of Pathology; Department of Medicine, University of Chicago, Illinois 60637; and Protein Design Laboratories, Mountain View, California 94043

Summary

Anti-CD3 monoclonal antibodies (mAbs) are potent immunosuppressive agents used in clinical transplantation. However, the activation-related adverse side effects associated with these mAbs have prompted the development of less toxic nonmitogenic anti-CD3 mAb therapies. At present, the functional and biochemical consequences of T cell exposure to nonmitogenic anti-CD3 is unclear. In this study, we have examined the early signaling events triggered by a nonmitogenic anti-CD3 mAb. Like the mitogenic anti-CD3 mAb, nonmitogenic anti-CD3 triggered changes in the T cell receptor (TCR) complex, including \( \zeta \) chain tyrosine phosphorylation and ZAP-70 association. However, unlike the mitogenic anti-CD3 stimulation, nonmitogenic anti-CD3 was ineffective at inducing the highly phosphorylated form of \( \zeta \) (p23) and tyrosine phosphorylation of the associated ZAP-70 tyrosine kinase. This proximal signaling deficiency correlated with minimal phospholipase C\( \gamma_1 \) phosphorylation and failure to mobilize detectable Ca\( ^{2+} \). Not only did biochemical signals delivered by nonmitogenic anti-CD3 resemble altered peptide ligand signaling, but exposure of Th1 clones to nonmitogenic anti-CD3 also resulted in functional anergy. Finally, a bispecific anti-CD3–anti-CD4 F(ab\(^9\))\(^2\) reconstituted early signal transduction events and induced proliferation, suggesting that defective association of lck with the TCR complex may underlie the observed signaling differences between the mitogenic and nonmitogenic anti-CD3.

OKT3, a murine antibody specific for the CD3e chain of the TCR complex, has been used clinically for over a decade in the treatment of steroid-resistant graft rejection (1). However, use of this antibody has been hampered by a toxic first dose reaction syndrome shown to be related to the initial T cell activation events and ensuing release of cytokines before the suppression of T cell responses (2, 3). Others have demonstrated that the mitogenic activity of OKT3 and other anti-CD3 mAbs depends upon extensive TCR–CD3 cross-linking via binding to FcR-positive cells (4). Therefore, recent efforts have been devoted to developing nonmitogenic forms of anti-CD3 by altering binding to Fc receptor. As a model system, an anti-murine CD3 mAb, 145-2C11, was genetically altered to eliminate FcR binding: its variable region gene was fused to a murine IgG3 Fc region, a mouse isotype with low affinity for murine FcR. This chimeric anti-CD3–IgG3 antibody has been shown to be nonmitogenic in vitro, and did not result in the serum cytokine elevation observed with the whole 145-2C11 mAb in vivo (5). However, the administration of nonmitogenic anti-CD3 mAbs was equally effective in prolonging graft survival as the parental 145-2C11 antibody (5). As similar non-FcR-binding mAbs derived from OKT3 are being tested clinically, it is important to gain further understanding of the mechanism(s) by which these nonmitogenic mAbs suppress T cell responses.

The mechanism of immunosuppression by anti-CD3 mAbs is complex. Mitogenic anti-CD3 mAbs modulate the TCR, induce apoptosis, and induce generalized long-term T cell unresponsiveness (6). Similarly, treatment of mice with the nonmitogenic anti-CD3 results in internalization of the TCR complex and depletion of T cells from the circulation and peripheral lymphoid organs. However, in contrast with the mitogenic antibodies, anti-CD3–IgG3 does not appear to induce global T cell unresponsiveness (5). Thus, the different anti-CD3 mAbs may suppress T cell responses by distinct mechanisms.

Currently, it is thought that TCR signaling results from...
a cascade of events requiring the recruitment and activation of nonreceptor tyrosine kinases. One of the earliest consequences of TCR engagement by mAb or peptide-MHC is the tyrosine phosphorylation of components of the TCR complex (8). The ζ chain of the TCR complex contains three immunoreceptor tyrosine-based activation motifs (D/EXXYXXL(X)_{2-5}YXXL) that become variably phosphorylated after TCR–CD3 ligation (9). It is thought that the activation-induced 21- and 23-kD phosphorylated bands evident on one-dimensional SDS-PAGE represent differentially phosphorylated forms of ζ (10). The other CD3 chains, γ, δ, and ε, containing one immunoreceptor tyrosine-based activation motif each, become tyrosine phosphorylated as well (8). It has been hypothesized that the src family kinases, lck or fyn, may be responsible for these early phosphorylation events (9). Within minutes, additional tyrosine phosphorylated proteins, including the ZAP-70 kinase, associate with the TCR–CD3 complex (11). These proximal events lead to a series of biochemical signals that activate downstream substrates in the PI-3 kinase, Ras, and phospholipase Cγ-1 (PLCγ-1) pathways, ultimately leading to activation of the T cell (9).

Until recently, it was thought that this cascade of events was always fully engaged after exposure to peptide-MHC ligand or mAbs and that different responses to stimuli reflected a quantitative addition of the number of receptors engaged. However, antigenic peptide analogues, designated as altered peptide ligands (APL), have illustrated that the TCR is not an on-off switch. Rather, stimulation with APL can result in qualitative differences in the early signals transduced through the TCR. Specifically, stimulation with APL results in a characteristic biochemical pattern involving partial ζ phosphorylation and ZAP-70 association in the absence of phosphorylation, ultimately leading to a lack of inositol-triphosphate (IP_3) turnover (10, 12, 13). The delivery of such a partial signal effectively shuts down T cell clones, resulting in the induction of unresponsiveness as manifested by an inability of the anergized T cell clones to produce IL-2 when rechallenged under optimal conditions.

In the present study, we examined the effect of nonmitogenic anti-CD3 mAbs on early signaling events in T cell clones. The anti-CD3–IgG3 chimeric antibody transduced a signal characterized by a altered pattern of ζ chain and ZAP-70 tyrosine phosphorylation, leading to defects in downstream signaling events. Nonmitogenic anti-CD3 also failed to induce higher order aggregation of the TCR into a cap. Similar signaling changes were observed in naive T cells at the level of TCR complex and PLCγ-1 tyrosine phosphorylation. Specific defects in early signal transduction and mitogenicity were restored by coaggregating CD4 (and thus presumably lck) with the TCR–CD3 complex. The nonmitogenic anti-CD3 induced T cell unresponsiveness in Th1 clones but not naive T cells. Together, these results suggest that administration of nonmitogenic anti-CD3 mAbs, especially in recipients undergoing acute transplant rejection, may result in the delivery of a partial T cell signal that renders activated T cells unresponsive.

Materials and Methods

Animals. 6- to 8-wk-old BALB/c, DBA/2, and B10.A mice were purchased from Frederick Cancer Research Institute Laboratories (Frederick, MD). DO 11.10 mice, transgenic for an OVA peptide (323–339)-specific, I-A^d-restricted αβ-TCR, were a gift of Drs. D. Loh and K. Murphy (Washington University, St. Louis, MO) (14). All mice were bred and maintained in a specific pathogen-free facility at the University of Chicago.

Cell Lines, Antibodies, and Reagents. The chicken OVA-specific Th1 clone pGL10 was provided by Dr. F. Fitch (University of Chicago, IL) and the pigeon cytochrome c (PCC)-specific Th1 clone, AE.7, was provided by Dr. M. Jenkins (University of Minnesota, Minneapolis, MN). OVA and PCC were purchased from Sigma Chem. Co. (St. Louis, MO). The following mAbs were used in this study: 145-2C11 (anti-CD3), AT 83A (anti-Thy-1) (provided by Dr. F. Fitch, University of Chicago, Chicago, IL); anti-CD3–IgG3 (5, 7); the anti-Ig antisera: goat anti–mouse IgG3 (Zymed, San Francisco, CA), rabbit anti–hamster IgG3 (Zymed, San Francisco, CA), rabbit anti-hamster (Cappel, Durham, NC); 145-2C11–FITC (Boehringer Mannheim, Indianapolis, Indiana); PV-1 (anti-CD28) (Dr. C. June, Naval Medical Research Institute, MD); H146 (anti-ε mAb containing supernatant) (Dr. F. Fitch); 4G10 (anti-phosphotyrosine) and anti-PLCγ-1 (mixed mAbs) (UBI, Lake Placid, NY); 12-222 (anti-ZAP-70 antisera) (Dr. A. Weiss, University of California, San Francisco, CA). Cyclosporine A was purchased from Sandoz (Basel, Switzerland). Indo-1 was purchased from Molecular Probes (Eugene, OR).

A anti-CD3–Fos × A anti-CD4–Jun Bispecific F(ab’-2)ipper Production. The anti-CD3 antibody was derived from hamster anti-mouse CD3 hybridoma 145-2C11 (15), and the anti-CD4 antibody from rat anti-mouse CD4 hybridoma GK1.5 (16). The VH and VL sequences for 145-2C11 (5), (GenBank accession no. U17870 and U17871) and GK1.5 (16, 16a) had been determined. Homodimers of anti-CD3–Fos and anti-CD4–Jun F(ab’-2)ippers were expressed by the genetic method described by Kostalny et al., (17). Anti-CD3–Fos and anti-CD4–Jun were individually purified from transfected Sp2/0 spent media by protein G-Sepharose affinity chromatography (18). The two homodimers were then reduced and reoxidized to form bispecific Fab’-2’ipper, as described (17). Bispecific Fab’-2’ipper was further purified by BAKER BOND Adbx column chromatography or hydrophobic interaction chromatography on a Bio-Gel® Pheny1-5 PW column.

Proliferation Assays. pGL10 and AE.7 T cell clones were maintained as previously described (19, 20). T cell clones were purified by Ficoll-Hypaque density centrifugation before use in all experiments. BALB/c spleens were lysed in hypotonic ACK buffer to remove erythrocytes. Proliferation and unresponsiveness assays were in 5 or 10% FCS supplemented DMEM. In a 96-well flat-bottomed plate, 2 × 10^4 splenocytes or 1 × 10^6 pGL10 T cells were incubated on ice for 10 min with anti-CD3 (final concentration of 1 μg/ml), and then for another 10 min on ice with the appropriate cross-linker (rabbit anti-IgG3 at 1:30 or goat anti-IgG3 at 1:100) or goat anti-hamster at 1:300, before being placed at 37°C. For anti-CD3–Fos homodimer or anti-CD3 × anti-CD4 heterodimer proliferation assays, antibodies were serially diluted in 96-well flat-bottomed plates, starting at 10 μg/ml. Assays were pulsed with 1 μCi/well of [3H]thymidine for the last 8 h of a 48-h incubation, harvested on a Filtermate 196 96-well plate harvester.

Abbreviations used in this paper: APL, altered peptide ligands; IP_3, inositol-triphosphate; PCC, pigeon cytochrome c; PLCγ-1, phospholipase Cγ-1.
For induction of unresponsiveness, 24-well plates were pre-blocked with 10% FCS-supplemented DMEM overnight to prevent soluble anti-CD3-IgG3 from adhering to the plastic. DO 11.10 lymph node cells (2 x 10^6) or pGL10 clones (1 x 10^6) were incubated 24 h in 1 ml media with or without 1-10 μg/ml soluble anti-CD3-IgG3, CsA (1 μg/ml), anti-CD28 (1 μg/ml), 2-3 x 10^10 T-depleted irradiated BALB/c splenocytes. Cells were then washed three times, and rested 72 h at 37°C. TCR reexpression was verified via FACS® analysis. For the secondary stimulation, 4-5 x 10^4 DO 11.10 lymph node cells or pGL10 cells were plated in the presence of 2-5 x 10^5 T-depleted (anti-Thy-1 plus complement) irradiated splenocytes and 10 μg/ml soluble 145-2C11 or 800 μg/ml OVA. Cultures were pulsed with [3H]thymidine after 48 h. For IL-2 production, 2.5 x 10^6 cells per well were stimulated in a 96-well flat-bottomed plate with immobilized anti-CD3 plus anti-CD28 at 1 μg/ml. 24 h supernatants from 3 wells were pooled and analyzed by ELISA (Endogen, Cambridge, MA). For AE.7 assays, 1 x 10^6 T cells per well were incubated for 24 h with 1 μg/ml of anti-CD3-IgG3 mAb, washed, resuspended, and then in a 96-well flat bottomed plate, 4 x 10^4 T cells were restimulated in the presence of 5 x 10^5 T-depleted irradiated B10.A splenocytes and 10 μM PCC.

Biochemistry. T cell clones or BALB/c lymph node cells were washed in PBS and then resuspended in ice-cold PBS at 1 x 10^6/ml or 2 x 10^6/ml, respectively. Anti-CD3-IgG3 was added at 4-5 μg/ml for a 10-min incubation on ice. An equal volume of anti-lg cross-linker or PBS prewarmed to 37°C was added and cells were incubated a further 2.5-5 min in a 37°C water bath. For anti-CD3-Fos homodimer and anti-CD3 x anti-CD4 heterodimer stimulations, cells were stimulated with 10 μg/ml of antibody. After the incubation, an equal volume of ice-cold 2x lysis buffer was added (final concentration: 0.5% Triton X-100, 50 mM Tris [pH 7.6], 100 mM NaCl, 5 mM EDTA, 1 mM NaVO_4, 10 μg/ml each leupeptin and aprotonin, 25 μM NPPG, and 1 mM PM SF). For immunoprecipitations, 20 μl of a 50% protein A-agarose bead slurry (Pharmacia-Upjohn, Uppsala, Sweden) were coated with 200 μl of mAb-containing supernatant or 2x of antisera for 1 h at 4°C. Lysates were added to the precoated protein A-agarose beads and incubated 1 h at 4°C. The samples were resolved on a 12% SDSPolyacrylamide gel for γ immunoprecipitations or an 8% gel for PLCγ-1, and then transferred to PVDF membrane (Millipore, Bedford, MA). Blots were blocked with 10% BSA (Sigma, St. Louis, MO). After incubation with primary antibody and the appropriate horseradish-peroxidase-coupled secondary antibody, blots were developed by Enhanced Chemilumi-}

**Results**

The anti-CD3-IgG3 mAb is nonmitogenic due to insufficient cross-linking of the TCR. Unlike the original 145-2C11 mAb, the anti-CD3-IgG3 chimeric antibody does not induce proliferation or IL-2 production in whole spleen cells (5). Also, soluble 145-2C11 failed to induce proliferation of T cell clones in the absence of FcR-mediated cross-linking (Bluestone, J.A. and J.A. Smith, unpublished observations; data not shown). To test directly the role of multivalent cross-linking, a secondary IgG3-specific cross-linking antibody was added to cultures containing the anti-CD3-IgG3 mAb. The addition of the cross-linking reagent reconstituted a mitogenic stimulus for both fresh murine splenocytes and a T cell clone (Fig. 1). Thus, the induction of proliferation by anti-CD3 requires a higher order of TCR aggregation that cannot be achieved by bivalent Ab binding alone.

Nonmitogenic Anti-CD3 Render T Cell Clones Hyporesponsive. Although insufficient for induction of T cell proliferation or cytokine production, the anti-CD3-IgG3

**Figure 1.** Non-FcR-binding anti-CD3 induces proliferation only in the presence of cross-linking anti-Ig antibody. Whole spleen (top) or pGL10 clone cells (bottom) were cultured with an anti-CD3-IgG3 chimeric antibody and a secondary rabbit anti-mouse IgG3 Ab mAb for 48 h. Results are expressed as the mean of triplicate determinations and are representative of four independent experiments.
mAb may deliver at least a partial signal, which alters T cell function. Therefore, the effects of anti-CD3–IgG3 on the functional responses of naive cells and T cell clones were examined. pGL10 T cells or DO 11.10 lymph node cells were incubated in the presence of splenic accessory cells (to compensate for the presence of non-T cells in the naive population) and nonmitogenic anti-CD3–IgG3. Previous studies have shown that treatment of T cells with anti-CD3–IgG3 resulted in downmodulation of TCR expression within 24 h (7). Therefore, after 24 h, the cells were washed and recultured for 3 d to allow reexpression of the TCR (data not shown). As seen in Fig. 2 A, upon restimulation with the mitogenic 145-2C11 mAb plus splenic APCs, thymidine incorporation by anti-CD3–IgG3-treated pGL10 was markedly reduced as compared with pGL10 cultured with media alone. In contrast, the functional responses of murine lymph node T cells were not affected by culture with anti-CD3–IgG3. The clonal unresponsiveness did not merely reflect decreased viability, because anti-CD3–IgG3-treated clones proliferated at the same rate in the presence of exogenously added IL-2 (data not shown). The effect of anti-CD3–IgG3 was not specific to the pGL10 clone, because the nonmitogenic anti-CD3 rendered the PCC-specific clone, AE.7, hyporesponsive as well (Fig. 2 B). To determine whether the reduced proliferation of anti-CD3–IgG3-treated T cell clones correlated with IL-2 production, pGL10 clones were cultured with or without anti-CD3–IgG3 for 24 h, rested, and then restimulated with immobilized anti-CD3 plus anti-CD28. Culture supernatants were analyzed by IL-2 ELISA. (C) pGL10 cells were incubated with nonmitogenic anti-CD3 in the presence of cyclosporine A, splenic APC, and anti-CD28 as indicated. 72 h after the primary culture, cells were restimulated with OVA antigen and APC. A, C, and D are representative of two separate experiments, and B is representative of four experiments. To examine whether the presence of CsA or CD28 co-stimulation would affect the nonmitogenic anti-CD3-induced unresponsiveness, pGL10 T cells were cultured with nonmitogenic anti-CD3 alone, or nonmitogenic anti-CD3 in the presence of CsA, or splenic APCs and anti-CD28 (Fig. 2 D). CsA partially blocked the induction of unresponsiveness by nonmitogenic anti-CD3, suggesting that this process may depend upon a calcium signal. In contrast, addition

Figure 2. T cell clones, but not lymph node T cells, are hyporesponsive after exposure to nonmitogenic anti-CD3. (A) DO 11.10 lymph node cells or pGL10 cells were incubated with either media alone or anti-CD3–IgG3 in the presence of irradiated T-depleted spleen cells for 24 h, washed, and rested for 72 h. The T cells were restimulated with mitogenic anti-CD3 (145-2C11) and fresh APC. (B) AE.7 clone cells were incubated with or without nonmitogenic anti-CD3, washed, and rested as above, and restimulated with the antigen PCC plus fresh APC. (C) pGL10 cells were incubated with or without nonmitogenic anti-CD3. After the 72-h rest, the pGL10 were restimulated with immobilized anti-CD3 plus anti-CD28. Culture supernatants were analyzed by IL-2 ELISA. (D) pGL10 cells were incubated with nonmitogenic anti-CD3 in the presence of cyclosporine A, splenic APC, and anti-CD28 as indicated. 72 h after the primary culture, cells were restimulated with OVA antigen and APC. A, C, and D are representative of two separate experiments, and B is representative of four experiments.
of anti-CD28 mAb in the primary culture failed to restore secondary responses.

Nonmitogenic Anti-CD3 Delivers a Partial TCR Signal. The functional consequences of culture with nonmitogenic anti-CD3 support the hypothesis that anti-CD3-IgG3 delivers a signal. Therefore, experiments were performed to determine the nature of the TCR signal triggered by nonmitogenic anti-CD3. Upon ligation of the TCR, one of the earliest events to occur is the tyrosine phosphorylation of components of the TCR complex (ζ and CD3ε, CD3δ, and CD3γ) (8). Phosphorylation of these chains allows subsequent association and phosphorylation of a variety of other proteins, including the protein tyrosine kinase, ZAP-70 (9). In the present study, the ability of nonmitogenic anti-CD3 to trigger these crucial proximal events in T cell clones was examined. T cells were stimulated with the anti-CD3-IgG3 mAb in the presence or absence of a secondary Ig cross-linker. The TCR complex was immunoprecipitated with anti-ζ and analyzed for tyrosine phosphorylation (Fig. 3 A). Stimulating T cells with anti-CD3 under cross-linking conditions induced both 21- and 23-kD forms of phosphorylated ζ (p21 and p23) as well as phosphorylation of CD3ε. The phosphorylated band below p21 (~18 kD) most likely represents another isoform of phosphorylated ζ (21). In contrast, the noncross-linked anti-CD3-IgG3 mAb induced similar levels of phosphorylated CD3ε and p21 ζ, but significantly less p23 ζ. Quantitation of the p21 and p23 bands by densitometry in multiple T cell clone experiments (n = 4) revealed a consistent correlation between the degree of anti-CD3 cross-linking and the p23/p21 ratio; conditions that promote cross-linking increased the relative level of p23 expression (Fig. 3 D).

Examination of the phosphoproteins that coprecipitated with the ζ chain in darker exposures or greater cell number revealed further differences between anti-Ig cross-linked and noncross-linked conditions (Fig. 3 B). Unlike the cross-linked anti-CD3 stimulation, several of these phosphoproteins (bands between 30 and 46 kD as well as at 70 and 76 kD) were missing or reduced in the anti-ζ precipitations from T cells stimulated with the noncross-linked anti-CD3 mAb. The proximal signals triggered by nonmitogenic anti-CD3 in lymph node T cells were similar to those induced in clones in that (a) nonmitogenic anti-CD3 induces phosphorylation of TCR chains and (b) in the absence of cross-linking, several TCR-associated phosphotyrosine containing proteins are missing or reduced in intensity (Fig. 3 C).

Thus, in both T cell clones and naïve T cells, the anti-CD3-IgG3-induced TCR activation complex (with asso-
associated signaling molecules) contained fewer tyrosine phosphorylated molecules than a mitogenically stimulated TCR complex. These results suggest that although nonmitogenic anti-CD3 induces some tyrosine phosphorylation of ζ, and the CD3 chains, it is deficient in triggering other proximal signaling events.

Previous studies have shown that the 70-kD band observed in anti-ζ precipitates represents the TCR-associated tyrosine kinase, ZAP-70 (22). The reduced intensity of this band in the immunoprecipitates from nonmitogenic anti-CD3-treated cells could either represent a failure of ZAP-70 association or deficient phosphorylation. To address this issue, T cells were stimulated with cross-linked or noncross-linked anti-CD3, and the lysates were immunoprecipitated with anti-ζ. The Western blot was probed with an anti-ZAP-70 antisera. As seen in Fig. 4, the noncross-linked anti-CD3–IgG3 induced similar levels of ZAP-70 recruitment to the TCR complex. These results demonstrate that the downstream signaling events of PLC-γ are dependent upon extensive cross-linking of the TCR–CD3 complex, but it is not efficiently phosphorylated.

Defects in Downstream Events in the Absence of TCR–CD3

The differences in proximal signal transduction observed in the absence of cross-linking were likely to be reflected in critical downstream biochemical events, such as the tyrosine phosphorylation (and thus activation) of PLC-γ-1. To examine this event, pGL10 were stimulated with anti-CD3–IgG3 in the presence or absence of cross-linker (Fig. 5 A). The dramatic increase in PLC-γ-1 phosphorylation observed in the presence of a secondary cross-linking Ab was not observed following anti-CD3–IgG3 stimulation alone. Similarly, cross-linking with anti-IgG enhanced PLC-γ-1 tyrosine phosphorylation induced by the nonmitogenic anti-CD3 in naive cells (Fig. 5 B).

Because anti-CD3–IgG3 was unable to induce significant PLC-γ-1 phosphorylation, it was anticipated that one of the events that depends upon PLC-γ-1 activation, Ca²⁺ mobilization, would likewise be impaired. T cell clones were loaded with the calcium-sensitive dye indo-1 and then analyzed by FACStar Plus® for calcium flux. A calcium flux was observed within 1 min (Fig. 5 C). Anti-IgG Abs in the absence of anti-CD3 did not result in a calcium flux (data not shown). These results demonstrate that the downstream signaling events of PLC-γ-1 activation and the ensuing Ca²⁺ flux are dependent upon extensive cross-linking of the TCR–CD3 by anti-CD3 mAbs.

Aggregation of the TCR complex has been shown to correlate with T cell activation; Kupfer et al. (23) demonstrated that when T cells encounter antigen–MHC on APCs, the TCR redistributes on the cell surface to form an aggregated activation cap. This redistribution is a signaling-dependent process most likely involving reorganization of the cytoskeleton (24, 25). To test whether the addition of a cross-linking Ab to anti-CD3 results in an aggregated TCR cap, confocal microscopy was performed on pGL10 and purified DO11.10 T cells incubated with anti-CD3 (2C11–FITC) under cross-linking versus noncross-linking conditions (Fig. 6). In the presence of cross-linking Ab, anti-

Figure 4. Stimulation with noncross-linked anti-CD3 is sufficient for TCR association, but not phosphorylation of ZAP-70. 2 × 10⁷ pGL10 T cells were stimulated with PBS, goat anti-IgG3 alone, anti-CD3–IgG3, or anti-CD3–IgG3 plus anti-IgG, for 2.5 min at 37°C, lysed, and immunoprecipitated with anti-ζ. Blots were probed with anti-ZAP-70 (bottom), and then stripped and reprobed with anti-phosphotyrosine (top).

Figure 5. Impaired PLC-γ-1 activation and Ca²⁺ flux in the absence of anti-CD3 cross-linking. (A) 2 × 10⁷ pGL10 cells were stimulated for 5 min at 37°C as indicated. Samples were precipitated with anti-PLC-γ-1, and then resolved on an 8% gel. The Western blot was probed with anti-phosphotyrosine (top), stripped, and then reprobed with anti-PLC-γ-1 (bottom). (B) 4 × 10⁷ lymph node cells were stimulated for 5 min with PBS, anti-CD3–IgG3, or anti-CD3–IgG3 plus goat anti-IgG3 and analyzed as in A. Densitometry performed on this experiment showed a fourfold increase in PLC-γ-1 phosphorylation (with compensation for protein amount) upon anti-CD3 cross-linking. (C) T cell clones were loaded with the calcium-sensitive dye indo-1, stimulated with anti-CD3–IgG3 (left), or anti-CD3–IgG3 followed by rabbit anti-IgG3 (right). Cells were analyzed on a FACStar Plus® for calcium flux. The rise in relative intracellular calcium concentration is indicated by an increase in the 405/495 nm emission ratio. (A–C) Data is representative of two separate experiments.
CD3 stimulation induces aggregation of the TCR into a cap on one side of the cell. However, in the absence of cross-linker, the anti-CD3 remained diffusely distributed on the cell surface. Thus, the signal delivered by nonmitogenic anti-CD3 appeared insufficient for the redistribution of TCRs into an aggregated cap.

Recruitment of CD4–lck into the Complex Reconstitutes Complete Proximal Signal Transduction and Mitogenicity. The inability of nonmitogenic anti-CD3 to trigger specific downstream events and proliferation most likely stems from the defective proximal events observed involving \( \zeta \) and ZAP-70. Previous studies have suggested that the src family kinase, lck, plays a crucial role in the phosphorylation of \( \zeta \), which subsequently allows association and phosphorylation of ZAP-70 (26, 27). Thus, it was possible that the differences in \( \zeta \) and ZAP-70 phosphorylation seen upon the addition of cross-linker to anti-CD3 may have reflected increased lck activation or enhanced recruitment to the TCR. Initial experiments examining lck activation by monitoring lck tyrosine phosphorylation revealed no differences between cross-linking and noncross-linking conditions. It is clear that CD4 associates with lck and can interact with the TCR complex in-diculby upon TCR ligation of antigen-M HC. Thus, artificially bringing CD4–lck into the TCR complex might reconstitute a mitogenic anti-CD3 stimulus, even in the absence of a secondary cross-linking Ab. To test this hypothesis, we took advantage of a bispecific anti-CD3 × anti-CD4 reagent prepared by a molecular approach to insure the presence of monovalent arms specific for CD3 and CD4 (as described in Materials and Methods). T cells were incubated with anti-CD3–Fos homodimer or the anti-CD3 × anti-CD4 bispecific F(ab\(^\prime\))\(_2\), lysed, and the TCR–CD3 complex was then immunoprecipitated and analyzed. The bispecific construct induced significant p23 \( \zeta \), ZAP-70 phosphorylation, as well as association of the phosphoproteins between 30–46 kD (Fig. 7 A), even in the absence of a secondary cross-linking antibody. In contrast, the overall pattern induced by anti-CD3–Fos resembled the results seen in T cells stimulated with the anti-CD3–IgG3 mAb: specifically, a reduced association of phosphoproteins and barely detectable ZAP-70 phosphorylation. In the lysates of T cells stimulated with the bispecific anti-CD3 × anti-CD4 construct, a large tyrosine-phosphorylated protein was observed that migrated just above the heavy chain. This phosphoprotein is likely to be p56 lck based on protein size. This band never appeared in the cross-linked anti-CD3 studies. One possible explanation for this difference is that in the absence of CD4 coaggregation, lck may dissociate from the TCR complex after lck phosphorylates its substrates. Whereas under stimulation conditions using the bispecific antibody, lck remains in the complex longer due to stable association with coaggregated CD4.

The biochemical results suggested that the anti-CD3 × anti-CD4 bispecific antibody delivered a competent activating signal to the T cells. In fact, T cell clones or fresh murine T cells cultured in the presence of anti-CD3 × anti-CD4 heterodimer proliferated, whereas T cells cultured with the anti-CD3–Fos homodimer did not (Fig. 7 B). Thus, enhanced association of lck with the TCR complex reconstituted both early signaling events and a mitogenic stimulus in the absence of further Ab cross-linking.

### Discussion

In this study, we have shown that bivalent anti-CD3 delivers a partial TCR signal that renders Th1 clones hyporesponsive. This signal consists of phosphorylation of several components of the TCR complex, (bands representing CD3e, CD3\(\delta\)), ZAP-70 association, and partial phosphorylation of TCR \( \zeta \); in the absence of cross-linking, there is a relatively greater induction of the phosphorylated p21 \( \zeta \) as compared with the p23 \( \zeta \) band species evident in T cell clones. Presently, it is unclear what the p21 and p23 forms of \( \zeta \) represent. p21 induction appears to be sufficient for...
TCR may fail to induce the oligomerization required for a role for aggregation in that a shorter dwell time of the dissociation rate. However, this finding does not exclude a role for certain altered peptide ligands and a higher TCR activity. Lyson et al. (29) showed a correlation between antagonist aggregation and a higher TCR activity in that absence of lck almost completely abrogates tyrosine phosphorylation and Ca²⁺ flux. The correlation between components of the TCR complex that are in close proximity. For example, if lck binds one phosphorylated ZAP-70 through its SH2 domain, the lck would be in a prime position to phosphorylate a neighboring ZAP-70 molecule in the TCR aggregate. In the noncross-linked situation, lck might migrate away before phosphorylating more ZAP-70 molecules. Thus, aggregated TCR signal transduction may result in amplification of these signals, because one kinase may act on multiple substrates. This capacity for amplification would mean that proximal differences should become magnified as the signal is propagated. As seen in the present study, a relative reduction in ZAP-70 phosphorylation leads to a more dramatic deficiency in PLC-γ-1 phosphorylation and Ca²⁺ flux.

The observation that noncross-linked anti-CD3 induces less ZAP-70 phosphorylation and p23 induction and ZAP-70 phosphorylation appear to be interrelated events. Indeed, the low level of ZAP-70 phosphorylation observed in the noncross-linked situation correlates with the small amount of p23 ζ that is generated. In a recent study, Weist et al. (28) proposed that the p23 form of ζ observed in thymocytes upon in vitro stimulation depends on greater TCR aggregation. Our findings are consistent with this hypothesis. Higher orders of TCR aggregation also appear to be required for recruitment of other phosphotyrosine containing molecules to the TCR-CD3 complex in both T cell clones and bulk naive cells. If any of these tyrosine-phosphorylated molecules contain SH2 domains, they may require the fully phosphorylated p23 form of ζ for association. Alternatively, the p23 form may be required for docking of a kinase that phosphorylates these associated molecules.

There are several ways in which the localization of multiple TCR complexes within a large aggregate could enhance signaling. In the kinetic proofreading model proposed by McKethan (30), TCR signal transduction was modeled as a reversible multistep pathway containing sequential phosphorylation events. In this paradigm, aggregation of TCRs might enhance propagation of the signal by favoring phosphorylation over dephosphorylation. On a more mechanistic level, aggregation may aid in recruiting key signaling molecules; recruitment of molecules may be further stabilized if there are multiple potential contact points (catalytic sites, SH2 domains, or other recognition motifs) between components of the TCR complex that are in close proximity. For example, if lck binds one phosphorylated ZAP-70 through its SH2 domain, the lck would be in a prime position to phosphorylate a neighboring ZAP-70 molecule in the TCR aggregate. In the noncross-linked situation, lck might migrate away before phosphorylating more ZAP-70 molecules. Thus, aggregated TCR signal transduction may result in amplification of these signals, because one kinase may act on multiple substrates. This capacity for amplification would mean that proximal differences should become magnified as the signal is propagated. As seen in the present study, a relative reduction in ZAP-70 phosphorylation leads to a more dramatic deficiency in PLC-γ-1 phosphorylation and Ca²⁺ flux.

The redistribution of TCR complexes to one pole, within minutes upon addition of secondary cross-linker to anti-CD3, is likely to reflect changes in the underlying cytoskeleton. Others have shown that TCR engagement can lead to redistribution of cytoskeletal elements such as talin, vinculin, and actin (9, 24). The cross-linking Ab might be providing sufficient TCR aggregation to trigger a threshold signal for cytoskeletal mobilization. Studies by Valtelluti et al. (25) have suggested that the cytoskeleton also plays an active role in sustaining a TCR signal, because the addition of agents which disrupt the actin cytoskeleton (e.g., Cytocholasin D) can block the rise in intracellular Ca²⁺. The cross-linked anti-CD3 system may be useful for dissecting the role of the cytoskeleton in proximal signaling events.

Our experiments suggest that efficient recruitment of lck may be the pivotal event accomplished by aggregation. Lck has been shown to be important for proximal signaling in that absence of lck almost completely abrogates tyrosine phosphorylation events (9, 31). It is well established that coaggregating anti-CD3 and anti-CD4 antibodies or using anti-CD3/anti-CD4 heteroconjugate mAbs can result in enhanced tyrosine phosphorylation and calcium mobilization (32). Recently, it was shown that in circumstances in which lck is limiting, as in double-positive thymocytes,
ZAP-70 phosphorylation requires caggregation of TCR and CD4 (28). Furthermore, blockading CD4 and presumably its associated Ick molecules with anti-CD4 mAbs can convert a partial agonist signal into an antagonistic signal with its associated characteristic signaling deficits (33). Thus, impaired CD4 recruitment has been proposed as a mechanism for altered peptide/antagonist peptide signaling. The pivotal nature of Ick recruitment is underscored by our finding that secondary antibody induced aggregation can be dispensed with, if Ick is recruited by bringing CD4 into the complex artificially. Even in the absence of exogenous cross-linking, stimulation with a bivalent anti-CD3 × anti-CD4 reagent reconstituted both the early signaling events of ZAP-70 phosphorylation and association of other phosphorylated proteins with the complex. In turn, these early events lead ultimately to a mitogenic stimulus.

The partial signals delivered by nonmitogenic anti-CD3 correlated with the induction of functional anergy as defined by an inability to proliferate due to poor IL-2 production. The striking similarity between the signals delivered by altered peptide ligands and nonmitogenic anti-CD3 are perhaps indicative of a common mechanism of anergy induction. How these partial signals translate into an off signal that shuts down T cell clonal responsiveness has yet to be determined. In the classical model of anergy, involving a complete signal one (through the TCR) in the absence of signal two (costimulation), induction of unresponsiveness depends upon a successful calcium signal that can be blocked by CsA (34, 35). Similarly, CsA has been shown to block anergy induction by altered peptide ligands (12). In fact, an altered peptide ligand triggered calcium signal has been recently demonstrated using an exquisitely sensitive system (36). The ability of CsA to block nonmitogenic anti-CD3–induced functional anergy suggests that a calcium signal may be important in this process. It is possible that the lack of detectable calcium flux by nonmitogenic anti-CD3 reflects insufficient sensitivity. In contrast with the classical models of anergy, the presence of competent APC or anti-CD28 antibodies did not rescue T cell clones from nonmitogenic anti-CD3–induced unresponsiveness.

An unresolved observation was the finding that culture with the nonmitogenic anti-CD3 suppresses IL-2 production in clones, but it did not appear to impair significantly the responsiveness of bulk T cells. Our results suggest similar defects in signaling between naive cells and clones in terms of ZAP-70 phosphorylation and TCR–CD3 complex–associated phosphorylated molecules, as well as the downstream events of PLCγ-1 phosphorylation and TCR capping. It is possible that naive cells and clones differ in the way they respond to nonmitogenic anti-CD3 mAbs, either in the triggering of other biochemical signals, or in the integration of downstream nuclear signals.

The mitogenic forms of anti-CD3 currently in use severely suppress global T cell responses. This study suggests that nonmitogenic anti-CD3 may selectively induce unresponsiveness in activated T cell subsets. These findings bear promising implications for transplant therapy in that it would be beneficial to be able to suppress the alloreactive T cells that mediate graft rejection, while maintaining the responsiveness of other T cells. Further investigation into the mechanisms by which nonmitogenic anti-CD3 modulates T cell responses should shed some light on the applicability of this potential therapeutic to transplantation.

We would like to thank J. Auger for her assistance with the calcium flux experiments and B. Eischfelder for his assistance with confocal microscopy.

This work was supported by PO1AI29531-07, CA-14599, HL07605, and a grant from the Baxter Foundation.

Address correspondence to Dr. Jeffrey A. Bluestone, Committee on Immunology, 5841 S. Maryland Avenue, M.C. 1089, Chicago, IL 60637.

Received for publication 24 October 1996 and in revised form 4 February 1997.

References

1. Ortho Multicenter Transplant Study Group. 1985. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. N. Engl. J. Med. 313:337–341.
2. Charpentier, B., C. Hiesse, O. Lantz, C. Ferran, S. Stephens, D. O’Shaunessy, M. Bodmer, G. Benoit, J.F. Bach, and L. Chatenoud. 1992. Evidence that antihuman tumor necrosis factor monoclonal antibodies prevents OKT3-induced acute syndrome. Transplantation. 54:997–1002.
3. Ferran, C., K. Sheehan, M. Dv, R. Schreiber, S. Merite, P. Landais, L. Noell, G. Grau, J.A. Bluestone, J.F. Bach, and L. Chatenoud. 1990. Cytokine related syndrome following injection of anti-CD3 monoclonal antibody: further evidence for transient in vivo T cell activation. Eur. J. Immunol. 20:509–515.
4. Kan, E.A.R., S.D. Wright, K. Welte, and C.Y. Wang. 1986. Fc receptors on monocytes cause OKT3-treated lymphocytes to internalize T3 and to secrete IL-2. Cell Immunol. 98:181–187.
5. Alegre, M.L., J.Y. Tso, H.A. Sattar, J. Smith, F. Desalle, M. Cole, and J.A. Bluestone. 1995. An anti-murine CD3 monoclonal antibody with a low affinity for Fc gamma receptors suppresses transplantation responses while minimizing acute toxicity and immunogenicity. J. Immunol. 155:1544–1555.
6. Hirsch, R., M. Eckhaus, H. Auchincloss, Jr., D.H. Sachs, and
J.A. Bluestone. 1988. Effects of in vivo administration of anti-T3 monoclonal antibody on T cell function in mice. I. Immunosuppression of transplantation responses. J. Immunol. 140:3766–3772.

7. Alegre, M.L. 1993. Ph.D. thesis. The Role of Cellular Activation in the Functional Properties of Anti-CD3 Monoclonal Antibodies In Vivo. University of Chicago, Chicago, IL. 343 pp.

8. Qian, D., I. Griswold-Prenner, M.R. Rosner, and F.W. Fitch. 1993. Multiple components of the T cell antigen receptor complex become tyrosine phosphorylated upon activation. J. Biol. Chem. 268:4488–4493.

9. Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. Cell. 76:263–274.

10. Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen. 1993. Identification of a murine T cell receptor signal transduction pathway requires ZAP70 and Lck. J. Immunol. 151:5723–5733.

11. Straus, D.B., and A. Weiss. 1993. The CD3 chains of the T cell receptor are tyrosine phosphorylated without ZAP-70 recruitment in APL-induced T cell anergy. J. Exp. Med. 178:1523–1530.

12. Sloan-Lancaster, J., B.D. Evavold, and P.M. Allen. 1993. Interaction of T-cell antigen receptor with the ZAP-70 tyrosine kinase and ZAP-70 is tyrosine phosphorylated after receptor stimulation. J. Exp. Med. 178:1523–1530.

13. Madrenas, J., R.L. Wange, J.L. Wang, N. Isakov, L.E. Samelson, and R.N. Germain. 1995. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science (Wash. D.C.). 267:515–518.

14. Murphy, K.M., C.T. Weaver, M. Elish, P.M. Allen, and D.Y. Loh. 1989. Peripheral tolerance to allogeneic class II histocompatibility antigens expressed in transgenic mice: evidence against a clonal deletion mechanism. Proc. Natl. Acad. Sci. USA. 86:10034–10038.

15. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for the murine T3 T3 polypeptide. Proc. Natl. Acad. Sci. USA. 84:1374–1378.

16. Dialynas, D.P., Z.S. Quinn, K.A. Wall, A. Pierres, J. Quintans, M.R. Loken, and F.W. Fitch. 1987. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. J. Immunol. 131:2445–2451.

17. Kostelny, S.K., M.S. Cole, and J.Y. Tso. 1992. Formation of a bispecific antibody by the use of leucine zippers. J. Immunol. 148:1547–1553.

18. Carter, P., R.F. Kelly, M.L. Rodrigues, M. Covarrubias, M.D. Velligan, W.L.T. Wong, A.M. Rowlund, C.E. Kottos, M.E. Carver, M. Yang, I.H. Bourell, S.H.M. Henner, and D. Henner. 1992. High level Escherichia coli expression and high-level murine T cell antigen expression in mice. J. Exp. Med. 178:1523–1530.

19. Quidt, H., and R.H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified T cell molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J. Immunol. 138:3704–3712.

20. Reis e Sousa, C., E.H. Levine, and R.N. Germain. 1996. Partial signaling by CD8+ T cells in response to antagonist ligands. J. Exp. Med. 184:149–157.

21. Strauss, D.B., and A. Weiss. 1993. The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and tyrosine phospholipid after receptor stimulation. J. Exp. Med. 178:1523–1530.

22. Kupfer, A., S.J. Singer, C.A. Janeway, Jr., and S.L. Swain. 1987. Co-clustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. Proc. Natl. Acad. Sci. USA. 84:5888–5892.

23. Selliah, N., W.H. Brooks, and T.L. Rosman. 1996. Proteolytic cleavage of alpha-actinin by calpain in T cells stimulated with anti-CD3 monoclonal antibody. J. Immunol. 156:3215–3221.

24. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanaveccia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. J. Exp. Med. 181:577–584.

25. Iwashima, M., B.A. Irving, N.S. van Oers, A.C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science (Wash. D.C.). 263:1136–1139.

26. Strauss, D.B., A.C. Chan, B. Patai, and A. Weiss. 1996. SH2 domain function is essential for the role of the Lck tyrosine kinase in T cell receptor signal transduction. J. Biol. Chem. 271:9976–9981.

27. Wiest, D.L., J.M. Ashe, R. Abe, J.B. Bolen, and A. Singer. 1996. TCR activation of ZAP-70 is impaired in CD4+CD8+ thymocytes as a consequence of intrathymic interactions that diminish available p56lck. Immunity. 4:495–504.

28. Lyons, D.S., S.A. Lieberman, J. Hampl, Y.-H. Chien, L.J. Berg, and M.M. Davis. 1996. A TCR binds to antigen ligands with lower affinities and faster dissociation rates than to agonists. Immunity. 5:53–61.

29. McKelthan, T.W. 1995. Kinetic proofreading in T-cell receptor signal transduction. Proc. Natl. Acad. Sci. USA. 92:5042–5046.

30. van Oers, N.S., N. Killeen, and A. Weiss. 1996. Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J. Exp. Med. 183:1053–1062.

31. Ledbetter, J.A., C.H. June, P.S. Rabinovitch, A. Grossmann, T.T. Tsu, and J.B. Imboden. 1988. Signal transduction through CD4 receptors: stimulatory vs. inhibitory activity is regulated by CD4 proxility to the CD3/T cell receptor. Eur. J. Immunol. 18:525–532.

32. Mannie, M.D., J.M. Rosser, and G.A. Wite. 1995. Autoantigenic rat myelin basic protein is a partial agonist that is converted into a full antagonist upon blockade of CD4. Evidence for the integration of efficacious and nonefficacious signals during T cell antigen recognition. J. Immunol. 154:2642–2654.

33. Jenkins, M.K., C.A. Chen, G. Jung, D.L. Mueller, and R.H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. J. Immunol. 144:16–22.

34. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J. Exp. Med. 165:302–319.