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# Inhibition of T Cell Receptor Expression and Function in Immature CD4<sup>+</sup>CD8<sup>+</sup> Cells by CD4

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- Most immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes express only a small number of T cell receptor (TCR) molecules on their surface, and the TCR molecules they do express are only marginally capable of transducing intracellular signals. TCR expression and function was not intrinsically low in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, but was found to be actively inhibited by CD4-mediated signals. Indeed, release of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from CD4-mediated signals resulted in significant increases in both TCR expression and signaling function. These results suggest that, in CD4<sup>+</sup>CD8<sup>+</sup> cells developing in the thymus, increased TCR expression and function requires release from CD4-mediated inhibition.

**T**HYMIC SELECTION OF THE DEVELOPING T cell repertoire occurs in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, with the fate of individual thymocytes determined by the T cell antigen receptors (TCR) they express (1). Thus, it seems paradoxical that most CD4<sup>+</sup>CD8<sup>+</sup> thymocytes express few TCR molecules that are only marginally capable of transducing intracellular signals (2). Identifying the regulatory mechanisms involved in either inducing or inhibiting TCR expression and function in developing CD4<sup>+</sup>CD8<sup>+</sup> thymocytes would enhance our understanding of T cell differentiation. The TCR<sup>lo</sup> phenotype of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is due to the low fractional survival of newly synthesized and assembled TCR

complexes in these cells, which can be quantitatively increased by in vivo administration of monoclonal antibody (MAb) to CD4 (3). CD4, when cross-linked, activates tyrosine kinases that phosphorylate various intracellular substrates, including TCR- $\zeta$  (4, 5), a subunit of the TCR complex that appears to be important for both TCR expression and TCR signal transduction (6). We now directly assess the influence of CD4-mediated signals on TCR expression and function in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. TCR expression and function in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes was inhibited by CD4-mediated signals, and the presence of these inhibitory signals correlated with the reported phosphorylation status of TCR- $\zeta$  in these cells (5).

Experimentally, TCR expression can be polyclonally increased in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes either by in vivo administration of MAb to CD4 (7) or by in vitro culture of thymocytes in single-cell suspension (8). In vitro, TCR<sup>lo</sup> thymocytes spontaneously increase their expression of TCR when cultured in single-cell suspension at 37°C but not at 4°C (Fig. 1A), with TCR profiles of thymocytes from suspension cultures at

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4°C identical to those of freshly explanted thymocytes. Three-color flow cytometry (FCM) of cultured thymocytes revealed that TCR expression increased only in CD4<sup>+</sup>CD8<sup>+</sup> cells, with no significant change in any other thymocyte subset (Fig. 1A). CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in suspension culture at 37°C for a short time not only increased surface expression of TCR but increased their total cellular expression of TCR as well (Fig. 1B). This increase in TCR expression required mRNA and protein synthesis, as it was blocked by actinomycin D, an inhibitor of transcription, and by cycloheximide and anisomycin, inhibitors of protein synthesis (9).

Spontaneous induction of TCR in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in suspension culture at 37°C results either from the release of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from inhibitory intrathymic signals or from their active stimulation by a reagent present in culture medium. Release from intrathymic inhibition seemed likely, because TCR induction does not occur in organ fragment cultures at 37°C for a short time in which thymocytes encounter culture medium but remain in their thymic microenvironment (Fig. 2A). TCR induction might specifically be due to release from CD4-mediated inhibitory signals, as MAb to CD4 administered in vivo increases TCR expression in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes resident in situ (7). Release from CD4-mediated intrathymic signals would also be consistent with the phosphorylation

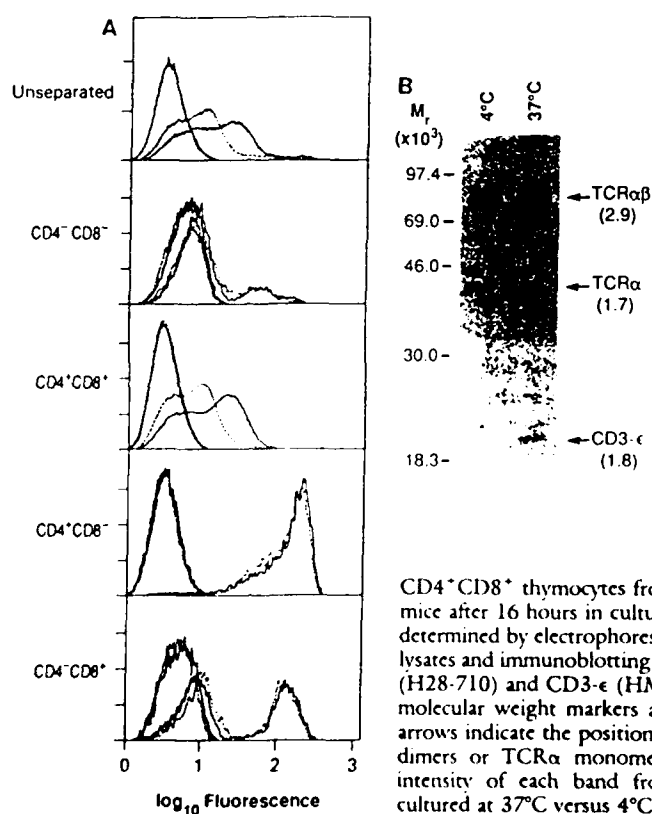
pattern of TCR-ζ in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, in that TCR-ζ chains are phosphorylated in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes resident in the thymus but spontaneously dephosphorylate upon culture in single-cell suspension at 37°C (5). Thus, because a known ligand for CD4 is Ia (10), the addition of Ia<sup>+</sup> stimulator cells to thymocyte suspension cultures would be predicted to engage thymocyte CD4 molecules and to generate signals inhibiting spontaneous TCR induction, as they do generate signals inhibiting spontaneous TCR-ζ dephosphorylation (5). Indeed, Ia<sup>+</sup> stimulator cells blocked the spontaneous increase in both surface and total cellular amounts of TCR occurring in thymocyte suspension cultures at 37°C (Fig. 2, B and C).

To determine whether stimulator cell Ia molecules were involved in the inhibition of thymocyte TCR induction, we used an Ia<sup>+</sup> cell line for which an Ia<sup>-</sup> variant existed. Thymocyte TCR induction was more effectively inhibited by the Ia<sup>+</sup> B lymphoma cell line M12.4.1 than by its Ia<sup>-</sup> variant M12.C3 (11) (Fig. 2D), even though both cell lines expressed equivalent amounts of cell surface major histocompatibility complex class I (9). In addition, MAb to Ia significantly blocked the inhibitory effect of Ia<sup>+</sup> M12.4.1 cells on TCR induction, whereas control MAb did not (Fig. 2D). The Ia-induced inhibitory effect on TCR expression was neither clonally restricted nor haplotype-specific, as TCR induction in

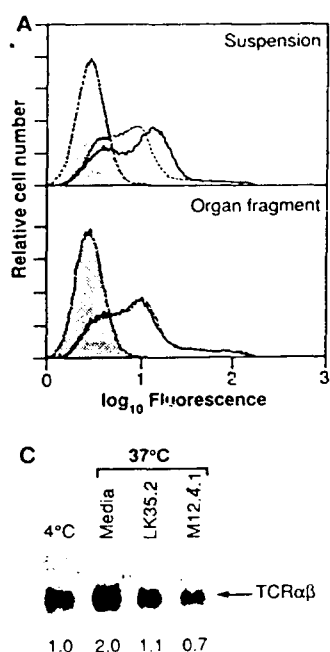
all TCR<sup>lo</sup> CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from all strains was inhibited by all Ia<sup>+</sup> stimulator cells tested, regardless of the haplotype or subregion encoding the Ia molecules they expressed. Thus, these data are most consistent with inhibition of TCR induction resulting from Ia engagement of clonally invariant CD4 molecules.

We next used MAb to CD4 to directly examine the effect of CD4-mediated signals on thymocyte TCR expression. Bivalent MAb to CD4, without further cross-linking, had no effect on TCR induction by thymocytes in suspension culture. We then added, as an immunoglobulin (Ig) cross-linker, low numbers of M12.C3 cells, which are FcR<sup>+</sup> but are Ia<sup>-</sup> and noninhibitory at the cell numbers used. In the presence of FcR<sup>+</sup> M12.C3 cells, immunoglobulin G (IgG) MAb to CD4 significantly inhibited TCR induction, whereas isotype matched MAb to CD8 did not, as determined both by FCM analysis of cell surface TCR expression and by immunoblotting for total cellular TCR expression (Fig. 3). Similar inhibition of TCR induction was observed with polyvalent IgM MAb to CD4, but not with polyvalent IgM MAb to CD8, in the absence of FcR<sup>+</sup> M12.C3 cells (9).

Finally, we wished to examine whether release of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from CD4-mediated inhibition was accompanied by improved TCR signaling, which is otherwise marginal in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (2). We assessed signaling by measuring intracellular calcium mobilization generated by cross-linking of MAb to TCR-αβ (Fig. 4). TCR signaling was marginal in uninduced CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from either organ fragment cultures (9) or 4°C suspension cultures, but was significantly enhanced in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from 37°C suspension cultures (Fig. 4, A and B) (12). The increased peak [Ca<sup>2+</sup>]<sub>i</sub> in 37°C suspension cultures was due to markedly increased responses by individual cells as well as to a somewhat greater fraction of cells responding. However, the addition of Ia<sup>+</sup> M12.4.1 cells to 37°C suspension cultures of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes inhibited the improvement in TCR signaling and did so more effectively than the addition of Ia<sup>-</sup> M12.C3 cells (Fig. 4A), indicative of Ia-induced negative regulation of TCR signaling ability. Improved TCR signaling ability in induced CD4<sup>+</sup>CD8<sup>+</sup> thymocytes could be related to increased TCR surface expression, decreased TCR-ζ phosphorylation, or both. Consequently, we examined TCR-signaling in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes that had been cultured in the presence of the transcription-inhibiting drug actinomycin D, which prevents TCR induction (9) but does not block TCR-ζ dephosphorylation



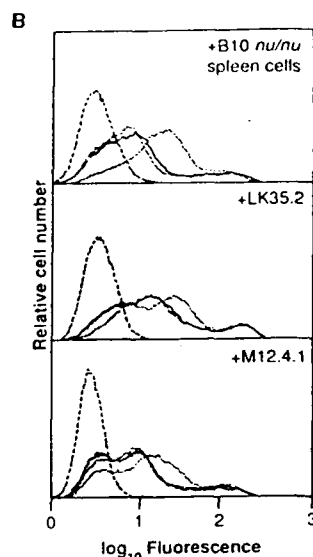
**Fig. 1.** In vitro induction of TCR in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. (A) Induction of surface TCR on CD4 and CD8 thymocyte subpopulations was assessed by three-color FCM on unseparated C57BL/10 (B10) thymocytes cultured for 4 hours in single-cell suspension ( $5 \times 10^6$  cells per milliliter) at either 37°C (solid lines) or 4°C (dashed lines). Surface TCR expression was determined by staining with MAb to CD3, with shaded areas representing background staining by control MAb (13). (B) Total cellular amounts of TCR in purified CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from 6-week-old C57BL/6 (B6) mice after 16 hours in culture at either 4°C or 37°C were determined by electrophoresis of detergent-solubilized cell lysates and immunoblotting with MAbs specific for TCRα (H28-710) and CD3-ε (HMT3-1) (14). The positions of molecular weight markers are indicated on the left, and arrows indicate the positions of TCRα (either in TCRαβ dimers or TCRα monomer) and CD3-ε. The relative intensity of each band from CD4<sup>+</sup>CD8<sup>+</sup> thymocytes cultured at 37°C versus 4°C is expressed in parentheses.



(Fig. 4B). TCR-induced calcium fluxes were significantly higher in actinomycin D-treated  $CD4^+CD8^+$  thymocytes than in untreated  $4^\circ C$  cultured thymocytes (Fig. 4B) whose surface TCR levels were similar but whose TCR- $\zeta$  chains remained phosphorylated; however, TCR-induced calcium fluxes in actinomycin D-treated  $CD4^+CD8^+$  thymocytes were not as great as in untreated  $CD4^+CD8^+$  thymocytes from  $37^\circ C$  suspension cultures (Fig. 4B) whose surface TCR levels were greater. Thus, release of  $CD4^+CD8^+$  thymocytes from intrathymic inhibition results in markedly improved TCR signaling, and that improvement is correlated with decreased TCR- $\zeta$  phosphorylation and with increased TCR expression. In fact, the improvement in TCR signaling is probably underestimated in these experiments, as uninduced  $CD4^+CD8^+$  thymocytes must be placed in single-cell suspension cultures at  $31^\circ C$  to be loaded with calcium-sensitive dyes, during which time some increase in TCR expression and some decrease in TCR- $\zeta$  phosphorylation occurs.

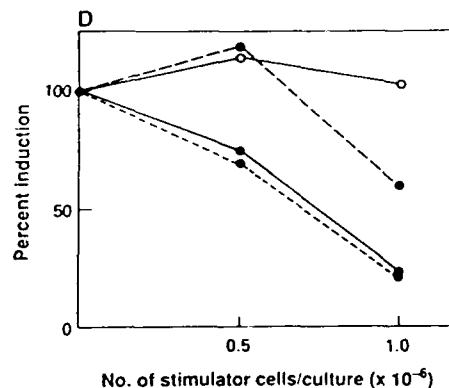
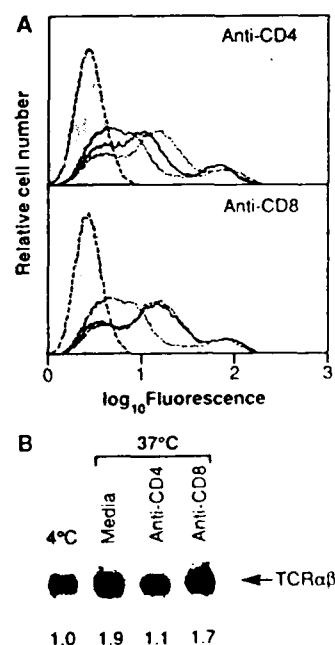
Our data demonstrate that CD4-mediated signals in  $CD4^+CD8^+$  thymocytes, induced either by Ia engagement or multivalent cross-linking of MAb to CD4, mimic the in vivo thymic microenvironment in inhibiting TCR expression and function, possibly to avoid premature deletion of developing thymocyte clones. Release of developing  $CD4^+CD8^+$  thymocytes from CD4-mediated inhibition would then result in increased TCR expression and function for subsequent TCR-mediated selection events in the thymus.

It is uncertain whether the presence of phosphorylated TCR- $\zeta$  chains in  $TCR^{lo}$



CD4 $^+$ CD8 $^+$  thymocytes simply reflects the presence of CD4-mediated signals or whether tyrosine phosphorylation of TCR- $\zeta$  chains is itself responsible for the low TCR expression and TCR signaling in these cells. In either case, the in vivo inductive effects of MAb to CD4 on thymocyte TCR expression (7) can now be understood as resulting from blockade of intrathymic CD4-ligand interactions that generate inhibitory signals in  $CD4^+CD8^+$  thymocytes, presumably because efficient multivalent cross-linking of MAb to CD4 on  $TCR^{lo}$   $CD4^+CD8^+$  thymocytes does not occur in vivo. An explanation is needed, however, for the inability of

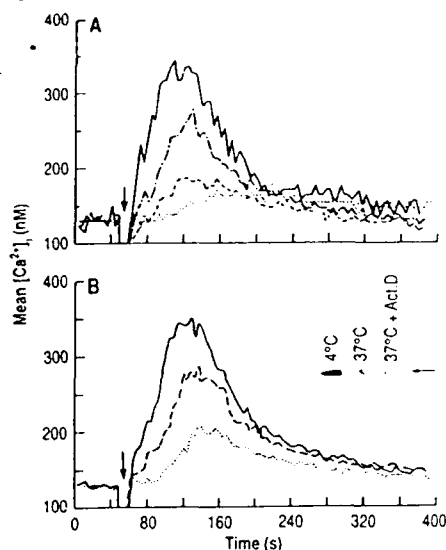
**Fig. 3. Multivalent cross-linking of CD4 inhibits thymocyte TCR induction.** (A) Effect of antibody-induced cross-linking of CD4 on cell surface TCR induction. B10.BR thymocytes were cocultured for 8 hours with  $5 \times 10^3$  FcR $^+$  M12.C3 cells without antibody at either  $4^\circ C$  (dashed line with left peak) or  $37^\circ C$  (dashed line with right peak), or with either MAb to CD4 (anti-CD4, MAb GK1.5) or to CD8 (anti-CD8, MAb 53-6.72) (solid lines) (18), and were assessed for surface TCR expression (16). (B) Effect of antibody-induced cross-linking of CD4 on induction of total cellular amounts of TCR in purified  $CD4^+CD8^+$  thymocytes. Total amounts of TCR were determined from cells lysed with NP-40 by immunoprecipitating and immunoblotting TCR with MAb H28-710 (anti-TCR $\alpha$ ). The relative intensity of the TCR $\alpha\beta$  band (arrow) is listed under each lane. Exposure time was 2 days.



**Fig. 2. Inhibitory effect of  $Ia^+$  cells on thymocyte TCR induction.** (A) Single cells or organ fragments ( $1 \text{ mm}^3$ ) from B6 thymuses were cultured for 4 hours at either  $37^\circ C$  (solid line) or  $4^\circ C$  (dashed line) and assessed for surface CD3 expression. Cell survival was  $>95\%$  in all cultures. Quantitation of CD3 fluorescence in fluorescence units (FU) (15) revealed that CD3 fluorescence changed  $+17,400$  FU in suspension culture (A, upper panel), whereas CD3

fluorescence changed  $-800$  FU in organ fragment culture (A, lower panel). (B) Single-cell suspensions of thymocytes were cultured alone for 8 hours at either  $4^\circ C$  (dashed lines with left peak) or  $37^\circ C$  (dashed lines with right peak), or were cocultured with  $Ia^+$  stimulator cells for 8 hours at  $37^\circ C$  (solid lines), and then assessed for surface TCR expression (16). B6 ( $H-2^b$ ) thymocytes were cocultured with  $3 \times 10^7$  B10 *nu/nu* spleen cells (B, upper panel); C3H ( $H-2^h$ ) thymocytes were cocultured with  $3 \times 10^6$  LK35.2 ( $H-2^{dk}$ ) cells (B, middle panel); Balb/c ( $H-2^d$ ) thymocytes were cocultured with  $3 \times 10^6$  M12.4.1 ( $H-2^d$ ) cells (B, lower panel). (C) Total cellular amounts of TCR $\alpha\beta$  in purified B6  $CD4^+CD8^+$  thymocytes cocultured with  $Ia^+$  stimulator cells were determined by immunoprecipitating and immunoblotting TCR from cells lysed with NP-40 with MAb to H28-710 (anti-TCR $\alpha$ ). The relative intensity of the TCR $\alpha\beta$  band (arrow) is listed under each lane. Exposure time was 16 hours. (D) DBA/2 ( $H-2^d$ ) thymocytes ( $1 \times 10^7$ ) were cocultured for 8 hours at  $37^\circ C$  with graded doses of either  $Ia^+$ -expressing M12.4.1 ( $H-2^d$ ) cells (closed symbols) or  $Ia^-$  M12.C3 ( $H-2^d$ ) cells (open symbols) without antibody (solid lines) or with  $20 \mu\text{g}$  of MAb per milliliter specific for either  $Ia^+$  (MAB M5-114) (long dash) or Fc receptor (FcR) (MAB 2.4G2) (short dash), both of which are rat IgG2b antibodies. To compare the effects of different stimulator cells on TCR induction, CD3 fluorescence for each curve was quantitated in FU (15), and percent induction was defined as the increase in CD3 fluorescence of thymocytes cocultured with stimulator cells relative to that of thymocytes cultured alone (17).

MAbs to Ia that are administered in vivo to increase TCR expression in  $CD4^+CD8^+$  thymocytes (7), as such antibodies would be expected to block inhibitory intrathymic CD4-Ia interactions. One interesting possibility is that there may exist on thymic stromal cells an alternative, possibly primitive, CD4 ligand in addition to Ia. Indeed, inhibition of TCR expression and function



**Fig. 4.** TCR-induced calcium mobilization in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in response to TCR cross-linking by MAb to TCR $\alpha\beta$ , and its relation to TCR expression and TCR- $\zeta$  phosphorylation. (A) Purified B6 CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured alone for 4 hours in single-cell suspension at either 37°C (solid line) or 4°C (dotted line) and then assessed for TCR-induced calcium mobilization by MAb to TCR $\alpha\beta$  (MAb H57-597) (19) that was cross-linked by avidin (25  $\mu$ g) at the indicated times (arrow) (20). Alternatively, cells from the same purified B6 CD4<sup>+</sup>CD8<sup>+</sup> thymocyte populations were cocultured at 37°C with  $1 \times 10^6$  Ia<sup>+</sup> M12.4.1 (dashed line) or Ia<sup>+</sup> M12.C3 (dash-dot) cells, and then assessed for TCR-induced calcium fluxes (21). Mean intracellular Ca<sup>2+</sup> concentrations are displayed versus time. (B) Effect of actinomycin D on TCR signaling and TCR- $\zeta$  phosphorylation in cultured CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Purified B6 CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured for 4 hours in suspension at either 37°C (solid line) or 4°C (dotted line), or were cultured at 37°C in the presence of actinomycin D (10  $\mu$ g/ml) (dashed line), and then assessed for TCR-induced calcium mobilization. (Inset) The same cell populations were also assessed for tyrosine-phosphorylated TCR- $\zeta$  (arrow) by immunoblotting with antibody to phosphotyrosine (22) (Act. D, actinomycin D).

in developing CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is probably the earliest function performed by CD4 and appears to be unique to immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, as CD4 performs other functions in more differentiated T cells.

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13. CD3 fluorescence profiles of the CD4 and CD8 thymocyte subpopulations were obtained by computer gating of three-color immunofluorescence data. Staining reagents were fluorescein isothiocyanate (FITC) conjugated to MAb 145-2C11 (anti-CD3) [O. Leo, M. Foo, D. H. Sachs, L. E. Samelson, J. A. Bluestone, *Proc. Natl. Acad. Sci. U.S.A.* 84, 1374 (1987)]; phycoerythrin (PE) conjugated to MAb GK1.5 (anti-CD4) [D. P. Djalynas *et al.*, *Immunol. Rev.* 74, 29 (1983)]; biotin conjugated to MAb 53-6.72 (anti-CD8) [J. A. Ledbetter and L. A. Herzenberg, *ibid.* 47, 63 (1979)]; and Texas red-streptavidin. Leu4-FITC was the control MAb.
14. CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were isolated before culture by adherence to plates coated with MAb to Lyt2.2, and were >96% CD4<sup>+</sup>CD8<sup>+</sup> as described (5). Nonidet-P40 (NP-40) lysates of cultured thymocytes ( $2 \times 10^6$ /lane) were applied to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in nonreducing conditions, subjected to electrotransfer, immunoblotting with MAb to TCR $\alpha$  (MAb H28-710) and to CD3- $\epsilon$  (MAb HMT3-1), and visualization with the use of <sup>125</sup>I-labeled protein A [T. Nakayama, R. T. Kubo, H. Kishimoto, Y. Asano, T. Tada, *Int. Immunol.* 1, 50 (1989); J. S. Spencer and R. T. Kubo *J. Exp. Med.* 169, 625 (1989)].
15. FU = cell frequency above control  $\times$  median intensity above control. Median intensity above control was calculated by converting median log channel

number above control to linear units with an empirically derived calibration curve for each three-decade logarithmic amplifier used.

16. Harvested cells were stained with both anti-CD3-FITC and 30-H12-biotin (anti-Thy1.2), then Texas red streptavidin. CD3 profiles were obtained by electronic gating on Thy1.2<sup>+</sup> cells to exclude the stimulator cells from the FCM analysis.
17. Percent induction is  $100 \times (\text{change in FU of thymocytes cultured with stimulator cells}) / (\text{change in FU of thymocytes cultured alone})$ .
18. The presence of MAb to CD4 or CD8 in these cultures resulted in marked down-modulation of surface CD4 and CD8, respectively, on the harvested cells, which indicated that both antibody preparations were active.
19. R. T. Kubo, W. Born, J. W. Kappler, P. Marrack, M. Pigeon, *J. Immunol.* 142, 2736 (1989).
20. Cells were incubated with a saturating amount of biotin conjugated to MAb H57-597 (anti-TCR $\alpha\beta$ ) at 4°C, then warmed to 37°C for 10 min before analysis. Indo-1 Ca<sup>2+</sup> measurements were done as in P. S. Rabinovitch, C. H. June, A. Grossmann, and J. A. Ledbetter [*J. Immunol.* 137, 952 (1986)].
21. Stimulator cells were excluded from analysis on the basis of cell size by electronic gating on forward light scatter.
22. Cultured CD4<sup>+</sup>CD8<sup>+</sup> thymocytes ( $3 \times 10^6$  per lane) were solubilized in 0.5% Triton X-100 lysis buffer with protease and phosphatase inhibitors. After immunoprecipitation with MAb to CD3- $\epsilon$  (MAb 145-2C11), 13% SDS-PAGE in reducing conditions, and electrotransfer, phosphorylated TCR- $\zeta$  was detected by immunoblotting with antibodies to phosphotyrosine as described (5). Exposure time was 4 days.
23. Both MAb to TCR $\alpha$  (MAb H28-710) and to CD3- $\epsilon$  (MAb HMT3-1) were generated and provided by R. Kubo, Denver, CO. We thank R. Kubo, R. Klausner, and J. Bonifacio for gifts of reagents and helpful discussions.

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| Distribution/      |                                     |
| Availability Codes |                                     |
| Dist               | Avail and/or Special                |
| A-1                | 20                                  |