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Kelly P. Kearse

Yousuke Takahama

Jennifer Punt
Haverford College

Susan O. Sharrow

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Early Molecular Events Induced by T Cell Receptor (TCR) Signaling in Immature CD4⁺CD8⁺ Thymocytes: Increased Synthesis of TCR- α Protein Is an Early Response to TCR Signaling That Compensates for TCR- α Instability, Improves TCR Assembly, and Parallels Other Indicators of Positive Selection

By Kelly P. Kearse, Yousuke Takahama, Jennifer A. Punt, Susan O. Sharrow, and Alfred Singer

From the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-1360

Summary

Differentiation of immature CD4⁺CD8⁺ thymocytes into mature CD4⁺ or CD8⁺ T cells occurs within the thymus and is dependent upon expression of antigen receptor complexes (T cell receptor [TCR]) containing clonotypic α/β proteins. We have recently found that CD4⁺CD8⁺ thymocytes express low levels of surface TCR because of limitations placed on TCR assembly by the instability of nascent TCR- α proteins within the endoplasmic reticulum (ER) of immature thymocytes. Because TCR- α/β expression increases during development, a molecular mechanism must exist for increasing the number of assembled TCR complexes present in immature CD4⁺CD8⁺ thymocytes that have been signaled to differentiate into mature T cells, although no such mechanism has yet been described. In the current report we have examined the molecular consequences of intracellular signals generated by engagement of surface TCR complexes on immature CD4⁺CD8⁺ thymocytes. Isolated TCR engagement generated signals that increased TCR- α RNA levels and increased synthesis of TCR- α proteins, which, in turn, significantly increased assembly of complete TCR- α/β complexes in CD4⁺CD8⁺ thymocytes. Increased TCR- α protein levels in TCR-signaled CD4⁺CD8⁺ thymocytes was the result of increased synthesis and not increased stability of TCR- α proteins, indicating that TCR engagement compensates for, but does not correct, the inherent instability of TCR- α proteins in the ER of immature thymocytes. Consistent with the delivery by TCR engagement of a positive selection signal, TCR engagement also increased CD5 expression, decreased RAG-1 expression, and decreased CD4/CD8 coreceptor expression in immature CD4⁺CD8⁺ thymocytes. These data identify amplified TCR- α expression as an initial response of immature CD4⁺CD8⁺ thymocytes to TCR-mediated positive selection signals and provide a molecular basis for increased surface TCR density on developing thymocytes undergoing selection events within the thymus.

Most lymphocytes of the T cell lineage develop in the thymus and express antigen specific receptors (TCR) containing clonotypic TCR- α and TCR- β polypeptides. Intrathymic differentiation of immature precursor cells into mature α/β T cells occurs via a series of ordered steps that are best characterized by variable expression of CD4/CD8 coreceptor molecules and increasing surface density of TCR- α/β (1). The CD4/CD8 developmental pathway of α/β T cells contains two major transitions: (a) differentiation of TCR- α/β ⁻CD4⁻CD8⁻ thymocytes into TCR- α/β ^{lo}CD4⁺CD8⁺ thymocytes, and (b) differentiation of TCR- α/β ^{lo}

CD4⁺CD8⁺ thymocytes into mature TCR- α/β ^{hi}CD4⁺ or TCR- α/β ^{hi}CD8⁺ T cells. The signals that induce the developmental transitions of α/β T cells along the CD4/CD8 developmental pathway are not known with certainty, but each appears to involve signaling via surface TCR complexes. Transition of CD4⁻CD8⁻ precursor cells into CD4⁺CD8⁺ thymocytes is associated with expression of TCR- β proteins in the absence of TCR- α chains (2, 3), while differentiation of CD4⁺CD8⁺ thymocytes involves signals transduced by surface TCR- α/β complexes (4).

Assembly of murine TCR- α/β complexes occurs in the

endoplasmic reticulum (ER)¹ via a series of sequential steps involving (a) assembly of CD3- γ , δ , ϵ proteins into partial CD3 complexes, (b) assembly of α/β proteins with CD3 components to form intermediate TCR complexes ($\alpha\beta\gamma\delta\epsilon$), and (c) association of ζ chains with intermediate TCR complexes to form complete TCR complexes ($\alpha\beta\gamma\delta\epsilon\zeta$), (5). Transport of clonotypic α/β proteins to the cell surface is dependent upon their assembly with CD3 components and ζ chains. Unassembled TCR chains and partial complexes of CD3 components are retained within the ER and, depending upon the particular protein, degraded (5). Intermediate TCR complexes not containing ζ exit the ER but are targeted to lysosomes for degradation. Only complete TCR complexes are efficiently transported to the cell surface (5).

Expression of the TCR- α/β complex is quantitatively regulated during development with immature CD4⁺CD8⁺ thymocytes expressing only 10% the number of surface TCR complexes as are expressed on mature T cells (6). Low TCR expression on immature CD4⁺CD8⁺ thymocytes results from the rapid degradation of nascent TCR- α proteins within the ER of CD4⁺CD8⁺ thymocytes that severely limits the formation of TCR- α/β dimers and assembly of complete TCR complexes (6). Because expression of TCR- α complexes by CD4⁺CD8⁺ thymocytes increases during T cell development, the differentiation of immature CD4⁺CD8⁺ thymocytes into mature CD4⁺ or CD8⁺ T cells must involve increased steady state levels of nascent TCR- α/β complexes. However, it is not known how such quantitative regulation of TCR- α/β expression in developing thymocytes is accomplished. In the current study we have examined the effects of TCR signaling on assembly of TCR proteins in developing thymocytes. Increased assembly of nascent TCR complexes was observed in TCR-signaled CD4⁺CD8⁺ thymocytes relative to untreated CD4⁺CD8⁺ thymocytes, resulting from amplified expression of a single TCR component, the TCR- α chain. Amplified expression of TCR- α chains in TCR-signaled CD4⁺CD8⁺ thymocytes was reflected in both increased levels of TCR- α RNA transcripts and increased TCR- α protein synthesis. In addition, TCR signaling decreased RAG-1 expression in CD4⁺CD8⁺ thymocytes and, interestingly, decreased expression of both CD4 and CD8 coreceptor molecules on a subset of CD4⁺CD8⁺ thymocytes showing increased CD5 expression in response to TCR engagement. These results demonstrate that engagement of surface TCR on immature CD4⁺CD8⁺ thymocytes induces molecular events in a subset of CD4⁺CD8⁺ thymocytes characteristic of an initial positive selection signal, defined by amplified expression of the TCR- α chain and increased assembly of nascent TCR complexes within the ER.

Materials and Methods

Animals and Cell Preparation. 6–8-wk-old C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

¹ Abbreviations used in this paper: CsA, cyclosporin A; ER, endoplasmic reticulum; HSA, heat stable antigen.

CD4⁺CD8⁺ thymocytes were isolated by their adherence to plastic plates coated with anti-CD8 mAb (83-12-5), and were typically >96% CD4⁺CD8⁺ as described (7). Purified splenic T cells were obtained by incubating single cell suspensions of spleen cells on rabbit anti-mouse Ig (Organon Technika-Cappel, Malvern, PA)-coated tissue culture plates for 60 min at 37°C, followed by isolation of nonadherent cells. The resultant cell populations were >95% CD3 ϵ ⁺ as determined by surface staining with mAb to CD3 ϵ .

Pronase Treatment of CD4⁺CD8⁺ Thymocytes and Culture on Anti-TCR- β -coated Plates. Preparation of anti-TCR- β -coated tissue culture plates was performed by adding 500 μ l of a 10 μ g/ml solution of purified anti-TCR- β mAb (H57-597), (PharMingen) in PBS to each well of a 24-well tissue culture plate (Costar Corp., Cambridge, MA) which was incubated overnight at 4°C. Antibody solution was removed from wells the next day and saved for reuse. Precoated wells were rinsed 3 \times with RPMI medium containing 10% FCS before the addition of cells. Cells were cultured at 5 \times 10⁶/ml in either medium alone or in wells precoated with purified mAb to TCR- β mAb. Cell viability was typically 80–90% as determined by trypan blue exclusion (data not shown). For pronase treatment, cells were resuspended at a concentration of 5 \times 10⁶/ml in PBS containing 100 μ g/ml pronase (70 U/mg; Calbiochem-Novabiochem, San Diego, CA) for 15 min at 37°C, and washed 3 \times in complete medium before culture. Cyclosporin A (CsA), (Sandoz, Hanover, NJ) was used in culture at a final concentration of 1 μ g/ml and did not affect viable cell recovery.

Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis. For metabolic labeling, cells were resuspended at 10 \times 10⁶ cells/ml in methionine-free RPMI 1640 media (Biofluids, Inc., Rockville, MD) containing 10% FCS and 10 mCi [³⁵S]methionine (Trans ³⁵S label; ICN, Irvine, CA) for 30 min at 37°C. Immunoprecipitation and gel electrophoresis was performed as previously described (7). The following mAbs were used for immunoprecipitation experiments in this study: anti-CD3 ϵ 145-2C11 (8), anti-TCR- α H28-710 (9), anti-TCR- β H597 (10), and anti-CD4 GK1.5 (11); the following antiserum was used: anti- ζ 551 antiserum (12).

RNA Analysis. Total cellular RNA was prepared from the indicated cell populations and equal amounts of RNA were denatured, subjected to electrophoresis on agarose gels, and transferred to nylon membranes. Blots were hybridized with the indicated probes as previously described (7).

Flow Cytometry. Staining for CD3 ϵ was performed with mAbs 145-2C11 conjugated with FITC; staining for CD4 with FITC-RM4.5 (PharMingen); staining for CD8 with either FITC- or Biotin-Lyt2.2 (Becton Dickinson & Co., Mountain View, CA); and staining for CD5 with Biotin-anti-Ly1 (Becton Dickinson & Co.) followed by Texas Red Streptavidin (Life Technologies, Gaithersburg, MD). Negative control staining was performed with FITC-Leu4 mAb to human CD3. Flow cytometry was performed with a modified dual laser FACSTAR PLUS[®] (Becton Dickinson & Co.). All fluorescence data were collected using logarithmic amplification on 50,000 viable cells as determined by forward light scatter intensity and propidium iodide exclusion.

Results

Impaired Assembly of TCR- α/β Complexes in Immature CD4⁺CD8⁺ Thymocytes. Low TCR expression on immature CD4⁺CD8⁺ thymocytes results from inefficient assembly of complete TCR complexes which, in turn, results from rapid degradation of nascent TCR- α chains within the

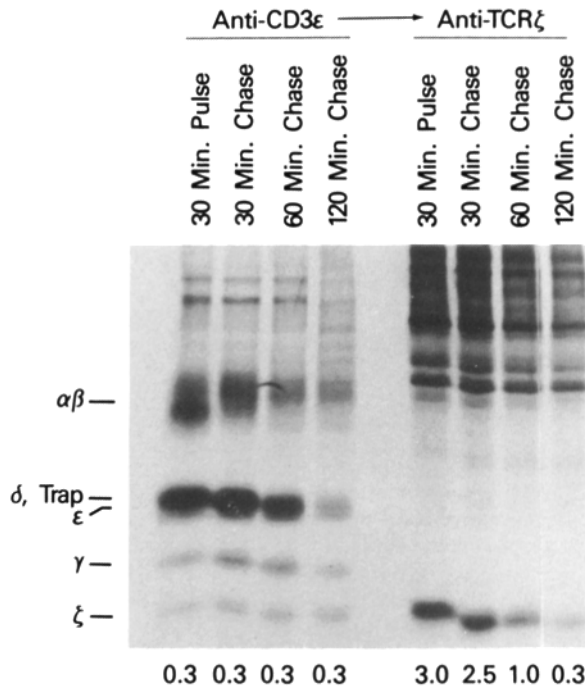


Figure 1. The formation of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes is severely limited in CD4⁺CD8⁺ thymocytes. CD4⁺CD8⁺ thymocytes were metabolically labeled for 30 min at 37°C and chased for the time period indicated. Digitonin lysates were sequentially precipitated with anti-CD3 ϵ mAb, followed by anti-TCR- ζ precipitation. Precipitation was complete as verified by the absence of radiolabeled material in subsequent precipitations and was specific in that TCR proteins were not precipitated by irrelevant control Abs (data not shown). Immunoprecipitated material was resolved on SDS-PAGE gels under reducing conditions. The numbers under each lane represent the optical density of the ζ band in arbitrary densitometry units. Multiple autoradiographs were scanned to ensure linearity.

ER (6). Inefficient formation of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes in CD4⁺CD8⁺ thymocytes is shown by metabolic labeling in Fig. 1. As demonstrated, most nascent ζ chains synthesized during a 30-min pulse period were not assembled into TCR complexes in CD4⁺CD8⁺ thymocytes as they were not immunoprecipitated with mAb to CD3 ϵ (Fig. 1); rather, they existed as unassembled ζ chains captured in

subsequent anti- ζ precipitations (Fig. 1). The small fraction of ζ chains that were assembled into TCR in CD4⁺CD8⁺ thymocytes were stable throughout the chase period, whereas unassembled ζ proteins were degraded in CD4⁺CD8⁺ thymocytes within 120 min of their synthesis (Fig. 1). These results demonstrate that assembly of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes is markedly inefficient in immature CD4⁺CD8⁺ thymocytes, but complete TCR complexes that are assembled in CD4⁺CD8⁺ thymocytes are stable.

We have recently found that impaired formation of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes in CD4⁺CD8⁺ thymocytes results from the uniquely rapid degradation of nascent TCR- α proteins in these cells, which severely limits the formation of TCR $\alpha\beta\gamma\delta\epsilon$ intermediates required for ζ addition (6). As shown in two different experiments presented in Fig. 2, nascent TCR- α proteins radiolabeled during a 30-min pulse period existed in two forms in CD4⁺CD8⁺ thymocytes and splenic T cells: as α/β dimers and α monomers. In CD4⁺CD8⁺ thymocytes most radiolabeled TCR- α monomers disappeared during a short chase period, without a commensurate increase in TCR- α/β dimers (Fig. 2, A and B). Thus, the majority of unassembled TCR- α monomers are rapidly degraded in CD4⁺CD8⁺ thymocytes with a calculated half-life of \sim 15 min (6). In contrast, in mature splenic T cells most nascent TCR- α proteins radiolabeled during the pulse survived the chase periods (Fig. 2, A and B). Unlike CD4⁺CD8⁺ thymocytes, the disappearance of TCR- α monomers in chase groups of splenic T cells resulted primarily from their assembly into α/β dimers and not from degradation (Fig. 2, A and B). Thus, nascent TCR- α chains are significantly less stable in immature CD4⁺CD8⁺ thymocytes than in mature T cells, with most nascent unassembled TCR- α proteins undergoing rapid degradation in the ER of CD4⁺CD8⁺ thymocytes.

Engagement of Surface TCR of CD4⁺CD8⁺ Thymocytes Results in Increased Assembly of Nascent TCR Complexes within the ER. Because TCR- α/β expression is quantitatively regulated during T cell differentiation, we wished to determine if TCR engagement affected the assembly of nascent TCR components into TCR complexes in immature CD4⁺CD8⁺ thymocytes. Purified CD4⁺CD8⁺ thymocytes were cultured

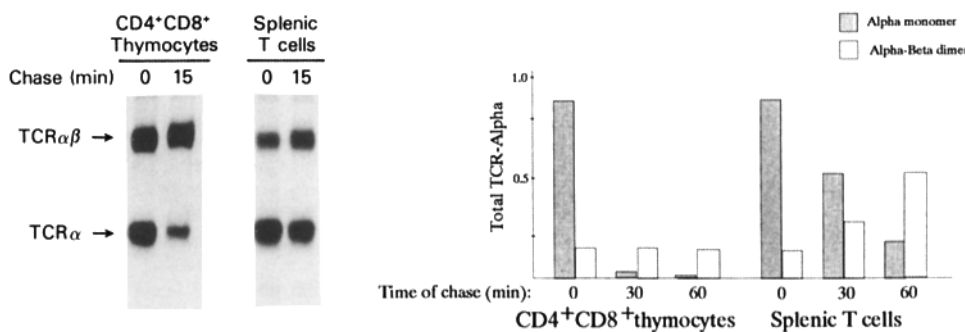


Figure 2. Rapid degradation of nascent unassembled TCR- α chains in immature CD4⁺CD8⁺ thymocytes but not mature T cells. CD4⁺CD8⁺ thymocytes and splenic T cells were radiolabeled for 30 min and chased for the time period indicated. (A) NP-40 lysates were immunoprecipitated with anti-TCR- α mAb; precipitated material was analyzed on one-dimensional SDS-PAGE gels under nonreducing conditions. It should be noted that the absolute amount of α/β dimerization occurring during the pulse period in splenic T cells

is markedly underestimated in this experiment because of unlabeled TCR- α protein pools that exist in mature splenic T cells but that do not exist in immature CD4⁺CD8⁺ thymocytes (16). (B) NP-40 lysates were precipitated with anti-TCR- α mAb to capture total TCR- α proteins. The relative amounts of TCR- α proteins existing as α/β dimers and α monomers in each cell type were determined by densitometric analysis and are expressed as a fraction of total TCR- α proteins in arbitrary densitometric units.

in medium alone or with platebound anti-TCR- β mAb to engage surface TCR complexes. Cells were then metabolically labeled and lysates precipitated with anti-CD3 ϵ mAb. In response to TCR engagement, assembly of nascent α/β and nascent ζ proteins with CD3 increased approximately fourfold, demonstrating that TCR signaling increased formation of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes in CD4 $^+$ CD8 $^+$ thymocytes (Fig. 3 A). That these events occurred in immature thymocytes was confirmed by the sensitivity of these cells to treatment with mAb to heat stable antigen (HSA) plus complement (Fig. 3), which eliminates immature CD4 $^+$ CD8 $^+$ thymocytes but not mature thymocytes (13). Augmentation of TCR- α/β assembly resulted from mAb-induced TCR signaling, and not from mAb-induced internalization or increased turnover of surface TCR complexes, as it was abrogated by CsA which inhibits TCR-signaling (14–16) but does not affect TCR-internalization or TCR turnover (Table 1). Thus, these data demonstrate that signaling via surface TCR complexes on immature CD4 $^+$ CD8 $^+$ thymocytes significantly increases assembly of nascent TCR component chains into multisubunit TCR complexes.

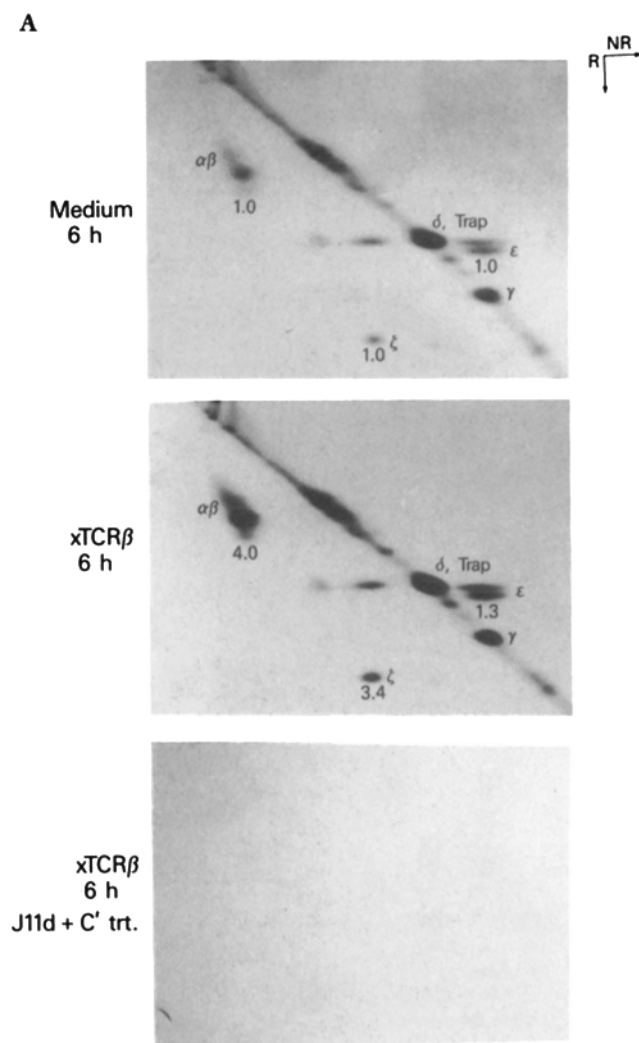


Table 1. Increased TCR Assembly in Response to TCR Cross-linking Is Abrogated by CsA

	Treatment*		
	CsA	\times TCR- β	\times TCR- β + CsA
CD3-assembled α/β dimer †	1.0	6.6	0.7

* CD4 $^+$ CD8 $^+$ thymocytes were cultured for 6 h in the presence or absence of platebound anti-TCR- β mAb and then metabolically labeled with [35 S]methionine for 30 min. Digitonin lysates were immunoprecipitated with anti-CD3 ϵ mAb and immunoprecipitated material was analyzed on two-dimensional nonreducing \times reducing gels. CsA was used at a final concentration of 1 μ g/ml. Note that internalization of surface TCR of CD4 $^+$ CD8 $^+$ thymocytes is unaffected by CsA treatment (Fig. 7 A). † The relative amounts of CD3-assembled dimer were quantitated by densitometry and are expressed in arbitrary densitometric units.

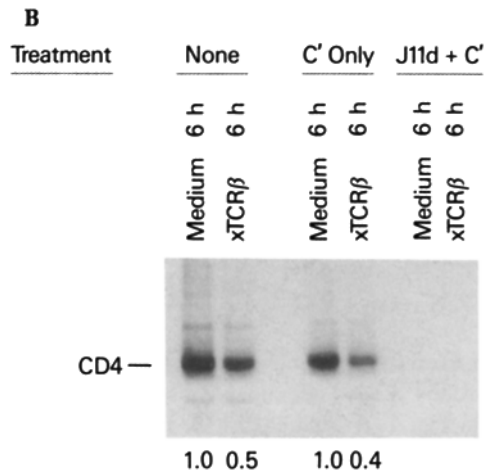


Figure 3. Increased assembly of nascent TCR complexes and decreased CD4 protein synthesis in TCR-signaled CD4 $^+$ CD8 $^+$ thymocytes. (A) CD4 $^+$ CD8 $^+$ thymocytes were cultured for 6 h in the presence or absence of platebound anti-TCR- β mAb and then metabolically labeled with [35 S]methionine for 30 min. Viability was unaffected by TCR cross-linking and was $>90\%$. Cells were solubilized with 1% digitonin; lysates were immunoprecipitated with anti-CD3 ϵ mAb and analyzed by two-dimensional nonreducing (NR)-reducing (R) SDS-PAGE. Where indicated, cells were treated with mAb to heat stable antigen (HSA) (mAb J11d; 19) plus C' before radiolabeling. mAb J11d plus C' treatment cytolytically eliminated $>99\%$ of CD4 $^+$ CD8 $^+$ thymocytes (data not shown). The positions of TCR proteins are indicated. The relative amounts of CD3 ϵ , TCR- α/β , and ζ proteins present in anti-CD3 ϵ immunoprecipitates of lysates of cells cultured for 6 h in the absence or presence of platebound anti-TCR- β mAb were quantitated by densitometry and are expressed in arbitrary densitometric units. Multiple exposures of the autoradiographs were scanned to ensure linearity. (B) The identical cell lysates shown in A were sequentially immunoprecipitated with anti-CD4 mAb and analyzed by one-dimensional SDS-PAGE under reducing conditions. Relative amounts of radiolabeled CD4 protein were determined by densitometry and are expressed in arbitrary densitometry units.

It should be appreciated that the relative amounts of nascent CD3 components synthesized in untreated and TCR-engaged CD4⁺CD8⁺ thymocytes were approximately equal (Fig. 3 A), indicating that increased assembly of complete TCR complexes in TCR-signaled CD4⁺CD8⁺ thymocytes was not the result of a general increase in protein synthesis. Moreover, synthesis of CD4 protein in the same samples was decreased in TCR-signaled CD4⁺CD8⁺ thymocytes relative to untreated groups (Fig. 3 B).

Increased Association of TCR- α and - β Proteins in TCR-signaled CD4⁺CD8⁺ Thymocytes. To determine the molecular basis for increased formation of complete TCR complexes in TCR-signaled CD4⁺CD8⁺ thymocytes, the assembly of nascent TCR- α with TCR- β proteins was examined. As demonstrated, equal amounts of newly synthesized TCR- β proteins were present in anti-TCR- β precipitates of untreated and TCR-signaled CD4⁺CD8⁺ thymocytes (Fig. 4); however, the relative amounts of nascent TCR- α proteins coprecipitated with TCR- β chains were four times greater in TCR-signaled CD4⁺CD8⁺ thymocytes than in untreated CD4⁺CD8⁺ thymocytes, indicating increased assembly of nascent TCR- α chains with TCR- β chains (Fig. 4). Thus, increased assembly of TCR- α with TCR- β proteins provides a molecular basis for enhanced formation of intermediate and complete TCR complexes in TCR-signaled CD4⁺CD8⁺ thymocytes (Fig. 3).

Amplified TCR- α Protein Synthesis in TCR-signaled CD4⁺CD8⁺ Thymocytes. We reasoned that several possibilities existed for increased α/β assembly after TCR engagement in immature CD4⁺CD8⁺ thymocytes: (a) increased synthesis of nascent TCR- α proteins; (b) increased stability of nascent TCR- α proteins, or both. As shown in Fig. 5, both TCR- α monomers (*thin arrow*) and α/β dimers (*thick arrow*) were present in anti-TCR- α precipitates of CD4⁺CD8⁺ thymocyte lysates at the end of a 30-min metabolic labeling period (Fig. 5). Importantly, antibody engagement of sur-

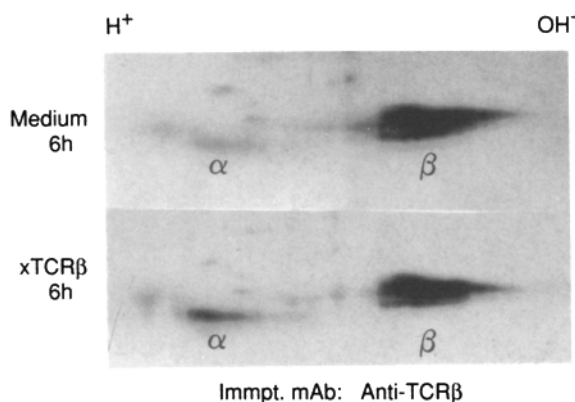


Figure 4. Increased association of TCR- α and - β proteins in TCR-signaled CD4⁺CD8⁺ thymocytes. CD4⁺CD8⁺ thymocytes were cultured for 6 h in the presence or absence of platebound anti-TCR- β mAb, harvested, and metabolically labeled with [³⁵S]methionine for 30 min. NP-40 lysates were immunoprecipitated with anti-TCR- β mAb and analyzed by two-dimensional NEPHGE/SDS-PAGE under reducing conditions. The positions of TCR- α and TCR- β proteins are indicated.

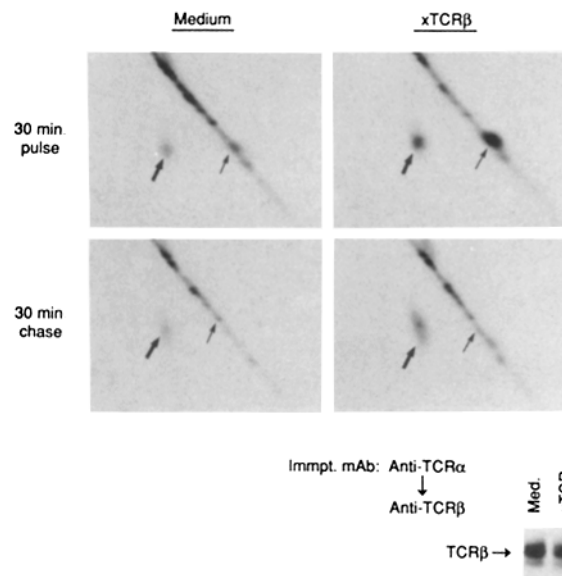


Figure 5. Amplified TCR- α protein synthesis in TCR-signaled CD4⁺CD8⁺ thymocytes. CD4⁺CD8⁺ thymocytes were cultured for 6 h in the presence or absence of platebound anti-TCR- β mAb and then metabolically labeled with [³⁵S]methionine for 30 min and chased in medium containing excess unlabeled methionine for an additional 30 min. NP-40 lysates were immunoprecipitated with anti-TCR- α mAb and analyzed by two-dimensional nonreducing (NR)-reducing (R) SDS-PAGE. The positions of TCR- α/β heterodimers (*thick arrows*) and monomeric TCR- α chains (*thin arrows*) are indicated. (Bottom) Supernatants from anti-TCR- α precipitates (pulse groups only) were sequentially immunoprecipitated with anti-TCR- β mAb to isolate nascent radiolabeled TCR- β proteins. Samples were analyzed on one-dimensional SDS-PAGE under reducing conditions. The position of TCR- β proteins is indicated. Note that the relative amounts of radiolabeled TCR- β chains are identical in untreated and TCR-signaled CD4⁺CD8⁺ thymocytes in contrast to radiolabeled TCR- α proteins that show amplified synthesis upon TCR engagement.

face TCR complexes resulted in an approximately fourfold increase in appearance of both TCR- α monomers and TCR- α/β dimers in TCR-signaled CD4⁺CD8⁺ thymocytes relative to untreated CD4⁺CD8⁺ thymocytes, indicating an approximately fourfold increase in overall TCR- α protein synthesis (Fig. 5, compare *left and right upper panels*). TCR signaling in CD4⁺CD8⁺ thymocytes specifically increased TCR- α protein synthesis as TCR- β protein synthesis was not affected (Fig. 5, *bottom*). Interestingly, even though TCR signaling increased TCR- α synthesis in CD4⁺CD8⁺ thymocytes, TCR signaling did not improve TCR- α stability as monomeric TCR- α proteins were degraded with similar kinetics in untreated and TCR-signaled CD4⁺CD8⁺ thymocytes (Fig. 5). The failure of TCR signaling to affect stability of unassembled TCR- α monomers is quantitated in Table 2.

We conclude that engagement of surface TCR complexes on CD4⁺CD8⁺ thymocytes amplifies TCR- α protein synthesis which, in turn, results in increased formation of α/β dimers that are assembled into complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes. However, because TCR signaling does not affect the median survival time of unassembled monomeric TCR- α proteins, we further conclude that TCR signaling compensates

Table 2. *Survival and Assembly of Nascent TCR- α Proteins in Cultured CD4⁺CD8⁺ Thymocytes*

Pulse	Chase	Treatment*					
		Total TCR- α †		Monomeric TCR- α ‡		Dimeric TCR- α/β §	
		Medium	\times TCR- β	Medium	\times TCR- β	Medium	\times TCR- β
	<i>min</i>						
30	0	100	100	52	68	48	32
	30	44	46	8	8	36	38

* CD4⁺CD8⁺ thymocytes were cultured for 6 h at 37°C on tissue culture plates containing medium or platebound anti-TCR- β mAb (\times TCR- β).

† The relative amount of TCR- α proteins is expressed as 100% in each treatment group. The amount of total TCR- α proteins in \times TCR- β groups was approximately fourfold greater than the amount of total TCR- α proteins in medium groups.

‡ Relative amounts of TCR- α proteins existing as monomers is expressed as a percent of total radiolabeled TCR- α proteins detected at the conclusion of the 30-min pulse period. The amount of TCR- α monomers in \times TCR- β groups was fivefold greater than the amount of TCR- α monomers in medium groups.

§ Relative amounts of TCR- α proteins assembled into heterodimers is expressed as a percent of total radiolabeled TCR- α proteins detected at the conclusion of the 30-min pulse period. The amount of TCR- α/β dimers in \times TCR- β groups was threefold greater than the amount of TCR- α/β dimers in medium groups. Multiple autoradiographs of different exposures were scanned to ensure linearity.

for, but does not correct, the inherent instability of unassembled TCR- α proteins in the ER of immature thymocytes.

It is noteworthy that unlike nascent TCR- α proteins, unassembled TCR- β proteins are relatively stable in the ER of immature CD4⁺CD8⁺ thymocytes (6). Thus, a sizeable intracellular pool of unlabeled TCR- β proteins exists in CD4⁺CD8⁺ thymocytes available for pairing with nascent TCR- α proteins. Indeed, anti-TCR- α immunoprecipitates of untreated and TCR-signaled CD4⁺CD8⁺ thymocyte lysates contained labeled TCR- α proteins but did not contain labeled TCR- β proteins (data not shown), reflecting the pairing of nascent TCR- α proteins with preexistent TCR- β chains.

Differential Effect of TCR Engagement on RNAs Encoding Immunologically Important Molecules in CD4⁺CD8⁺ Thymocytes. To identify other early molecular responses of CD4⁺CD8⁺ thymocytes to TCR signaling, we examined the effect of TCR engagement on RNA transcripts encoding a number of immunologically important molecules. TCR engagement resulted specifically in increased levels of TCR- α and CD5 RNAs in immature CD4⁺CD8⁺ thymocytes, whereas RNAs encoding other TCR components such as TCR- β , CD3 ϵ , and ζ molecules were either unchanged or decreased (Fig. 6). Consistent with a positive selection signal, TCR signaling resulted in decreased RAG-1, CD4, and CD8 RNA levels in CD4⁺CD8⁺ thymocytes. It might be noted that decreased CD4 RNA levels in TCR-signaled CD4⁺CD8⁺ thymocytes (Fig. 6) is consistent with decreased CD4 protein synthesis in TCR-signaled CD4⁺CD8⁺ thymocytes (Fig. 3 B). These effects were specific for TCR engagement in that they were not observed upon antibody engagement of other surface molecules on CD4⁺CD8⁺ thymocytes including CD4, CD8, and CD28 (data not shown). Thus, TCR engagement on CD4⁺CD8⁺ thymocytes results in significantly increased levels of RNAs encoding TCR- α chains, but not other TCR component chains. In addition, these data show that engagement of surface TCR complexes on immature CD4⁺CD8⁺

thymocytes results in induction of molecular events consistent with a positive selection signal as it decreased expression of RAG-1, CD4, and CD8 RNAs, and increased expression of CD5 RNA.

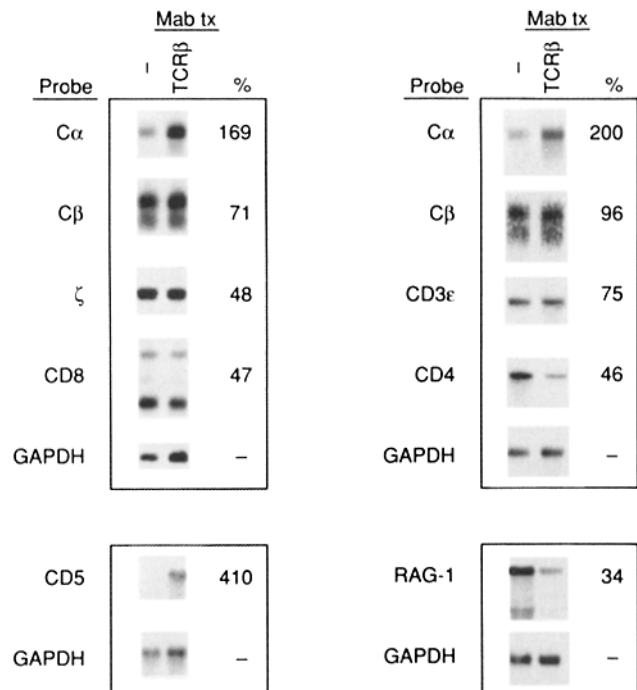


Figure 6. Effect of TCR cross-linking on steady state RNAs in CD4⁺CD8⁺ thymocytes. CD4⁺CD8⁺ thymocytes were cultured for 6 h at 37°C in the presence or absence of anti-TCR- β mAb and RNA analyzed by Northern blot hybridization using the indicated probes. The relative amounts of RNAs encoding the indicated proteins were quantitated by densitometry and are expressed in arbitrary densitometric units normalized to GAPDH. Multiple exposures of autoradiographs were scanned to ensure linearity.

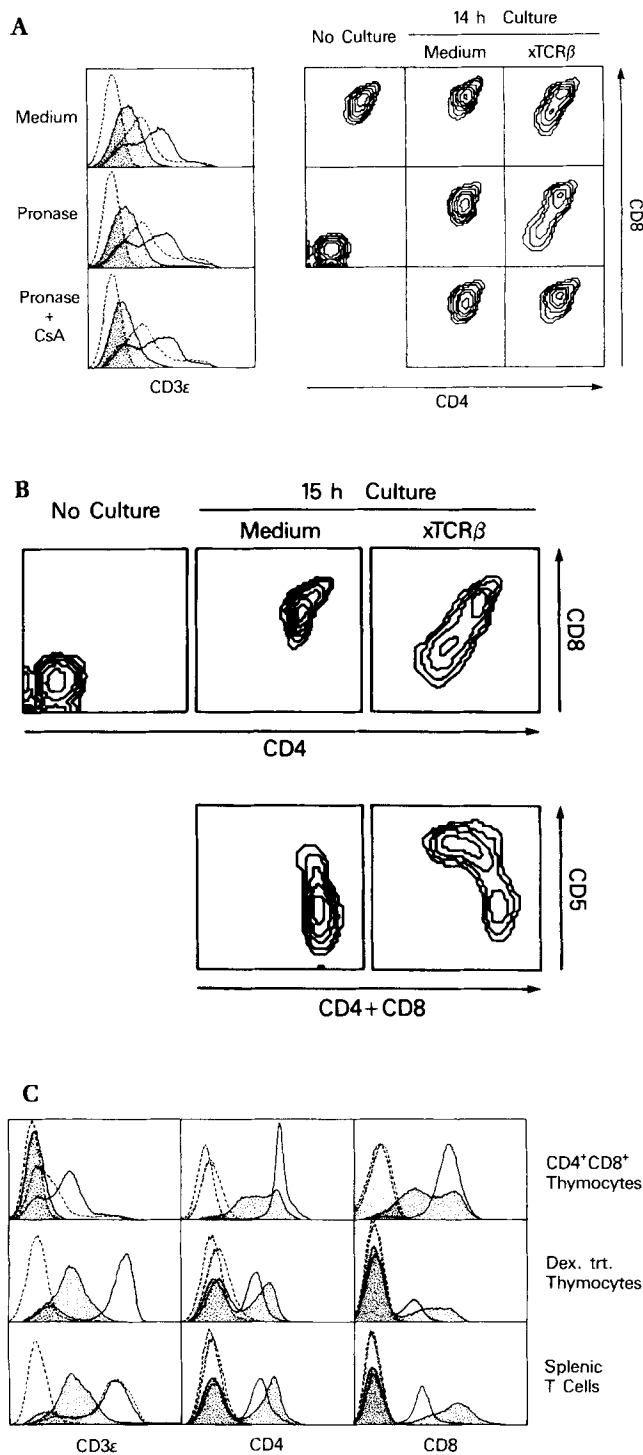


Figure 7. TCR engagement reduces surface expression of CD4/CD8 coreceptor molecules and increases CD5 levels on a single subpopulation of CD4⁺CD8⁺ thymocytes. (A) Purified CD4⁺CD8⁺ thymocytes were placed in 37°C single-cell suspension cultures for 14 h in the presence or absence of platebound mAb to TCR-β. Where indicated, CD4⁺CD8⁺ thymocytes were treated with pronase to remove preexisting surface CD4 and CD8 molecules before being placed into culture. Also where indicated, cultures contained the drug CsA. After culture, cells were stained with specific mAbs for surface expression of CD3ε, CD4, and CD8. One-color staining histograms of CD3ε expression include cells cultured in medium alone at either 4°C (dashed line) or 37°C (solid line); and cells

Identification of CD4⁺CD8⁺ Thymocyte Subpopulations Responding to TCR Engagement. To determine if TCR signaling decreased CD4/CD8 RNA levels and increased CD5 RNA levels in all CD4⁺CD8⁺ thymocytes or only in a subset of CD4⁺CD8⁺ thymocytes, we studied expression of CD4, CD8, and CD5 molecules by individual TCR-signaled CD4⁺CD8⁺ cells. As previously reported, CD4⁺CD8⁺ thymocytes placed in 37°C single cell suspension cultures spontaneously increase surface TCR expression as revealed by CD3ε staining (17), (Fig. 7 A). However, TCR engagement by mAb caused internalization of cross-linked TCR complexes and consequently reduced surface TCR levels. Interestingly, TCR engagement also caused decreased expression of both CD4 and CD8 coreceptor proteins on approximately 50% of CD4⁺CD8⁺ thymocytes (Fig. 7 A, top row).

To specifically examine the effect of TCR engagement on expression of newly synthesized CD4/CD8 proteins, CD4⁺CD8⁺ thymocytes before culture were treated with pronase to remove existing surface CD4/CD8 molecules (Fig. 7 A, middle row). As demonstrated, pronase stripping removed CD4 and CD8 molecules but did not affect surface TCR expression (Fig. 7 A, middle row). During 37°C culture, pronase-stripped cells reexpressed CD4 and CD8 proteins to nearly original levels (Fig. 7 A, middle row); CD4 and CD8 reexpression required de novo protein synthesis as it was blocked by protein synthesis inhibitors such as cyclohexamide (data not shown). Interestingly, TCR engagement inhibited surface CD4/CD8 reexpression on ~50% of pronase-stripped CD4⁺CD8⁺ thymocytes, without affecting CD4/CD8 reexpression on the remaining CD4⁺CD8⁺ thymocytes (Fig. 7 A, middle row). Inhibition of CD4/CD8 reexpression on pronase-stripped cells by TCR engagement required TCR signaling as inhibition of reexpression was blocked by CsA (Fig. 7 A, bottom row). As demonstrated, surface CD5 expression increased on precisely the same CD4⁺CD8⁺ thymocytes whose expression of newly synthesized CD4/CD8 molecules was inhibited by TCR signaling (Fig. 7 B), indicating that the consequences of TCR signaling occurred in a subset of individual thymocytes. Thus, individual CD4⁺CD8⁺ thymocytes respond to TCR engagement by decreased CD4/CD8 expression and increased CD5 expression and

cultured at 37°C with platebound TCR-β mAb (stippled curve). Negative control staining (shaded curve) is also indicated. Note that pronase treatment effectively removed surface CD4 and CD8 molecules but did not affect CD3ε expression. (B) Purified CD4⁺CD8⁺ thymocytes were treated with pronase before culture in the presence or absence of platebound anti-TCR-β mAb. After culture, cells were stained with specific mAbs for expression of CD4, CD5, and CD8. Unlike CD4/CD8 expression, CD5 expression was not affected by pronase treatment (data not shown). (C) Purified populations of CD4⁺CD8⁺ thymocytes, dexamethasone-resistant thymocytes, and splenic T cells were pronase treated and then cultured for 14 h with or without platebound anti-TCR-β mAb. After culture, cells were stained for surface expression of CD3ε, CD4, and CD8. One-color staining histograms include cells cultured in medium alone at either 4°C (dashed line) or 37°C (solid line); and cells cultured at 37°C with platebound anti-TCR-β mAb (stippled curve). Negative control staining (shaded curve) is also indicated.

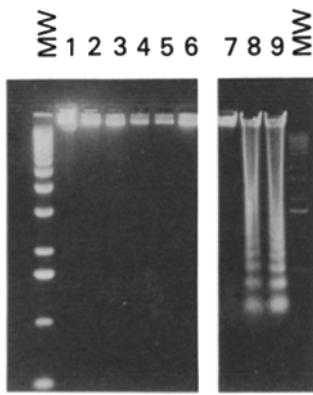


Figure 8. TCR cross-linking does not induce DNA degradation in CD4⁺CD8⁺ thymocyte subpopulations responding to TCR signaling. CD4⁺CD8⁺ thymocytes were cultured for 16 h in the presence or absence of platebound anti-TCR- β mAb. After culture, electronic cell sorting was used to separate CD4⁺CD8⁺ thymocytes into CD5^{hi} and CD5^{lo} cell populations. Genomic DNA was prepared and analyzed by agarose gel electrophoresis. CD4⁺CD8⁺ thymocytes were cultured in medium alone at either 4°C (lane

1) or 37°C (lane 2). At the conclusion of 37°C cultures with platebound anti-TCR- β mAb, CD4⁺CD8⁺ thymocytes were either unsorted (lane 3) or sorted into CD5^{lo} (lane 5) or CD5^{hi} cells (lanes 4 and 6). All lanes contain genomic DNA from 10⁶ cells with the exception of lanes 4 and 5, which contain genomic DNA from 4 × 10⁵ cells. As a positive control for DNA degradation, unsorted thymocytes were cultured for 4 h in PBS alone (lane 7), PBS containing dexamethasone at 10 μ g/ml (lane 8), or 100 μ g/ml (lane 9). MW, molecular weight markers.

represent a subpopulation of ~50% of CD4⁺CD8⁺ thymocytes.

Finally, we determined if the responses of CD4⁺CD8⁺ thymocytes to TCR signaling were developmentally regulated by comparing the effects of TCR signaling on CD4 and CD8 reexpression in immature CD4⁺CD8⁺ thymocytes, mature dexamethasone-resistant thymocytes, and mature splenic T cells (Fig. 7 C). TCR engagement decreased CD4 and CD8 reexpression on a subpopulation of immature CD4⁺CD8⁺ thymocytes, but, in contrast, it increased CD4 and CD8 expression on mature thymocytes and mature splenic T cells (Fig. 7 C). Thus, decreased CD4/CD8 coreceptor expression in response to TCR signaling is developmentally regulated as it occurs on a subset of immature CD4⁺CD8⁺ thymocytes but not on mature T cells.

Isolated TCR Engagement Does Not Induce DNA Fragmentation in Immature CD4⁺CD8⁺ Thymocytes. In certain circumstances TCR signaling in immature CD4⁺CD8⁺ thymocytes can induce apoptosis and DNA fragmentation (18–20). However, it has been uncertain whether isolated TCR signaling, in the absence of additional signals generated by interaction with antigen presenting cells, is sufficient to induce apoptosis in immature thymocytes. In fact, recent studies have demonstrated that induction of apoptosis in immature CD4⁺CD8⁺ thymocytes requires simultaneous signaling through both TCR and costimulator molecules (21, 22). As shown in Fig. 8, dexamethasone, a known mediator of apoptosis, did induce DNA fragmentation in cultured CD4⁺CD8⁺ thymocytes (Fig. 8, lanes 8 and 9); however, isolated TCR engagement did not induce significant DNA fragmentation in cultured CD4⁺CD8⁺ thymocytes (Fig. 8, lanes 1–3). To directly examine DNA fragmentation in the subset of CD4⁺CD8⁺ thymocytes responding to TCR engagement, TCR-signaled CD4⁺CD8⁺ thymocytes were sorted into CD5^{hi} and CD5^{lo} populations. As demonstrated,

isolated TCR signalling did not induce apoptosis in immature CD4⁺CD8⁺ thymocytes as DNA fragmentation was not detected in either CD5^{hi} or CD5^{lo} cells (Fig. 8, lanes 4–6).

Discussion

The present study has examined the molecular consequences of isolated TCR engagement on immature CD4⁺CD8⁺ thymocytes. We found that TCR signaling did not induce apoptosis of CD4⁺CD8⁺ thymocytes, but instead generated signals that significantly increased assembly of nascent TCR proteins into TCR complexes within the ER of CD4⁺CD8⁺ thymocytes. While TCR signaling in CD4⁺CD8⁺ thymocytes induced substantial increases in both TCR- α RNA levels and TCR- α protein synthetic rates, it did not improve the stability of nascent TCR- α proteins. Thus TCR signals compensate for, but do not correct, limitations placed on TCR assembly in developing thymocytes by the rapid degradation of nascent TCR- α proteins within the ER. Consequently, it is not until CD4⁺CD8⁺ thymocytes further differentiate into mature CD4⁺ or CD8⁺ T cells that the ER stability of nascent TCR- α proteins improves. In addition we found that individual immature CD4⁺CD8⁺ thymocytes also responded to TCR signaling by decreasing expression of RAG-1, CD4, and CD8, and by increasing expression of CD5. Taken together, these results indicate that isolated TCR engagement induces molecular events in a subset of CD4⁺CD8⁺ thymocytes characteristic of an initial positive selection signal and provide a molecular basis for increased surface TCR density on developing thymocytes that are undergoing positive selection within the thymus.

Assembly of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes competent for transport to the cell surface is severely limited in immature CD4⁺CD8⁺ thymocytes due to the unique instability of nascent TCR- α proteins within the ER of CD4⁺CD8⁺ thymocytes (6). As TCR- α/β expression increases during development, a molecular mechanism must exist for increasing the number of assembled TCR complexes present in immature CD4⁺CD8⁺ thymocytes that have been signaled to differentiate into mature T cells. The present finding that TCR signals in CD4⁺CD8⁺ thymocytes amplify TCR- α protein synthesis, which increases assembly of TCR complexes, provides a molecular basis for upregulation of TCR- α/β expression during positive selection of immature CD4⁺CD8⁺ thymocytes (23, 24). Indeed, we found that TCR signals in immature CD4⁺CD8⁺ thymocytes resulted in an approximately fourfold increase in synthesis of TCR- α proteins and a commensurate fourfold increase in assembly of complete TCR $\alpha\beta\gamma\delta\epsilon\zeta$ complexes. Thus, TCR signals increase TCR- α synthesis in CD4⁺CD8⁺ thymocytes and permit them to upregulate TCR expression despite the dramatic degradation of nascent TCR- α proteins occurring within their ER.

Interestingly, the median survival time of ~15 min for nascent TCR- α proteins was unchanged after TCR engagement in CD4⁺CD8⁺ thymocytes. Thus, whereas TCR signaling compensates for impaired formation of TCR complexes

in CD4⁺CD8⁺ thymocytes by increasing transcription and synthesis of nascent TCR- α chains, TCR engagement does not correct the limited survival of nascent TCR- α proteins within the ER of CD4⁺CD8⁺ thymocytes. Instability of unassembled TCR- α proteins in CD4⁺CD8⁺ thymocytes may relate to the recent suggestion that TCR- α proteins are ineffectively inserted into the ER lipid bilayer because their transmembrane region is unstable as an α -helix (25). Because the TCR- α transmembrane domain contains an ER degradation motif (26), failure to insert the transmembrane region into the lipid bilayer might expose the degradation motif to proteolytic enzymes in the ER lumen and result in accelerated degradation of nascent TCR- α proteins. Thus, the stability within the ER of nascent TCR- α proteins may be dependent upon their interaction with other ER proteins such as the molecular chaperone calnexin, which may promote retention of TCR- α chains in the lipid bilayer (27). It is possible that interaction of nascent TCR- α proteins with ER proteins such as calnexin may be relatively impaired in CD4⁺CD8⁺ thymocytes and it is an impairment of such intermolecular interactions that is primarily responsible for the instability of nascent TCR- α proteins in the ER of immature thymocytes. This possibility is currently under investigation.

It has recently been reported that the rate of synthesis of TCR- α chains is lower in immature CD4⁺CD8⁺ thymocytes than in mature thymocytes (28). We do not think this is correct because nascent TCR- α proteins are remarkably unstable in immature CD4⁺CD8⁺ thymocytes. Indeed, the rate of TCR- α synthesis in developing thymocytes is difficult to determine with accuracy as most of the nascent TCR- α proteins radiolabeled during the pulse period would have been degraded. In fact, the rate of TCR- α protein synthesis is probably equivalent in immature and mature T cells because these cells have comparable amounts of TCR- α RNA (29).

Amplified TCR- α protein synthesis in TCR-signaled CD4⁺CD8⁺ thymocytes resulted from increased TCR- α RNA levels which were specifically increased by TCR engagement as RNAs encoding other TCR component chains were not increased. In fact the number of ζ RNA transcripts were decreased in TCR-signaled CD4⁺CD8⁺ thymocytes

relative to untreated groups. The significance of this decrease in ζ RNA is unclear since ζ protein is synthesized in excess in CD4⁺CD8⁺ thymocytes due to limited formation of TCR $\alpha\beta\gamma\delta\epsilon$ intermediate complexes (6), (Fig. 1). It is notable that in the present study TCR signaling decreased expression of both CD4 and CD8 coreceptor molecules in responding CD4⁺CD8⁺ thymocytes rather than decreasing expression of only one coreceptor molecule. Perhaps the loss of transcripts encoding both coreceptor molecules reflects the absence of any concurrent coreceptor signals in our experimental system. Alternatively, it is possible that rapid elimination of RNA transcripts encoding both CD4 and CD8 coreceptor molecules reflects the initial consequence of TCR-mediated differentiation signals, and that CD4 or CD8 RNA expression is selectively reinduced in responding CD4⁺CD8⁺ thymocytes by subsequent signals transduced by remaining CD4 or CD8 surface proteins. Even though decreased expression of both CD4 and CD8 surface molecules has been reported in CD4⁺CD8⁺ thymocytes undergoing clonal deletion upon in vitro stimulation with antigen-presenting cells (18), the results of the current study demonstrate that decreased CD4/CD8 expression in response to TCR signaling is not restricted to developing thymocytes undergoing clonal deletion.

Finally, we found that only 50% of CD4⁺CD8⁺ thymocytes respond to TCR engagement. The 50% of CD4⁺CD8⁺ thymocytes that failed to respond might have been unresponsive because they express insufficient amounts of surface TCR, or, alternatively, because their surface TCR are not functionally coupled to intracellular signaling pathways. Expression of uncoupled TCR complexes might either be a result of developmental immaturity or a result of previous in vivo signaling events.

In conclusion, the present study demonstrates that TCR signals induce increased TCR- α RNA levels and increased TCR- α protein synthesis in a subset of immature CD4⁺CD8⁺ thymocytes, which in turn, result in increased assembly of complete TCR complexes within the ER. These results define amplified TCR- α expression and increased formation of nascent TCR complexes as an early event in the intrathymic selection of developing thymocytes.

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Address correspondence to Dr. Kelly P. Kearse, Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bldg. 10, Rm. 4B-17, Bethesda, MD 20892.

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