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Critical Relationship Between TCR Signaling Potential and TCR Affinity During Thymocyte Selection

Paul E. Love,* Jan Lee,[1†] and Elizabeth W. Shores[2†]

Whether a developing thymocyte becomes positively or negatively selected is thought to be determined by the affinity/avidity of its TCR for MHC/peptide ligands expressed in the thymus. Presumably, differences in affinity translate into differences in the potency of the ensuing TCR-mediated signals, and these differences in signal strength determine the outcome of thymocyte selection. However, there is little direct evidence establishing a relationship between TCR-ligand affinity and signal strength during positive and negative selection. The TCR complex contains multiple signaling motifs, known as immunoreceptor tyrosine-based activation motifs (ITAMs) that are required for T cell activation. To examine the effects of TCR signal strength on selection, the signaling potential of the TCR was modified by substituting transgenic TCR ζ-chains containing either three, one, or zero ITAMs for endogenous (3-ITAM) ζ-chain. These ζ-chain variants were then bred into different $\alpha\beta$TCR transgenic backgrounds. We report that reductions in TCR signaling potential have distinct effects on the selection of thymocytes expressing different TCRs, and that the requirement for ζ-chain ITAMs critically depends upon the specificity and apparently, affinity, of the TCR for its selecting ligand(s). ***The Journal of Immunology*****, 2000, 165: 3080–3087.**

As T cells develop in the thymus, they undergo a selection process resulting in the survival of functionally competent, self MHC-restricted cells. Current theories posit a major role for the affinity of the interaction between the TCR and intrathymic self-ligands in controlling the outcome of thymocyte selection (reviewed in Refs. 1 and 2). According to these theories, the majority of thymocytes express $\alpha\beta$TCRs with negligible specificity for self-MHC/ligand and eventually die through a process termed "death by neglect". At the other extreme, thymocytes that express TCRs with high affinity for self-ligands are negatively selected (physically or functionally deleted from the TCR repertoire). Finally, cells that express $\alpha\beta$TCRs with the appropriate specificity and affinity are positively selected and mature to either $CD4^+CD8^-$ (CD4-single positive (SP)[3]) $\alpha\beta TCR^{high}$ or $CD4^-CD8^+$ (CD8-SP) $\alpha\beta TCR^{high}$ cells.

It has been presumed that differences in the affinity/avidity of TCR-ligand interaction are translated into differences in TCR-mediated signal strength, which in turn, ultimately regulate the outcome of thymocyte selection. However, there is little direct evidence linking the affinity/avidity of the TCR-ligand interaction with the potency of the ensuing proximal TCR signals required for positive vs negative selection. One approach to address this relationship would be to directly measure biochemical differences in TCR signal potency upon thymocyte stimulation with ligands known to cause their positive or negative selection. However, the interpretation of such studies is limited by the need to examine responses of bulk populations of cells where only a small subset of cells may be undergoing selection

In this study, we approached this question from a different perspective, asking how alterations in TCR signaling potential effect selection of thymocytes expressing TCRs that bind selecting ligands with different affinities. Immunoreceptor tyrosine-based activation motifs (ITAMs) present within the invariant TCR subunits (CD3 γ-, δ-, ϵ-, and ζ-chain) are critical for TCR signaling (3–6). ζ-chain possesses three ITAMs, and because it exists in the TCR as a dimer, contributes 6 of the 10 potential ITAMs to a TCR (7). For these studies, we used mice that were genetically altered to express different numbers of TCR ζ-chain ITAMs (8). These ζ-chain variants were then bred into different $\alpha\beta$TCR transgenic backgrounds, and the effects on thymocyte selection were compared. Our results demonstrate that alterations in the number of ITAMs within the TCR complex have distinct effects on thymocyte selection depending upon the specificity, and presumably affinity, of the TCR for its selecting ligand(s). These data provide direct evidence in support of the idea that differences in TCR signaling potential can regulate the outcome of thymocyte selection. In addition, our results demonstrate that the number of TCR ITAMs required for efficient positive or negative selection varies depending upon the affinity of the TCR/ligand interaction.

Materials and Methods

Transgenic mice

The generation of $\zeta\eta$-deficient (designated $\zeta^{-/-}$; Ref. 9), ζ-3 ITAM transgenic, ζ-1 ITAM transgenic, and ζ-0 ITAM transgenic mice (8) has been described previously. Although multiple founder lines were generated for each ζ-variant transgene, those founder lines expressing similar levels of surface TCR were selected for these studies. It should be noted that T cells from each ζ-variant transgenic line expressed slightly higher levels of surface TCR than did T cells from mice expressing endogenous ζ-chain (8). Therefore, it is important to compare thymocyte selection among the different ζ-variant transgenic lines (3, 1, or 0 ITAMs). H-Y (10), DO11.10 (11), 2C (12), and P14 (13) $\alpha\beta$TCR transgenes were bred into the $\zeta^{-/-}$, ζ-ITAM background in our animal facility. For some studies, the $\zeta^{-/-}$,

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[3] Abbreviations used in this paper: SP, single positive; ITAMs, immunoreceptor tyrosine-based activation motifs; FCM, flow cytometry; QR, Red670; DN, double negative; DO11, DO11.10 mice.

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ζ-ITAM αβTCR transgenic genotype was bred into a $RAG^{-/-}$ background (14). All mice were housed in microisolator cages and fed sterile food and water.

Proliferation assays

Single-cell suspensions were prepared from lymph nodes from normal ($\zeta^{+/+}$) or transgene-reconstituted ($\zeta^{-/-}$) mice. CD4 T cells were purified by panning after incubating lymph node cells with Abs to CD8, B220, and MAC-1 for 30 min. Alternatively, CD8 T cells were purified by panning after incubating lymph node cells with Abs to CD4, B220, and MAC-1 for 30 min. Cells were washed, placed on rabbit anti-mouse Ig-coated plates at 25°C 5%CO_2, and collected after 1–2 h. Accessory cells and APCs were prepared from spleen cell suspensions of C57BL/6 or B10.D2 mice. APCs were depleted of T cells with anti-Thy1.2 + C′ and irradiated with 3000 rad. Responder T cells (1×10^5) were combined with 5×10^5 accessory cells in flat-bottom 96-well plates in the presence or absence of the indicated stimulants. Peptides $OVA_{323–339}$ (ISQAVHAAHAEINEAGR), p33 (KAVYNFATM), A4Y (KAVANFATM), and A6F (KAVYNAATM) were synthesized in the Food and Drug Administration Core Facility (Bethesda, MD) and were added to culture at the indicated concentrations. Following stimulation for 48 h, cells were pulsed for 12 h with 1 μCi [^{3}H]thymidine and harvested. Abs used for panning, including anti-CD8 (2.43), anti-CD4 (GK1.5), anti-B220 (6B2), and anti-MAC-1 (M1/70), were purified with protein G from tissue culture supernatant generated from B cell hybridomas grown on an artificial capillary system (Cellco, Germantown, MD).

IL-2 production

Cells were stimulated as described above and culture supernatants were harvested after 48 h. IL-2 ELISAs were performed using the reagents and protocol obtained from PharMingen (San Diego, CA).

Flow cytometry

For multicolor flow cytometry (FCM), thymocytes or lymph node cells were first incubated with Ab to the Fc receptor (mAb 2.4G2) to prevent FcR binding. For two- and three-color FCM, cells were incubated with FITC-conjugated Abs, PE-conjugated Abs, and Red670 (QR)-conjugated Abs. The FCM was performed on a Becton Dickinson (Mountain View, CA) FACScan using standard CellQuest (Becton Dickinson) software. Data were collected on $10–20 \times 10^4$ viable cells as determined by forward and side light scatter. The majority of the mAbs used for FCM analysis were purchased from PharMingen and included biotinylated anti-CD4 (RM4.5) and PE-anti-CD8 (53-6.7), FITC and PE-anti-CD3ε (145-2C11), and FITC-anti-Vα2 (B20.6). Anti-2C TCR (1B2), anti-DO11.10 TCR (KJ126), and anti-H-Y clonotypic receptor (T3.70) were purified from cell culture supernatants and labeled with FITC in our laboratory.

Induction of cell surface CD69 expression

Total thymocytes or lymph node cells were prepared from DO11 transgenic mice. T cell-depleted APC were prepared as described above and were >98% B220/MAC-1-positive. Thymocytes or lymph node cells (1×10^6) were incubated with 3×10^6 APC from C57BL/6 or B10.D2 mice overnight at 37°C. Cells were stained with the indicated Abs. To exclude APC from analysis, cells were stained with anti-B220-biotin and anti-MAC-1-biotin in conjunction with avidin-QR, and QR-negative cells were analyzed.

Results

Reduction in TCR signaling potential has distinct affects on positive selection depending on the specificity of the αβTCR

We began by comparing the effects of reduced TCR signaling potential (as a consequence of reduction in the number of ζ-chain ITAMs) on the selection of thymocytes expressing transgenes encoding distinct αβTCRs specific for peptides expressed in the context of $H\text{-}2^b$ class I molecules. Hence, the efficiency of positive selection could be monitored by examining the number of CD8-SP, TCR clonotype-positive thymocytes and peripheral T cells. Positive selection in H-Y TCR transgenic female mice is thought to be mediated by a relatively low affinity interaction between the TCR and its selecting ligand(s) as judged by the low numbers of clonotype-TCR positive CD8-SP thymocytes generated in these mice and the effects of CD8 over-expression on their development (15). Previous studies in a $RAG^{+/+}$ background (19) and confirmed in this paper in a $RAG^{-/-}$ background (Fig. 1*A*, Table I) demonstrate that the efficiency of positive selection for cells bearing the H-Y TCR varies directly with the number of ζ-chain ITAMs present in the TCR complex. Specifically, the number of CD8-SP, clonotype-TCR positive ($T3.70^+$) thymocytes and lymph node T cells is dramatically reduced in $\zeta^{-/-}$ mice reconstituted with the ζ-chain variant lacking ITAMs (ζ-0 ITAM) compared with H-Y mice reconstituted with the ζ-variant containing 3 ITAMs (ζ-3 ITAM). Interestingly, the efficiency of thymocyte selection was intermediate in $RAG^{-/-}$ ζ-1 ITAM mice. Together, these data demonstrate that decreasing the signaling potential of the TCR markedly impairs positive selection of H-Y-specific ($T3.70^+$) thymocytes.

We next examined thymocyte selection in mice expressing the 2C αβTCR (detected by the 1B2 clonotype-specific Ab), which is thought to have a higher affinity for its positively selecting ligand than the H-Y TCR (15, 17, 18). In contrast to results observed in H-Y TCR transgenic mice, thymocytes expressing the 2C TCR were capable of undergoing positive selection in the absence of ζ-chain ITAMs. Indeed, in 2C TCR transgenic mice, efficient positive selection was observed regardless of whether the ζ-chain contained 3, 1, or 0 ITAMS (Fig. 1*B*, Table I). In fact, the absolute number of $1B2^+$ CD8-SP thymocytes was higher in ζ-1 ITAM mice than in either $\zeta^{+/+}$ or ζ-3 ITAM mice, suggesting that reduction of the 2C TCR signaling potential actually enhanced the efficiency of positive selection (Table I). Similar findings were observed in the $RAG^{-/-}$ background, ruling out a contribution by other TCRs that might be coexpressed with the 2C TCR (data not shown).

Importantly, $2C\ TCR^+$ CD8-SP T cells were also observed in the peripheral lymphoid organs of all the ζ-variant reconstituted mice. These cells were found to be functionally competent (responding to their cognate Ag, L^d) and present in large numbers regardless of the number of ITAMs present in the ζ-chain subunit of the TCR (Figs. 1*B* and 2*A*, Table I). In contrast to CD8-SP $1B2^+$ cells, reduction in the number of TCR-ITAMs did affect the generation of $CD4^-CD8^-$ (double negative (DN)) $1B2^+$ T cells. Specifically, the number of DN $1B2^+$ T cells detected in the thymus and lymph nodes of mice expressing TCRs containing ζ-0 ITAM or ζ-1 ITAM was markedly reduced compared with those present in mice expressing endogenous ζ-chain or the equivalent ζ-3 ITAM transgene (Fig. 2*B*, Table II). These results are consistent with the interpretation that upon decreasing the TCR signaling potential (by reducing the number of ζ-chain ITAMs), T cell selection and/or survival becomes increasingly coreceptor dependent (19–20).

We also examined how reductions in TCR signaling potential influenced the selection of thymocytes expressing the P14 αβTCR transgene (detected by an anti-Vα2 Ab). Recent studies in a model system similar to our own reported the presence of peripheral CD8-SP, $V\alpha2^+$ T cells in mice that express TCRs containing ζ-chains lacking functional ITAMs (21). However, thymocytes were not examined in that study, an important point because the efficiency of positive selection may not necessarily be reflected by the number of peripheral T cells. Indeed, studies in unreconstituted $\zeta^{-/-}$ mice have shown that significant numbers of SP T cells can accumulate in the periphery despite their near absence in the thymus (9). Interestingly, examination of the thymus in our ζ-variant mice revealed that although positive selection of $V\alpha2^+$ thymocytes did not absolutely require ζ-chain ITAMs, the efficiency of positive selection was slightly reduced in ζ-0 ITAM mice compared with $\zeta^{+/+}$ or ζ-3 ITAM transgenic mice (Fig. 1*C*, Table I). Nonetheless, the lymph nodes of mice expressing ζ-chains with 3

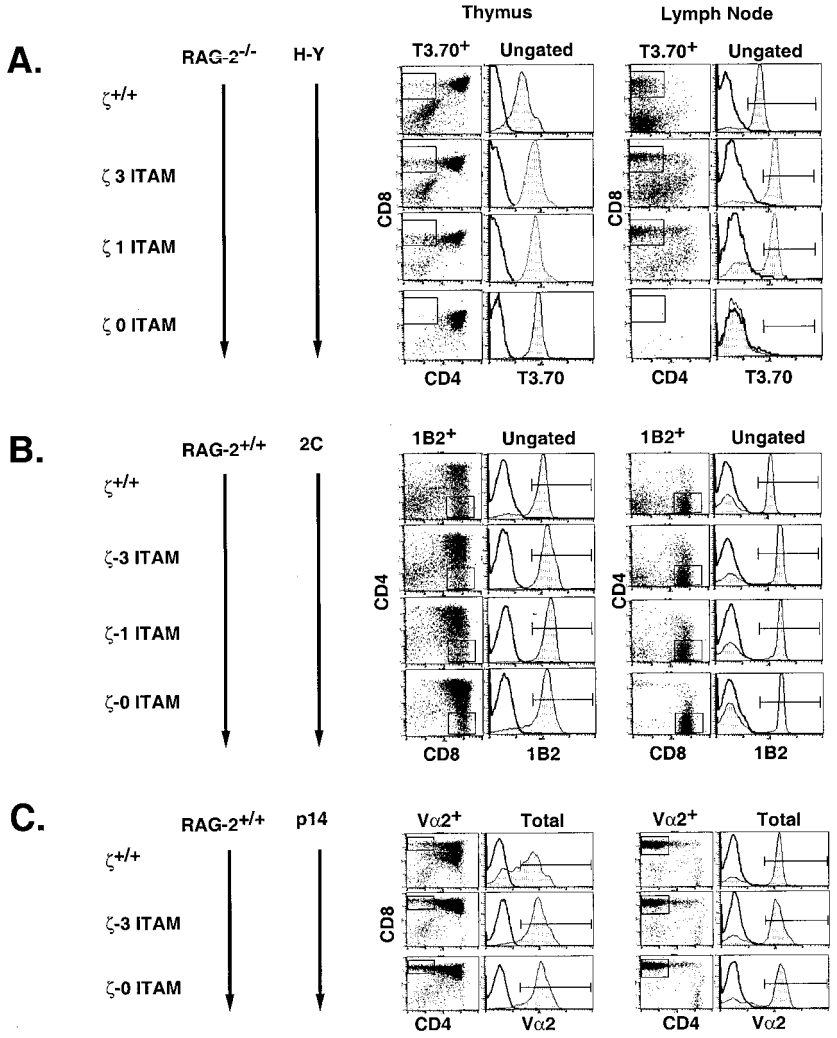

FIGURE 1. Positive selection of thymocytes expressing transgenic MHC H-2b class I-restricted $\alpha\beta$TCRs. Data show immunofluorescence and multicolor FCM analysis of thymocytes and lymph node T cells from H-2^b, adult (6- to 12-wk-old) mice. Three-color FCM analysis was performed on cells stained with anti-clonotypic Abs conjugated to FITC, anti-CD8-PE, and anti-CD4-QR. CD4 vs CD8 two-color profiles are displayed on either total cells (thymocytes or total lymph node cells) or on clonotype-positive cells, as indicated by the marker in the single-color histograms. Single-color profiles (shaded area) depict staining with anti-clonotypic Abs on total thymocytes or lymph node cells. Solid lines reflect staining with negative control Ab. *A*, Female RAG$^{-/-}$, H-Y$^+$ TCR transgenic mice. $\zeta^{+/+}$ or $\zeta^{-/-}$ mice reconstituted with transgenes that encode variant ζ-chains that contain 3 ITAMs (*ζ-3 ITAM*), a single ITAM (*ζ-1 ITAM*), or a ζ-chain that lacks ITAMs (*ζ-0 ITAM*) are shown. The clonotypic TCR is detected with the T3.70 Ab. *B*, RAG$^{+/+}$, 2C TCR transgenic mice. The 2C clonotypic TCR is detected with the 1B2 Ab. *C*, RAG$^{+/+}$ P14 TCR transgenic mice. The P14 clonotypic TCR is detected with the anti-Vα2 Ab.

or 0 ITAMs contained large numbers of Vα2$^+$ CD8-SP T cells, which were able to respond to their cognate Ag composed of a peptide of the lymphocytic choriomeningitis virus (p33) or its variants (A4Y and A64) in the context of H-2D^b (Figs. 1*C* and 2*B*, Table I; Refs. 13, 22, and 23).

Reduction in TCR signaling potential has distinct affects on selection in a manner dependent on the nature of the selecting ligand

We next examined thymocyte selection under conditions in which the specificity of the $\alpha\beta$TCR was held constant, but the ligand mediating thymocyte selection was varied. For these studies we used mice expressing the MHC class II-restricted DO11.10 (DO11) transgenic $\alpha\beta$TCR, where positive selection is scored by the generation CD4-SP thymocytes expressing the clonotypic TCR (detected by the KJ126 Ab). Thymocytes expressing this TCR are positively selected in A^d mice, but undergo negative selection in A^b mice, presumably because the DO11 TCR binds with higher affinity to A^b (+ self-ligand) than to A^d (+ self-ligand; Refs. 24 and 25). Consistent with its higher affinity for A^b than A^d, coculture of cells from DO11 A$^{d/d}$ transgenic mice with APC from C57BL/6 (H-2$^{b/b}$) but not B10.D2 (H-2 $^{d/d}$) mice stimulated upregulation of the early activation marker, CD69, on both double positive and CD4-SP thymocytes and lymph node T cells (Fig. 3).

Table I. *Generation of CD8-SP$^+$ thymocytes and lymph node T cells*

	ζ Transgene	H-Y RAG$^{-/-}$ T3.70$^+$ CD8-SP	2C RAG$^{+/+}$ 1B2$^+$ CD8-SP	P14 RAG$^{+/+}$ Vα2$^+$ CD8-SP
Thymus[a]	$\zeta^{+/+}$	31.2 ± 12 (n = 2)	15.5 ± 12 (n = 7)	6.5 ± 1.3 (n = 4)
	ζ-3 ITAM	42.3 ± 6 (n = 5)	13.3 ± 3.2 (n = 7)	6.2 ± 2.4 (n = 6)
	ζ-1 ITAM	18.4 ± 7 (n = 5)	19.1 ± 3.5 (n = 7)	ND
	ζ-0 ITAM	0.7 ± 0.2 (n = 7)	15.0 ± 1.6 (n = 9)	4.8 ± 1.4 (n = 8)
Lymph Node[b]	$\zeta^{+/+}$	19.6 ± 9 (n = 3)	31.2 ± 5.0 (n = 7)	63.0 ± 5.3 (n = 4)
	ζ-3 ITAM	33.8 ± 2 (n = 5)	47.4 ± 3.0 (n = 6)	57.1 ± 12.1 (n = 6)
	ζ-1 ITAM	16.6 ± 7 (n = 5)	51.7 ± 1.5 (n = 7)	ND
	ζ-0 ITAM	0.3 ± 0.1 (n = 7)	44.9 ± 3.2 (n = 9)	35.9 ± 6.7 (n = 8)

[a] Absolute number (× 10^5) of CD8-SP, RAG$^{-/-}$, H-Y clonotype-positive thymocytes. Absolute number (× 10^6) of CD8-SP, RAG$^{+/+}$, 2C, or P14 clonotype-positive thymocytes.

[b] Percent of CD8-SP clonotype-positive lymph node cells.

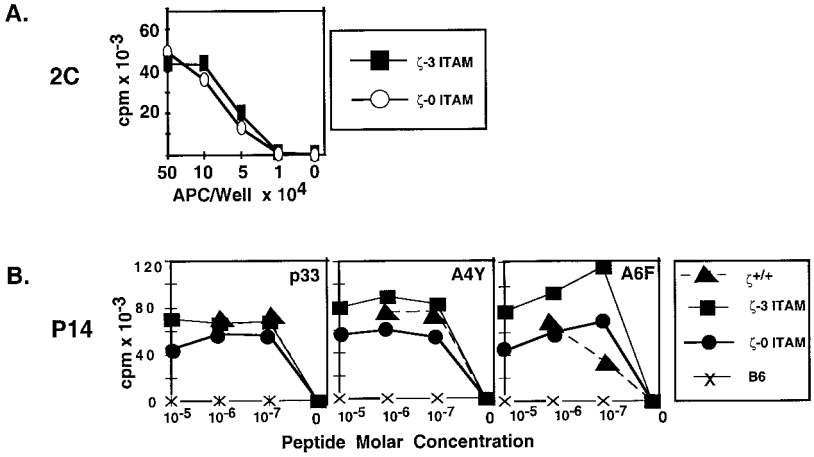

FIGURE 2. Functional response of T cells from 2C and P14 TCR transgenic mice. $CD8^+$ lymph node T cells from normal C57BL/6, 2C, or P14 transgenic mice were prepared as described in *Materials and Methods*. *A*, $CD8^+$ lymph node T cells from 2C TCR transgenic mice were cultured with indicated numbers of T-depleted irradiated APC from the spleen of B10.D2 mice. *B*, $CD8^+$ lymph node T cells were cultured with T-depleted APC from C57BL/6 mice in the absence or presence of varying concentration of p33, A4Y, or A6F. Following stimulation for 48 h, cells were pulsed for 12 h with 1 μCi [^{3}H]thymidine and harvested.

We first examined positive selection of thymocytes expressing the DO11 TCR in an $A^{d/d}$ background. Positive selection was markedly impaired in $A^{d/d}$ DO11 ζ-0 ITAM mice (Figs. 4A and 5) compared with $\zeta^{+/+}$ or ζ-3 ITAM mice, which contained large numbers of DO11 TCR^{high} ($KJ126^{high}$) CD4-SP thymocytes (Figs. 4*A* and 5). An intermediate phenotype was observed in DO11 ζ-1 ITAM mice (Figs. 4*A* and 5). Interestingly, although positive selection of DO11 TCR transgenic thymocytes was impaired in the absence of ζ-chain ITAMs, it was not completely abrogated. Indeed, the peripheral lymph nodes of DO11 ζ-0 ITAM mice contained significant numbers of CD4-SP $KJ126^+$ T cells, albeit at a reduced frequency compared with $\zeta^{+/+}$, ζ-3 ITAM, or ζ-1 ITAM mice in which nearly all of the CD4-SP T cells expressed the transgenic TCR (KJ126; Fig. 4*A*). Nonetheless, the $KJ126^+$ LN T cells from ζ-0 ITAM mice were able to respond to their cognate Ag, $A^d + OVA_{323–329}$ (11, 26), proliferating and producing cytokines in a manner similar to those from ζ-3 ITAM DO11 transgenic mice (Table III). The slightly reduced response generated by T cells from ζ-0 ITAM mice is likely due to the fact that the CD4-SP T cell compartment in ζ-0 ITAM mice contains a lower percentage of $KJ126^+$ cells than do CD4-SP T cells from ζ-3 ITAM or ζ-1 ITAM mice (Fig. 4*A*, *far right panels*).

Because the DO11 TCR is reported to have a higher affinity for A^b than A^d (24, 25), we predicted that the presence of A^b might improve positive selection in ζ-0 ITAM mice. Indeed an increase in the percentage and total numbers of $KJ126^+$ $CD4^+$ thymocytes was noted in (heterozygous) $A^{b/d}$ $DO11^+$ ζ-1 ITAM and ζ-0 ITAM mice (Figs. 4*B* and 5). In addition, nearly all lymph node CD4-SP T cells from ζ-0 ITAM mice expressed the transgenic TCR, whereas only ~50% of CD4-SP T cells were $KJ126^+$ in $A^{d/d}$ mice (Fig. 4, *A* and *B*). Moreover, cells from $A^{b/d}$ ζ-0 ITAM mice were able to respond to Ag by proliferating and producing cytokines (data not shown). In contrast, thymocytes from $A^{b/d}$ $\zeta^{+/+}$ and ζ-3 ITAM DO11 TCR transgenic mice exhibited impaired positive selection and instead appeared to be undergoing negative selection. (Fig. 4*B*). Consistent with a shift from positive to partial negative selection, <50% of CD4-SP lymph node T cells expressed the clonotypic TCR in $A^{d/b}$ mice, whereas in $A^{d/d}$ mice, nearly 100% of CD4-SP cells were $KJ126^+$ (Fig. 4, *A* and *B*)

Finally, we assessed thymocyte selection in $A^{b/b}$ DO11 TCR transgenic mice. Regardless of the number ITAMs contained in the TCR ζ-chain, thymi obtained from $A^{b/b}$ mice had reduced cellularity (10–39%) compared with thymi from $A^{d/d}$ DO11 TCR transgenic mice (data not shown), consistent with the idea that A^b promotes negative selection of DO11 TCR^+ thymocytes. However, thymocytes and lymph node T cells from mice expressing the endogenous or 3 ITAM transgenic ζ-chain ($\zeta^{+/+}$ or ζ-3 ITAM mice) exhibited a dramatically different phenotype from those obtained from ζ-0 ITAM mice (Fig. 4*C*). First, whereas only a small percentage of thymocytes from $A^{b/b}$ $\zeta^{+/+}$ or ζ-3 ITAM mice expressed KJ126, most thymocytes from $A^{b/b}$ ζ-0 ITAM mice were $KJ126^+$ (Fig. 4*C*, *left panels*). Second, and most importantly, although low numbers of $KJ126^+$ CD4-SP thymocytes were present in $\zeta^{+/+}$ and ζ-3 ITAM mice, these cells did not survive as they were nearly absent in the periphery (Fig. 6*C*, *right panels*). In fact, the predominant populations of lymph node T cells expressing the DO11 TCR in $\zeta^{+/+}$ and ζ-3 ITAM mice consisted of $CD4^-CD8^-$ and $CD8^+$ T cells (data not shown), indicating that most $KJ126^+$ CD4-SP thymocytes undergo negative selection in $A^{b/b}$ mice. In contrast, the lymph nodes of $A^{b/b}$ ζ-0 ITAM mice contained relatively large numbers of $KJ126^+$ CD4-SP T cells (Fig. 4*C*) that were able to proliferate in response to cognate Ag (data not shown). Together, these data indicate that in $A^{b/b}$ mice, T cells expressing the DO11 TCR are negatively selected when the TCR contains a ζ-chain with 3-ITAMs but in mice expressing TCRs with a signaling-deficient ζ-chain, negative selection is impaired to the extent that $KJ126^+$ CD4-SP cells are positively selected in the thymus and can populate the peripheral lymphoid organs.

Discussion

Current models of thymocyte selection presume that alterations in TCR-ligand binding strength (affinity) are translated into quantitative difference is TCR-mediated signal strength and it is the potency of this signal that regulates the outcome of positive and negative selection. Nonetheless, little direct evidence exists to support this notion. In this study we have sought to examine the relationship between binding strength and signal strength during thymocyte selection. Specifically, we have examined how alterations

Table II. *Generation of $CD4^-CD8^-$ (DN) thymocytes and lymph node T cells*

ζ Transgene		2C $1B2^+$ DN
Thymus[a]	$\zeta^{+/+}$	5.3 ± 1.0 (n = 7)
	ζ-3 ITAM	6.2 ± 1.2 (n = 7)
	ζ-1 ITAM	3.8 ± 0.9 (n = 7)
	ζ-0 ITAM	1.0 ± 0.6 (n = 9)
Lymph Node[b]	$\zeta^{+/+}$	12.7 ± 3.2 (n = 7)
	ζ-3 ITAM	12.7 ± 2.3 (n = 6)
	ζ-1 ITAM	5.9 ± 2.0 (n = 7)
	ζ-0 ITAM	1.0 ± 0.5 (n = 9)

[a] Absolute number ($\times 10^6$) of DN clonotype-positive thymocytes.
[b] Percent of DN clonotype-positive lymph node cells.

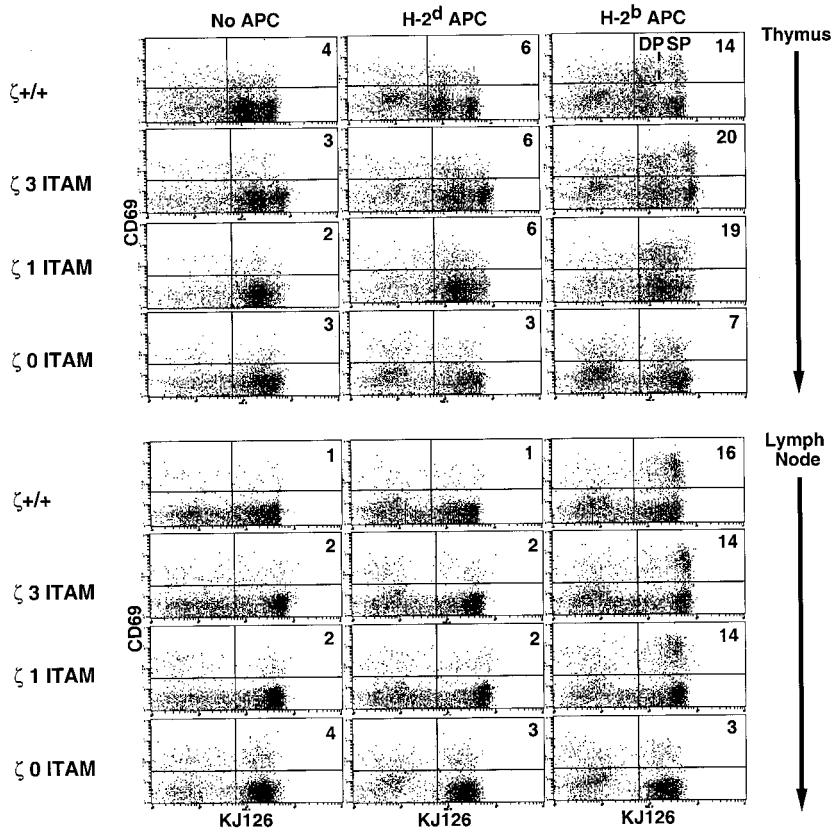

FIGURE 3. CD69 up-regulation stimulated by H-2^b but not H-2^d APC. Total thymocytes or lymph node cells were prepared from H-2$^{d/d}$ DO11 TCR$^+$ $\zeta^{+/+}$, ζ-3 ITAM, ζ-1 ITAM, and ζ-0 ITAM transgenic mice. T cell-depleted APC were prepared as described in *Materials and Methods*. Thymocytes or lymph node cells (1×10^6) were incubated at 37°C for 18 h in the absence (*No APC*) or presence of 3×10^6 APC from C57BL/6 (*H-2^b APC*) or B10. D2 (*H-2^d APC*) mice. Analysis of APC revealed that they were >98% positive upon staining with anti-B220 and anti-MAC-1 Abs. Therefore, to exclude APC from analysis, cells were stained with anti-B220-biotin and anti-MAC-1-biotin in conjunction with avidin-QR, and the data shown represent QR-negative cells. Cultured cells were also stained with the clonotype-specific Ab, KJ126 (FITC) and anti-CD69-PE. The numbers in the quadrants represent KJ126$^+$ CD69$^+$ cells. Two populations of cells defined by intensity of KJ126 staining can be observed. In analysis not shown, it was observed that cells staining most intensely with KJ126 (*upper right panel*) represent predominantly CD4-SP cells (*SP*), whereas cells staining with less intensity represent predominantly double positive thymocytes (*DP*).

in TCR signal strength affect the requirement for the affinity of the TCR ligand interaction required to attain the outcome of either positive or negative selection. We used two experimental strategies to address this issue. In the first approach, we compared thymocyte selection among mice expressing a variety of class I-restricted $\alpha\beta$TCR, which are thought to have different affinities for their intrathymic ligands and examined effects on selection when the TCR signaling potential was decreased. In the second strategy, we examined thymocyte selection in mice expressing a single class II-restricted $\alpha\beta$TCR transgene but introduced thymic ligands that are thought to bind with distinct affinities to that $\alpha\beta$TCR. Our results demonstrate that reduction of the TCR signaling potential has distinct effects on both positive and negative selection in a manner dependent on the specificity, and presumably affinity, of the TCR for self-ligands.

These findings are consistent with a model for thymocyte selection in which a direct relationship exists between the affinity of the TCR for its selecting ligand and the strength of the TCR-mediated signals that mediate the outcome of thymocyte selection. (Fig. 6). A prediction of this model is that the effect on positive selection of reducing the TCR signaling potential might vary among TCRs that bind with different affinities to their selecting ligand(s). Thus a TCR that binds with very low affinity to its selecting ligand (Fig. 6, TCR #1) may require the full compliment of TCR ITAMs to initiate signals for positive selection. Positive selection of thymocytes expressing other TCRs that bind with higher affinity to their selecting ligands might be unaffected upon impairment of the TCR signaling apparatus (TCR #2). Finally, for TCRs that bind to self-ligands with relatively high affinity, reduction of the TCR signaling potential may enable these thymocytes to escape negative selection, seemingly improving positive selection (TCR #3). In our current studies, we believe we have identified such receptors, H-Y, P14, and 2C, respectively. At present, the relative affinity of the H-Y, P14, and 2C TCRs for their selecting ligands has not been established. However, experimental data exists to support the notion that the affinity of 2C TCR for its selecting ligand(s) is higher than that of the H-Y TCR for its ligand. When a transgene encoding CD8 (resulting in higher surface expression of CD8) was introduced into these backgrounds (15, 17, 18) positive selection of H-Y$^+$ CD8-SP cells was enhanced, whereas introduction of the same transgene into 2C TCR transgenic mice resulted in negative selection. Based on these probable differences in TCR-ligand affinities, we predicted that positive selection of 2C transgenic thymocytes might be less dependent on ζ-chain ITAMs than thymocytes from H-Y transgenic mice, a prediction borne out in our current studies.

To complement studies using mice expressing different $\alpha\beta$TCRs, thymocyte selection was also examined under conditions in which the $\alpha\beta$TCR was held constant but the nature of the thymic ligand mediating selection was varied. Positive selection of thymocytes expressing the DO11 TCR occurs in mice expressing I-A^d but this receptor has been purported to have a higher affinity for I-A^b (24). Consequently, we examined the selection of thymocytes expressing the DO11 TCR in A$^{d/d}$, A$^{d/b}$, and A$^{b/b}$ backgrounds, predicting positive selection of thymocytes with impaired signaling function (ζ-0 ITAM) might be enhanced in the presence of a high affinity ligand. Indeed, although positive selection of DO11 transgenic thymocytes was poor in ζ-0 ITAM A$^{d/d}$ mice, the efficiency of selection was improved in A$^{d/b}$ mice. Conversely, positive selection was not enhanced in A$^{b/d}$ DO11 $\zeta^{+/+}$ or DO11 ζ-3 ITAM mice, possibly because their thymocytes already received optimal signals for positive selection. An alternative explanation is that while the higher affinity interaction of the DO11 TCR with A^b may indeed promote positive selection of a subset of thymocytes, it also promotes negative selection of other thymocytes. Consistent with the latter, the absolute numbers of DO11$^+$

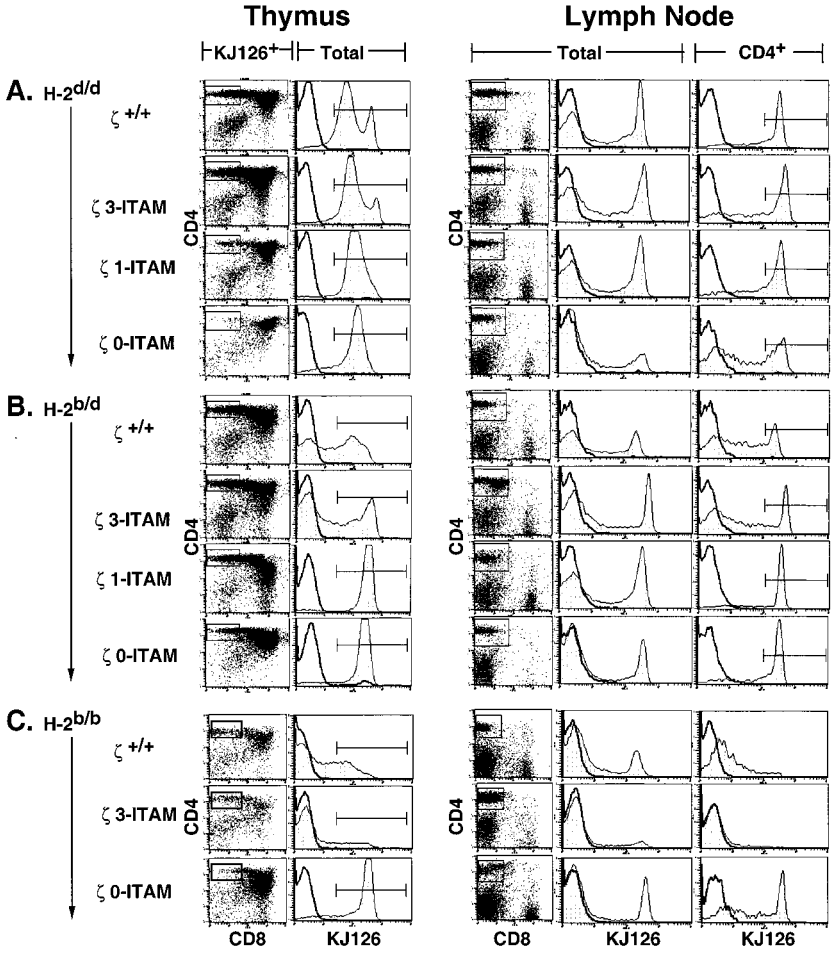

FIGURE 4. Phenotype of DO11 TCR transgenic mice. DO11 TCR$^+$, $\zeta^{-/-}$ mice were reconstituted with ζ-3 ITAM, ζ-1 ITAM, or ζ-0 ITAM transgenes. Also shown are data from an animal expressing endogenous ζ-chain ($\zeta^{+/+}$). Thymocytes and lymph node T cells from DO11 transgenic mice developing in an H-2$^{d/d}$ background (*A*), H-2$^{b/d}$ background (*B*) and H-2$^{b/b}$ background (*C*) were examined. Data show FCM analysis of thymocytes and lymph nodes from adult (6- to 12-wk-old) mice. Three-color FCM analysis was performed on cells stained with the DO11 clonotype-specific Ab, KJ126-FITC, anti-CD8-PE, and anti-CD4-QR. Regions shown in two-color profiles represent KJ126$^+$ CD4-SP cells and were used to calculate data shown in Fig. 5. Analysis of numbers and percentages of these thymocytes is shown in Fig. 5. Single-color profiles (shaded areas) depict KJ126 staining on total thymocytes or lymph node T cells. Also shown is KJ126 staining on CD4$^+$ lymph node T cells. Solid lines reflect staining with negative control Ab.

CD4-SP cells remained relatively constant between I-A$^{d/d}$ and A$^{b/d}$ mice, whereas the frequency of such cells increased in A$^{b/d}$ mice due to the reduction (~50%) in total thymocyte numbers. Such a result might be expected if large numbers of DO11 CD4$^+$CD8$^+$ thymocytes were being deleted in the thymus. Also consistent with negative selection was the dramatic decrease in KJ126$^+$ thymocytes and CD4-SP T cells in DO11 $\zeta^{+/+}$ or DO11 ζ-3 ITAM mice in H-2$^{b/b}$ mice. Thymocyte numbers were markedly reduced in all A$^{b/b}$ mice compared with corresponding A$^{d/d}$ mice, regardless of the number of ζ-chain ITAMs in the TCR. However, the phenotype of the remaining thymocytes and lymph node T cells varied significantly depending on the signaling potential of the TCR. In A$^{b/b}$ DO11, $\zeta^{+/+}$ and A$^{b/b}$ DO11, ζ-3 ITAM mice, the number and percentage of KJ126$^+$ thymocytes was greatly reduced compared with their A$^{d/d}$ counterparts. Moreover, lymph nodes from these animals were grossly deficient in KJ126$^+$ CD4-SP T cells. In contrast, despite the relatively low numbers of KJ126$^+$ CD4-SP cells in the thymus of A$^{b/b}$ DO11, ζ-0 ITAM mice, these cells were present in the periphery and were capable of responding to cognate Ag. Thus, in DO11 TCR transgenic mice, the majority of thymocytes expressing TCRs that contain a signaling competent ζ-chain are negatively selected upon exposure to high affinity ligand (A$^{b/b}$), whereas at least some thymocytes from ζ-0 ITAM mice are more resistant to negative selection and can in fact undergo positive selection in the A$^{b/b}$ background.

Data from several groups suggest that unique signals regulate positive and negative selection (27–30). In our current model, alterations in the most proximal region of the TCR signaling pathway (i.e., the number of ζ-chain ITAMs) was found to effect both positive and negative selection. Hence, these current findings are most consistent with the notion that negative and positive selection lie on a continuum of proximal TCR signals. Whether alterations

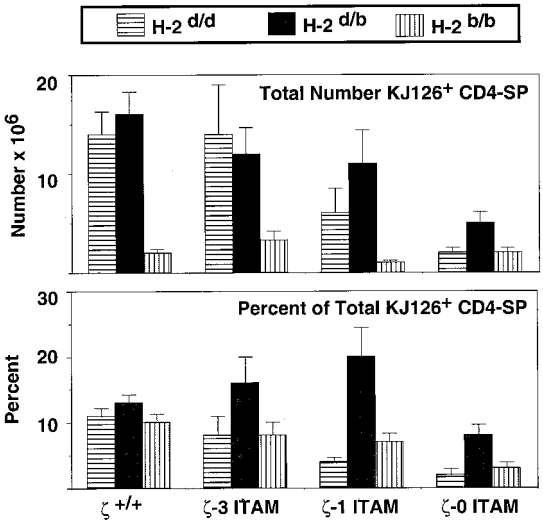

FIGURE 5. Total and percent KJ126$^+$/CD4-SP per thymus in H-2$^{d/d}$, H-2$^{b/d}$, and H-2$^{b/b}$ DO11 TCR transgenic mice. Percent KJ126$^+$/CD4-SP cells was determined by electronically gating on the region similar to that displayed in Fig. 4*A*. Total numbers were determined by multiplying the percent by the total thymus yield. Shown is mean (bar) and SE (line) for five to nine mice for each group.

Table III. *Proliferative and cytokine response of H-2$^{d/d}$ DO11 CD4-SP T cells*[a]

	$OVA_{323–329}$ (μg/ml)						IL-2 (pg/ml)					
	100.00	25.00	1.50	0.40	0.10	0	100.00	25.00	1.50	0.40	0.10	0
$\zeta^{+/+}$	12.4[b]	10.7	7.2	3.4	1.0	0.3	7095	6016	1488	322	91	<LOD
ζ-3 ITAM	23.2	20.5	12.4	11.9	7.1	0.4	5423	5821	1725	579	95	<LOD
ζ-1 ITAM	28.7	30.9	22.5	9.4	1.9	0.3	7505	5896	572	333	40	48
ζ-0 ITAM	14.9	12.7	6.9	3.3	0.6	0.4	8008	5976	1418	410	132	<LOD
B10.D2	0.6	0.3	0.2	0.2	0.2	0.3	188	193	<LOD	<LOD	<LOD	<LOD

[a] Results from a representative experiment are shown. $CD4^+$ lymph node T cells from H-2$^{d/d}$ DO11 TCR transgenic mice were cultured with T-depleted irradiated APC from spleens of B10.D2 mice in the absence or presence of varying concentrations of $OVA_{323–339}$. For proliferation assays, cells were stimulated for 40 h and then pulsed for 8 h with 1 Ci[^{3}H]thymidine and harvested. For cytokine assays, cells were stimulated as described above, in parallel cultures. Supernatants were obtained after 24 h and IL-2 secretion was assayed. LOD, Limit of detection.

[b] Values are expressed in cpm × 10^{-3}.

in numbers of ζ-chain ITAMs differentially effect the activation of specific downstream signaling pathway in this in vivo model is not currently known. Different TCR ITAMs have been found to preferentially interact with distinct substrates in vitro (31–33). Moreover, the pattern of ITAM phosphorylation may influence TCR signaling. For example, recent data suggest an inhibitory role for partially (mono) phosphorylated ITAMs in vitro (34). However, evidence of negative signaling has not been observed in in vivo studies (21, 35).

Our current and previous findings (16) together with those of Ardouin et al. (21), raise several interesting questions regarding the signaling requirements for thymocyte selection and mature T cell activation. Specifically, why, as in DO11 TCR transgenic mice, are not all thymocytes that express the same TCR affected similarly by attenuation of the TCR signaling potential, and why are mature T cell responses only minimally affected in the absence of ζ-chain ITAMs? We speculate that the pool of developing thymocytes may include naturally acquired or regulated variations in the expression of molecules that directly or indirectly participate in the TCR signaling response. These could include the TCR itself as well as CD4, CD8, and other surface receptors in addition to intracellular signaling molecules. One example of such a molecule is CD5, which functions to negatively regulate signaling by the TCR (36). We recently found that CD5 surface expression is itself positively regulated by the strength of the TCR signal during thymocyte development, providing a feed-back mechanism for fine tuning the TCR signaling response (37). Within a certain range, the ability of thymocytes to compensate for inappropriate TCR signals (i.e., too strong or too weak), either through natural or regulated variations in the expression of other regulatory molecules may explain why some, but not all thymocytes in DO11 and P14, ζ-0 ITAM mice are positively selected, and why these cells, once positively selected, are capable of responding to Ag despite their impaired TCR signaling potential.

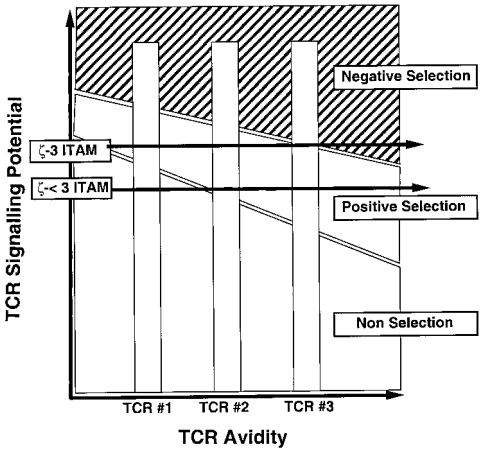

FIGURE 6. Proposed relationship between TCR affinity and signaling potential, and impact on outcome of thymocyte selection. The manner of display of the model is based on a model proposed by Jameson et al. (1). However, this current model addresses the relationship between signaling and affinity. TCR #1, TCR #2, and TCR #3 represent hypothetical TCRs with defined affinities for their selecting thymic ligands. TCR #1 has a low affinity for its thymic ligand, such that reducing the signaling ability of the TCR (reduced ITAMs) results in nonselection. TCR #2 has a higher affinity for its thymic ligand(s) and reducing the number of ζ-chain ITAMs has no effect on its ability to be positively selected. TCR #3 has a high affinity for thymic ligands, to the extent that some clonotypic thymocytes are negatively selected when the full complement of ITAMs are present in the TCR. Reducing TCR ITAMs in this case results in a net increase in the number of positively selected thymocytes.

Our current findings also speak to the question of why the TCR contains multiple signaling chains and motifs. Because reduction in the number of TCR ITAMs can dramatically affect the efficiency of positive selection while minimally impairing peripheral T cell function, the primary advantage of a multi-ITAM TCR structure appears to be in enabling the selection of a self MHC-restricted TCR repertoire. Currently, debate exists concerning the advantage of having an immune system that is selected to recognize self-MHC and hence is "autorecognizing," while simultaneously needing to avoid "autoreactivity." A delicate balance must exist during selection to regulate the development of an immune system with these features. The TCR complex, with its multiple signaling components and activation motifs appears ideally equipped to control the development of this immune recognition system.

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