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## Fine Tuning of TCR Signaling by CD5

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# Fine Tuning of TCR Signaling by CD5

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Current data indicate that CD5 functions as an inhibitor of TCR signal transduction. Consistent with this role, thymocyte selection in TCR transgenic/CD5<sup>-/-</sup> mice is altered in a manner suggestive of enhanced TCR signaling. However, the impact of CD5 deletion on thymocyte selection varies depending on the transgenic TCR analyzed, ranging from a slight to a marked shift from positive toward negative selection. An explanation for the variable effect of CD5 on selection is suggested by the observation that CD5 surface expression is regulated by TCR signal intensity during development and CD5 surface levels on mature thymocytes and T cells parallel the avidity of the positively selecting TCR/MHC/ligand interaction. In this study, we generated mice that overexpress CD5 during thymocyte development (CD5-tg), and then examined the effect of CD5 overexpression or CD5 deletion (CD5<sup>-/-</sup>) on selection of thymocytes that express the same TCR transgenes. The results demonstrate that the effect on thymocyte selection of altering CD5 expression depends on the avidity of the selecting interaction and, consequently, the level of basal (endogenous) CD5 surface expression. Substitution of endogenous CD5 with a transgene encoding a truncated form of the protein failed to rescue the CD5<sup>-/-</sup> phenotype, demonstrating that the cytoplasmic domain of CD5 is required for its inhibitory function. Together, these results indicate that inducible regulation of CD5 surface expression during thymocyte selection functions to fine tune the TCR signaling response. *The Journal of Immunology*, 2001, 166: 5464–5472.

**T**hymocyte selection marks a critical event in T cell development, resulting in the survival of self-restricted cells (positive selection), and the death of autoreactive cells (negative selection; Ref. 1–3). This cell fate decision is dictated primarily by the specificity of the TCRs expressed on immature thymocytes for self ligands (self-MHC + self-peptides) present in the thymus. In general, high-avidity TCR/MHC/ligand interactions have been shown to promote negative selection, whereas relatively low-avidity TCR/MHC/ligand interactions promote positive selection (1–3). Engagement of the TCR complex leads to the generation of intracellular signals that are required for mediating these selection events (4). Current models of selection maintain that the avidity of the TCR/self-ligand interaction determines the intensity of the TCR signaling response, resulting in the activation of downstream pathways that either promote cell survival and differentiation or initiate cell death (1–3).

Surface receptors other than the TCR also participate in thymocyte selection by directly or indirectly influencing the TCR signaling response. CD4 and CD8, which also bind to MHC, potentiate TCR signaling by increasing the avidity of TCR/MHC/ligand interaction and by recruitment of the Src family protein tyrosine kinase, Lck, to the proximity of the TCR complex (4, 5). CD45 also functions to augment TCR signaling by positively regulating the activity of both Lck and Fyn (4, 5). CD5, a monomeric cell surface glycoprotein expressed on thymocytes, T cells, and a sub-

set of B cells, has been shown to negatively regulate signaling through both the B cell and T cell Ag receptors (6, 7). In the absence of CD5, peritoneal B-1 cells that normally are triggered to undergo apoptosis in response to IgM cross-linking develop resistance to apoptosis and enter the cell cycle (7). Moreover, thymocytes from CD5<sup>-/-</sup> mice are hyperresponsive to stimulation through the TCR (6). The cytoplasmic domain of CD5 contains four potential tyrosine phosphorylation sites, including an imperfect immunoreceptor tyrosine-based activation motif (ITAM),<sup>2</sup> an immunoreceptor tyrosine-based inhibition motif (ITIM), and multiple potential Ser/Thr phosphorylation sites (8–10). The precise mechanism by which CD5 inhibits TCR signaling has not been elucidated. However, CD5 is tyrosine-phosphorylated after TCR engagement and associates with several effector molecules that could potentially influence the TCR signaling response, including Cbl, rasGAP, SHP-1, casein kinase 2, and TCR- $\zeta$ /ZAP-70 (11–15).

The function of CD5 in development, particularly during thymocyte selection, remains unclear. CD5<sup>-/-</sup> mice exhibit no obvious defects in T cell development (16). However, when cohorts of thymocytes expressing a defined (transgenic)  $\alpha\beta$ TCR were analyzed, thymocyte selection was found to be dramatically altered in mice lacking CD5 (6, 17). In positively selecting MHC backgrounds, CD4<sup>+</sup>CD8<sup>+</sup> (double-positive, or DP) thymocyte numbers are reduced in TCR-transgenic (tg)/CD5<sup>-/-</sup> mice relative to TCR-tg; CD5<sup>+/+</sup> mice suggesting a shift in cell fate from positive selection toward negative selection (6, 17). However, the impact of CD5 deficiency on positive selection varies depending on the specificity of the TCR transgene. For example, in P14 TCR-tg/CD5<sup>-/-</sup> mice and (A<sup>bd</sup>) DO10.10 (DO10)/TCR-tg/CD5<sup>-/-</sup> mice, few thymocytes express high levels of the clonotypic TCR, and few single-positive (SP) clonotype-TCR<sup>high</sup> cells are present in the periphery, suggesting that in the absence of CD5, most clonotype-TCR<sup>high</sup> thymocytes undergo negative selection

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<sup>2</sup> Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine based inhibition motif; DP, double positive (CD4<sup>+</sup>CD8<sup>+</sup>); SP, single positive (CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup>); tg, transgenic; DO10, DO10.10; hu, human; m, mutant.

(6, 17). In contrast, large numbers of clonotype-TCR<sup>high</sup> thymocytes and SP T cells are generated in H-Y TCR-tg/CD5<sup>-/-</sup> and (A<sup>d</sup>) DO10 TCR-tg/CD5<sup>-/-</sup> mice, indicating that thymocytes expressing these TCRs can still be positively selected in the absence of CD5 (6, 17).

A possible explanation for the variable effects of CD5 deletion on thymocyte selection is suggested by the recent observation that CD5 surface expression is regulated by the intensity of the TCR signal and by the avidity of the TCR/ligand interaction during selection (18). Relatively high-avidity positively selecting interactions result in high surface expression of CD5 on DP and SP thymocytes, whereas lower avidity interactions induce lower surface expression of CD5 (18). Consequently, the impact of CD5 deletion (or CD5 overexpression) on thymocyte selection might be predicted to be different depending on the avidity of the TCR for its selecting ligand.

To test this idea, we generated CD5-tg mice and then compared the effect of CD5 overexpression or CD5 deletion on thymocyte selection in different TCR-tg backgrounds. Significantly, the effect on thymocyte selection of altering CD5 surface levels critically depended on the avidity of the TCR for its selecting ligand. Moreover, the cytoplasmic domain of CD5 was required for its inhibitory function, as substitution of endogenous CD5 with a transgene encoding a mutant form of the protein lacking the imperfect ITAM and distal sequences failed to rescue the CD5<sup>-/-</sup> phenotype. Together, these results demonstrate that regulation of CD5 surface expression by TCR signaling serves as a mechanism for fine tuning the TCR signaling response during thymocyte selection.

## Materials and Methods

### Mice

The human (hu) CD2-CD5 transgene (CD5-tg) was generated by substituting murine CD5 coding sequences for the  $\zeta$  cDNA sequences in construct  $\zeta$ -CT108 (19). The huCD2-mutant CD5-tg (mCD5-tg) was generated in a similar fashion, except that the murine CD5 coding sequence was first mutated *in vitro* with a synthetic primer: 5'-CAT-GTG-GAC-AAT-TAA-TAC-AGC-CAG-CC-3' that substitutes a stop codon (TAA) for Glu<sup>428</sup> (GAA; see Fig. 5A). The CD5 and mCD5 cDNAs were sequenced before insertion into the hCD2 transgenic cassette. Four CD5-tg founder lines and four mCD5-tg founder lines were generated by zygote injection. CD5 expression was quantitated by Northern blotting, Western blotting, and flow cytometric analysis. CD5-tg and mCD5-tg founder lines that expressed similar levels of surface protein that fell within the high-normal range (see Figs. 1 and 6) were selected for use in the current experiments. CD5<sup>-/-</sup> mice (16) were obtained from Dr. Ron Schwartz, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). TCR-tg mice used in these studies included the MHC class I-restricted TCRs P14 (20) and H-Y (21) and the MHC class II-restricted TCRs AND (22) and DO10 (23). Mice were maintained in the H-2D<sup>b</sup> background by mating with C57BL/6 partners and were moved into or maintained in the H-2D<sup>d</sup> background by mating with B10.D2 partners.

### Antibodies

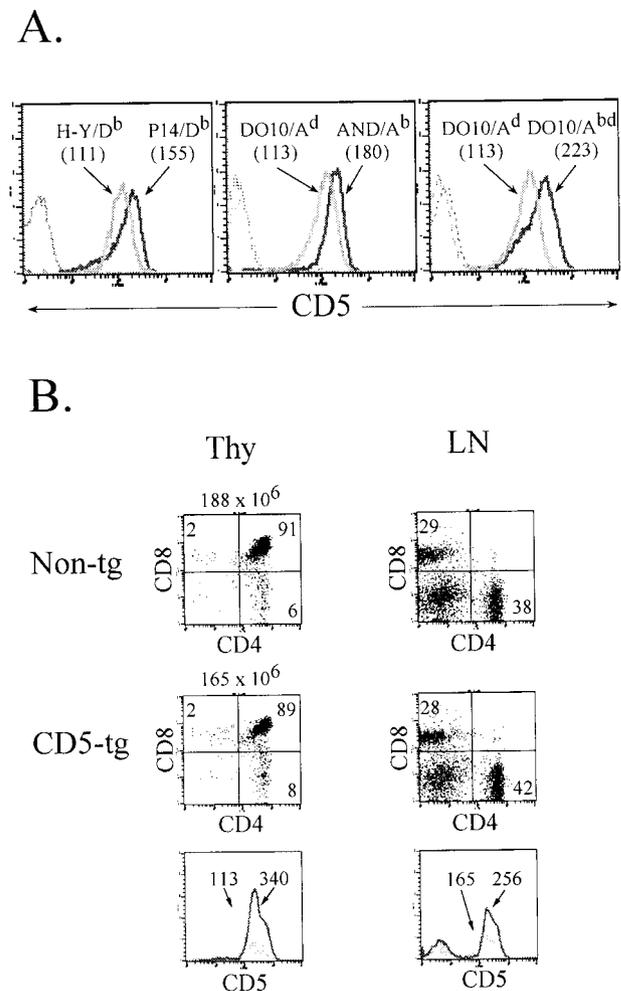
mAbs used for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA), unless noted otherwise, and included: FITC-, PE-, or CyChrome-conjugated anti-CD4 (H129.19), anti-TCR $\beta$ (H57-597), anti-CD8 (53-6.7), anti-CD3 (145-2C11), anti-CD5 (53-7.3), anti-V $\alpha$ 11 (RR8-1), and anti-V $\alpha$ 2 (B20.1). Unconjugated anti-CD16/CD32 (2.4G2) was used to block nonspecific Fc receptor binding. The anti-H-Y clonotypic receptor mAb (T3.70) and anti-DO10 clonotypic receptor mAb (KJ126) were purified from cell culture supernatants and labeled with FITC in our laboratory. In some experiments, Quantum red-conjugated anti-CD4 (H129.19) or anti-CD8 (53-6.7) (Sigma, St. Louis, MO) were used for flow cytometry. Abs for immunoprecipitation and Western blotting included anti-CD5 (53-7.3) (BD Pharmingen), Q-20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine-HRP (4G10) (Upstate Biotechnology, Lake Placid, NY), and polyclonal anti-goat-HRP (Santa Cruz Biotechnology).

### Flow cytometric analysis and measurement of calcium flux

Thymi and lymph nodes were excised from mice and single-cell suspensions were prepared in FACS buffer (HBSS (Life Technologies, Rockville, MD) plus 0.1% BSA). For multicolor flow cytometry, thymocytes or lymph node cells first were incubated with unlabeled Ab to the Fc receptor (mAb 2.4G2) to prevent nonspecific binding of Abs. Background staining was measured with fluorochrome-conjugated rat IgG2a (BD Pharmingen) and designated as control. For two- and three-color multicolor flow cytometry, cells were incubated with fluorochrome-conjugated Abs for 1 h at 4°C, washed, and resuspended in FACS buffer. Analysis was performed on a Becton Dickinson Immunocytometry Systems (Mountain View, CA) FACScan with standard CellQuest software. Data were collected on  $1 \times 10^4$  viable cells as determined by forward and side light scatter. Calcium flux measurements were performed as described (24).

### Western blot analysis

Thymocytes were enumerated, washed twice in ice-cold PBS, and resuspended in PBS at a concentration of  $1 \times 10^8$ /ml. Thymocyte stimulations, immunoprecipitations, Western blotting, and PAGE were performed as described (24, 25). Briefly, thymocytes were incubated for 5 min in medium



**FIGURE 1.** A, CD5 surface expression on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from mice of the indicated MHC types expressing MHC class I-restricted (H-Y and P14) or MHC class II-restricted (DO10 and AND) TCR transgenes. Numbers in parentheses show mean CD5 fluorescence. B, Phenotype of CD5-tg mice. Comparison of thymocytes (Thy) and lymph node (LN) cells from non-tg and CD5-tg littermates by FACS analysis. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show CD5 surface staining on total thymocytes or total lymph node cells. Non-tg (shaded), CD5-tg (solid lines). Numbers in histograms indicate mean CD5 fluorescence.

lacking (–) or containing (+) pervanadate (1 mM; Sigma). Thymocyte lysates were prepared and incubated at 4°C for 1 h in the presence of protein G beads plus anti-CD5 (53-7.3). Beads were washed and eluted proteins were resolved by 10% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes and blotted with antiphosphotyrosine-HRP and detected by ECL (Amersham, Arlington Heights, IL). For detection of CD5, blots were stripped and reprobed with polyclonal goat anti-mouse CD5 followed by polyclonal anti-goat-HRP (Santa Cruz Biotechnology).

## Results

### Effect of altered CD5 surface expression on thymocyte selection depends on the avidity of the TCR for selecting ligand

In this study, we used two MHC class I-restricted TCR-tg lines (H-Y and P14) and two MHC class II-restricted TCR-tg lines (DO10 and AND) to test the effect on thymocyte selection of altering the level of CD5 surface expression. In a previous report (Ref. 18; also shown in Fig. 1A), we observed that mean CD5 surface expression was higher on DP thymocytes and CD8-SP T cells from P14 TCR-tg mice than on equivalent populations of cells from H-Y TCR-tg mice. Likewise, CD5 surface levels were higher on DP thymocytes and CD4-SP T cells from AND TCR-tg mice than on similar cells from (A<sup>d</sup>) DO10 TCR-tg mice (Ref. 18 and Fig. 1A). Significantly, these differences in CD5 surface expression correlated with the presumed affinity/avidity of the individual TCRs for their positively selecting ligands in the thymus (i.e., P14 > H-Y and AND > (A<sup>d</sup>) DO10; Ref. 18).

To examine the effect of loss of CD5 surface expression on thymocyte selection, each of the TCR transgenes was mated into the CD5<sup>-/-</sup> background (16). Alternatively, CD5 surface expression was augmented by generating transgenic mice that express CD5 under the control of the huCD2 promoter/enhancer and then

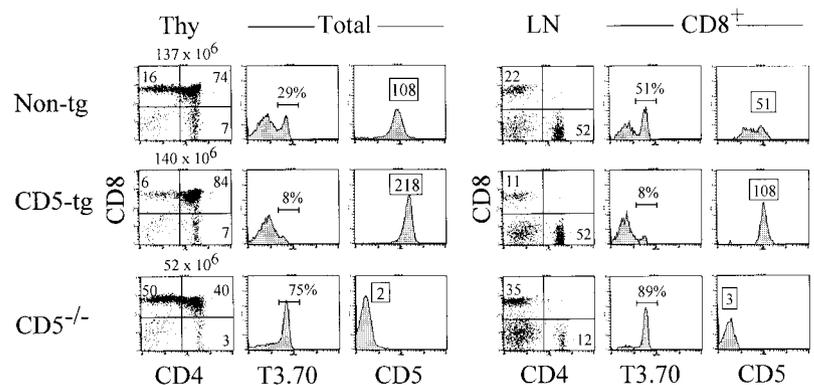
mating the CD5 transgene into the different TCR-tg backgrounds. For these studies, a transgenic founder line was selected that increased CD5 expression on thymocytes to levels within the high-normal range observed on total thymocytes from non-tg mice (Fig. 1B). As previously noted for non-TCR-tg/CD5<sup>-/-</sup> mice (16), no obvious alterations in T cell development were detected in non-TCR-tg/CD5-tg mice (Fig. 1B and data not shown).

Results from representative experiments performed with MHC class I-restricted TCR transgenes are shown in Fig. 2. The effect of the CD5 transgene on thymocyte selection was different in mice expressing the H-Y TCR or the P14 TCR transgenes (Fig. 2). Augmentation of CD5 surface expression markedly inhibited positive selection in H-Y TCR-tg mice as assessed by the selective reduction of clonotype-TCR<sup>high</sup> (T3.70<sup>high</sup>) thymocytes and T3.70<sup>high</sup> CD8-SP T cells in H-Y TCR-tg/CD5-tg mice (Fig. 2A). In contrast, applying the same criteria, positive selection was only slightly impaired in P14 TCR-tg/CD5-tg mice (Fig. 2B). In this respect, it is important to note that although introduction of the CD5 transgene increased the level of CD5 surface expression by the same increment in both H-Y and P14 TCR-tg mice, the level of baseline CD5 expression (i.e., in non-CD5-tg mice) was ~50% greater on thymocytes from P14 TCR-tg mice than on thymocytes from H-Y TCR-tg mice (Ref. 18 and Figs. 1A and 2).

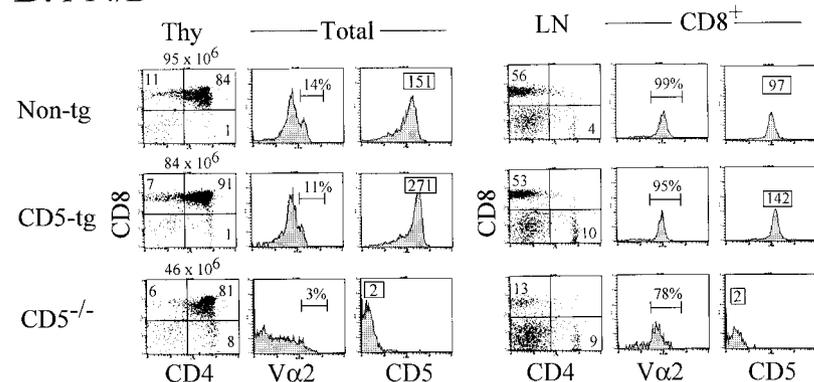
The effect of CD5 deletion on thymocyte selection also was different in H-Y TCR-tg and P14 TCR-tg mice. As reported previously (6) and shown in Fig. 2, total thymocyte numbers are reduced ~2- to 3-fold in both H-Y TCR-tg/CD5<sup>-/-</sup> and P14 TCR-tg/CD5<sup>-/-</sup> mice. Moreover, the reduction in thymocyte cellularity occurs predominantly in the DP compartment, suggesting that in both cases, there is a shift from positive toward negative selection

**FIGURE 2.** Effect of altering CD5 surface levels on positive selection of thymocytes in mice expressing MHC class I-restricted transgenic TCRs. **A.** Comparison of thymocytes (Thy) and lymph node (LN) T cells from H-2D<sup>b</sup>, H-Y TCR-tg female mice that are CD5<sup>+/+</sup> (non-tg), CD5-tg, or CD5<sup>-/-</sup>. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes or CD8<sup>+</sup> T cells with H-Y clonotype-specific Ab, T3.70 (the percentage of T3.70<sup>high</sup> cells is also shown) or CD5 surface expression on total thymocytes or CD8<sup>+</sup> T cells (mean CD5 fluorescence is shown in the boxes). **B.** Comparison of thymocytes and lymph node T cells from H-2D<sup>b</sup>, P14 TCR-tg mice that are CD5<sup>+/+</sup> (non-tg), CD5-tg, or CD5<sup>-/-</sup>. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes or CD8<sup>+</sup> T cells with the P14 TCR $\alpha$ -chain-specific Ab, V $\alpha$ 2 (the percentage of V $\alpha$ 2<sup>high</sup> cells is also shown) or CD5 surface expression on total thymocytes or CD8<sup>+</sup> T cells (mean CD5 fluorescence is shown in the boxes).

### A. H-Y/D<sup>b</sup>



### B. P14/D<sup>b</sup>



in the absence of CD5 (6). However, the number of T3.70<sup>high</sup> thymocytes and T3.70<sup>high</sup> CD8-SP T cells was not reduced in H-Y TCR-tg/CD5<sup>-/-</sup> mice relative to H-Y TCR-tg/CD5<sup>+/+</sup> mice (Fig. 2A). In contrast, clonotype-TCR<sup>high</sup> (V $\alpha$ 2<sup>high</sup>) thymocytes were nearly absent in P14 TCR-tg/CD5<sup>-/-</sup> mice and few V $\alpha$ 2<sup>high</sup> CD8-SP T cells were present in the peripheral lymphoid organs of these mice (Ref. 6 and Fig. 2B). Thus, the relative impact of CD5 deletion was much more severe in P14 TCR-tg mice than in H-Y TCR-tg mice, resulting in marked reduction in the number of clonotype-TCR<sup>high</sup> thymocytes and CD8-SP T cells in the former but not the latter.

To determine whether the effect on thymocyte selection of altering CD5 expression also varied according to TCR specificity in mice expressing MHC class II-restricted TCRs, we next examined (A<sup>d</sup>) DO10 TCR-tg and AND TCR-tg mice (Fig. 3). Previous data argue that the avidity of the positively selecting interaction is greater for AND TCR than for (A<sup>d</sup>) DO10 TCR (18). Significantly, the results obtained with DO10 TCR-tg mice were similar to those observed with H-Y TCR-tg mice. Positive selection was inhibited in (A<sup>d</sup>) DO10 TCR-tg/CD5<sup>-/-</sup> mice, as assessed by the selective reduction in clonotype-TCR<sup>high</sup> (KJ126<sup>high</sup>) CD4-SP thymocytes and peripheral T cells (Fig. 3A). In contrast, the results obtained with AND TCR-tg mice were similar to those obtained with P14 TCR-tg mice in that the CD5 transgene had minimal effect on the generation of clonotype TCR<sup>high</sup> (V $\alpha$ 11<sup>high</sup>) thymocytes and V $\alpha$ 11<sup>high</sup> CD4-SP T cells (Fig. 3B). Again, it is important to note that although introduction of the CD5 transgene increased CD5 surface levels by roughly the same increment in both cases, mean CD5 surface expression was ~50% greater on thymocytes from AND TCR-tg/CD5<sup>+/+</sup> mice than on thymocytes from (A<sup>d</sup>) DO10 TCR-tg/CD5<sup>+/+</sup> mice (Ref. 18 and Figs. 1 and 3). As in H-Y

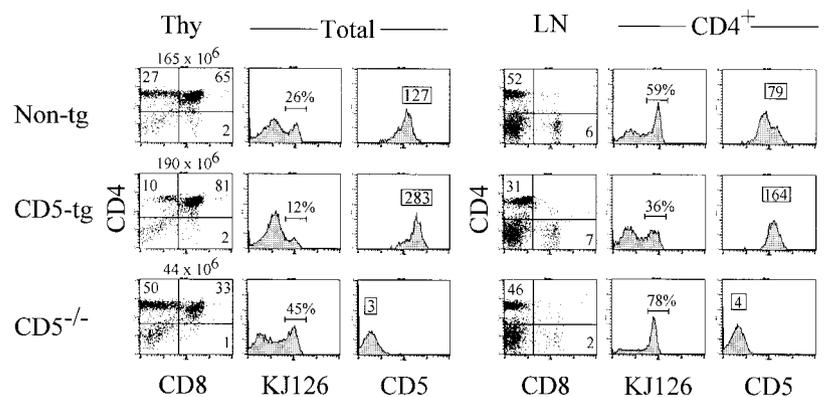
TCR-tg mice (Fig. 2A), loss of CD5 expression in (A<sup>d</sup>) DO10 TCR-tg mice led to a reduction in DP thymocytes, but did not lead to a reduction in the number of clonotype-TCR<sup>high</sup> (KJ126<sup>high</sup>) CD4-SP thymocytes or peripheral KJ126<sup>high</sup> CD4-SP T cells (Fig. 3A). However, in AND TCR-tg/CD5<sup>-/-</sup> mice, DP thymocytes and V $\alpha$ 11<sup>high</sup> thymocytes and T cells also were markedly reduced (Fig. 3B).

Taken together, these data indicate that thymocytes that express putative high-avidity TCRs (P14 and AND) undergo negative selection in CD5<sup>-/-</sup> mice. Consistent with this idea, most DP thymocytes from AND TCR-tg/CD5<sup>-/-</sup> mice and P14 TCR-tg/CD5<sup>-/-</sup> mice down-regulate their TCRs and express high surface levels of CD69 (Fig. 4B), events that have been associated with strongly activating signals (26). In contrast, the presence of normal numbers of clonotype-TCR<sup>high</sup> thymocytes and T cells in H-Y TCR-tg/CD5<sup>-/-</sup> and (A<sup>d</sup>) DO10 TCR-tg/CD5<sup>-/-</sup> mice demonstrates that loss of CD5 does not result in deletion of most thymocytes that express putative low-avidity TCRs. Conversely, forced overexpression of CD5 markedly attenuated positive selection of thymocytes expressing putative low-avidity TCRs (H-Y and (A<sup>d</sup>) DO10) but did not impair positive selection of thymocytes expressing putative high-avidity TCRs (P14 and AND).

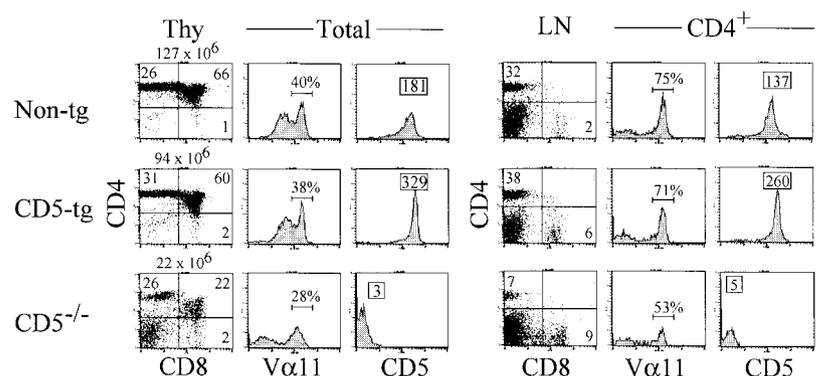
These results suggested that the impact of CD5 deletion or enhanced CD5 expression on thymocyte selection critically depends on the basal level of CD5 surface expression, which in turn is regulated by the avidity of the TCR for its selecting ligand (18). To test this hypothesis further, we examined the effect of CD5 deletion or overexpression on thymocyte selection under conditions in which the TCR specificity was held constant but the avidity of the selecting interaction was varied. Thymocytes that express DO10 TCR also are positively selected in the A<sup>b</sup> background; however,

**FIGURE 3.** Effect of altering CD5 surface levels on positive selection of thymocytes in mice expressing MHC class II-restricted transgenic TCRs. *A*, Comparison of thymocytes and lymph node T cells from A<sup>d</sup>, DO10 TCR-tg mice that are CD5<sup>+/+</sup> (non-tg), CD5-tg, or CD5<sup>-/-</sup>. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes or CD4<sup>+</sup> T cells with DO10 clonotype-specific Ab, KJ126 (the percentage of KJ126<sup>high</sup> cells is also shown), or CD5 surface expression on total thymocytes or CD4<sup>+</sup> T cells (mean CD5 fluorescence is shown in the boxes). *B*, Comparison of thymocytes and lymph node T cells from A<sup>b</sup>, AND TCR-tg mice that are CD5<sup>+/+</sup> (non-tg), CD5-tg, or CD5<sup>-/-</sup>. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes or CD4<sup>+</sup> T cells with the AND TCR $\alpha$ -chain-specific Ab, V $\alpha$ 11 (the percentage of V $\alpha$ 11<sup>high</sup> cells is also shown), or CD5 surface expression on total thymocytes or CD4<sup>+</sup> T cells (mean CD5 fluorescence is shown in the boxes).

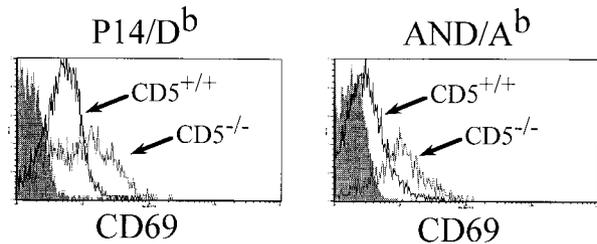
### A. DO10/A<sup>d</sup>



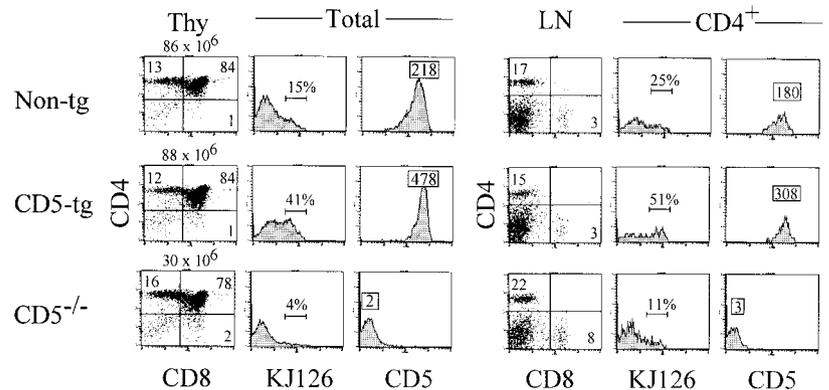
### B. AND/A<sup>b</sup>



A.



**FIGURE 4.** A, CD69 surface expression on gated CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from P14 TCR-tg and AND TCR-tg mice and their genetic variants lacking CD5. Shaded plots show staining with control Ab. B, Comparison of thymocytes and lymph node T cells from A<sup>bd</sup>, DO10 TCR-tg mice that are CD5<sup>+/+</sup> (non-tg), CD5-tg, or CD5<sup>-/-</sup>. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes or CD4<sup>+</sup> T cells with DO10 clone-type-specific Ab, KJ126 (the percentage of KJ126<sup>high</sup> cells is also shown) or CD5 surface expression on total thymocytes or CD4<sup>+</sup> T cells (mean CD5 fluorescence is shown in the boxes).

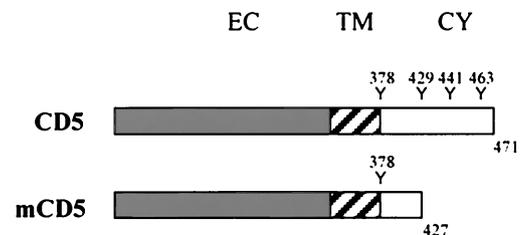
B. DO10/A<sup>bd</sup>

the DO10 TCR is alloreactive against I-A<sup>b</sup> (27, 28). The small thymus size and reduction in KJ126<sup>high</sup> thymocytes observed in (A<sup>b</sup>) DO10 TCR-tg mice relative to (A<sup>d</sup>) DO10 TCR-tg mice is consistent with the induction of partial negative selection resulting from the increase in avidity of the selecting TCR/MHC-ligand interaction in the A<sup>b</sup> background (27, 28). An intermediate phenotype is observed in A<sup>bd</sup> mice (i.e., a less dramatic reduction in thymocyte numbers and KJ126<sup>+</sup> thymocytes and CD4-SP T cells; Fig. 4). Significantly, CD5 surface expression is increased nearly 2-fold on thymocytes from (A<sup>bd</sup>) DO10 TCR-tg mice relative to (A<sup>d</sup>) DO10 TCR-tg mice (Ref. 18 and Figs. 1 and 4). Accordingly, the effects of CD5 deletion or overexpression on selection of thymocytes in (A<sup>bd</sup>) DO10 TCR-tg mice were found to more closely resemble those obtained with the putative higher-avidity TCRs (AND and P14) than those observed for the lower-avidity TCRs ((A<sup>d</sup>) DO10 and H-Y; Fig. 4). Specifically, deletion of CD5 in (A<sup>bd</sup>) DO10 TCR-tg mice resulted in a reduction in DP thymocyte numbers and loss of KJ126<sup>high</sup> thymocytes and KJ126<sup>high</sup> CD4-SP T cells (Fig. 4). In contrast, expression of the CD5 transgene did not impair, and in fact appeared to slightly improve, positive selection in (A<sup>bd</sup>) DO10 TCR-tg mice as assessed by the increase in KJ126<sup>high</sup> thymocytes and KJ126<sup>high</sup> CD4-SP T cells in (A<sup>bd</sup>) DO10 TCR-tg/CD5-tg mice relative to (A<sup>bd</sup>) DO10 TCR-tg mice (Fig. 4).

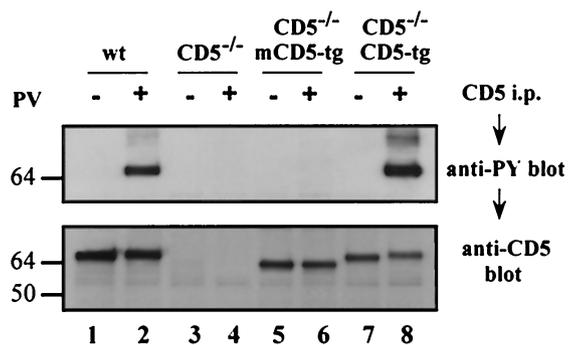
*The cytoplasmic domain of CD5 is required for negative regulation of TCR signaling during selection*

To determine whether signal transduction by CD5 is required for its inhibitory activity in thymocytes, we generated a transgene that expresses a mutant form of the protein (mCD5) lacking a large portion of the cytoplasmic tail, including the imperfect ITAM-(Tyr<sup>429</sup>-Tyr<sup>441</sup>) sequences (Fig. 5A). The wild-type and mCD5 transgenes then were used to reconstitute CD5 surface expression in CD5<sup>-/-</sup> mice by mating. Analysis of CD5 expression in thymocytes from in CD5<sup>-/-</sup>/CD5-tg and CD5<sup>-/-</sup>/mCD5-tg mice re-

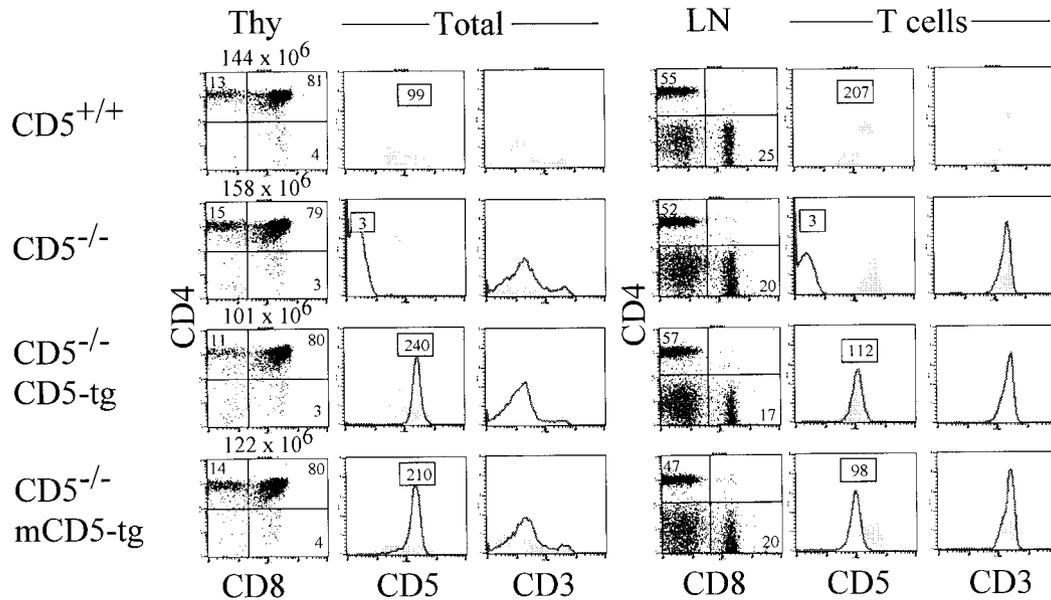
A.



B.



**FIGURE 5.** A, cDNA constructs used to generate the CD5 and mCD5 transgenes. The location of the four cytoplasmic tyrosine residues and the site of the truncation in construct mCD5 are shown. B, Analysis of CD5 and mCD5 protein tyrosine phosphorylation following stimulation with pervanadate. Thymocytes ( $1 \times 10^8$ ) were harvested from non-tg (wt) mice, CD5<sup>-/-</sup> mice, and CD5<sup>-/-</sup> mice reconstituted with wt CD5 (CD5-tg) or truncated/mutant CD5 (mCD5-tg) transgenes, then incubated for 5 min in medium lacking (-) or containing (+) pervanadate. Thymocyte lysates were prepared and incubated at 4°C for 1 h in the presence of protein G beads containing anti-CD5. Beads were washed and eluted proteins were resolved by 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and blotted with anti-phosphotyrosine mAb (upper panel). Lower panel, Blots were stripped and reblotted with anti-CD5 to detect the presence of the CD5 and mCD5 proteins.



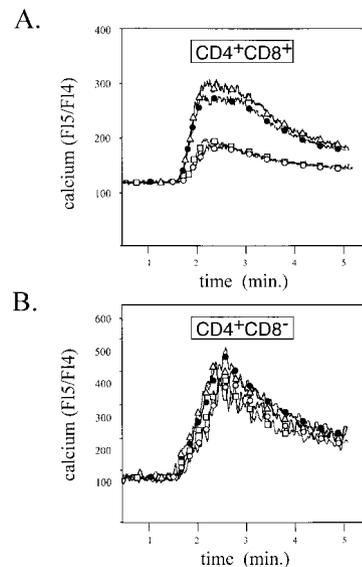
**FIGURE 6.** Reconstitution of  $CD5^{-/-}$  mice with transgenes encoding wild-type ( $CD5$ -tg) or truncated ( $mCD5$ -tg) transgenes. Two color plots show  $CD4/CD8$  staining profiles. Histograms show  $CD3$  and  $CD5$  surface staining on total thymocytes or total lymph node T cells. Numbers in boxes indicate mean  $CD5$  fluorescence. Shaded histograms depict results from  $CD5^{+/+}$  mice and are repeated in each panel for comparison.

vealed proteins of the predicted mobilities (67 and 62 kDa for  $CD5$  and  $mCD5$ , respectively; Fig. 5B). Moreover, although wild-type  $CD5$  was tyrosine phosphorylated after stimulation with pervanadate, tyrosine phosphorylation of  $mCD5$  was undetectable (Fig. 5B).

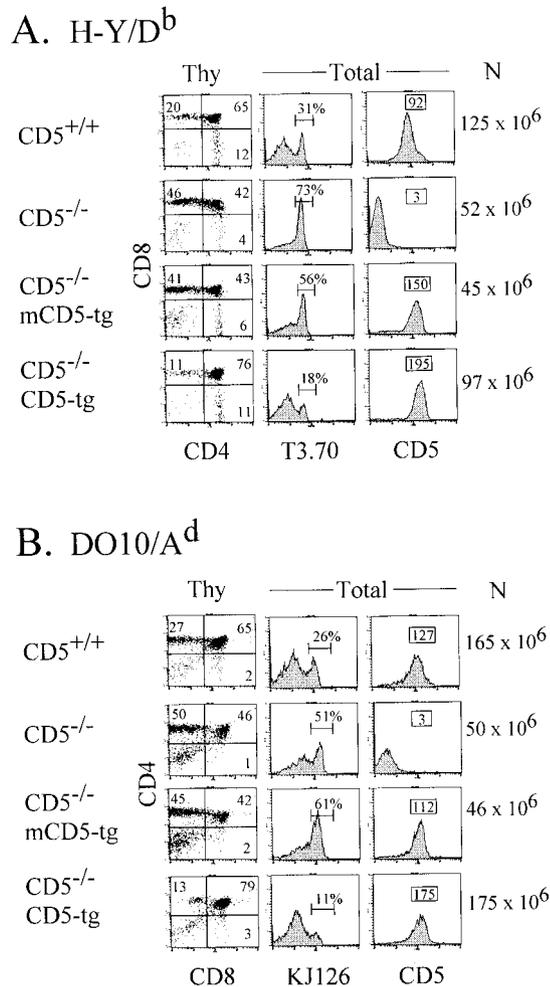
Thymocyte development appeared grossly normal in non-TCR-tg mice where the  $mCD5$  transgene was substituted for endogenous  $CD5$  (Fig. 6). Moreover, thymocytes from  $CD5^{-/-}/CD5$ -tg and  $CD5^{-/-}/mCD5$ -tg mice expressed surface levels of  $CD5$  that were within the high-normal range (Fig. 6). The narrow single peak of  $CD5$  expression in  $CD5^{-/-}/CD5$ -tg and  $CD5^{-/-}/mCD5$ -tg mice indicated further that the relatively broad range of  $CD5$  surface expression on thymocytes from  $CD5^{+/+}$  mice is attributable to regulation by endogenous  $CD5$  regulatory sequences (Fig. 6).  $CD5$  surface expression on all peripheral T cells (both  $CD4$ -SP and  $CD8$ -SP) from  $CD5^{-/-}/CD5$ -tg and  $CD5^{-/-}/mCD5$ -tg mice was similar to the levels normally expressed on  $CD8$ -SP T cells from  $CD5^{+/+}$  mice (i.e.,  $\sim 2$ -fold lower than normally present on the majority of  $CD4$ -SP T cells).

Thymocytes from  $CD5^{-/-}$  mice have been shown to be hyper-responsive to TCR stimulation relative to  $CD5^{+/+}$  mice as assessed by several criteria, including the TCR-mediated calcium mobilization response (6). To assess whether the cytoplasmic tail of  $CD5$  is required for its inhibitory activity, we compared the TCR-mediated calcium responses elicited by thymocytes from  $CD5^{-/-}$  mice and  $CD5^{-/-}$  mice reconstituted with either the  $CD5$  or  $mCD5$  transgenes. DP thymocytes from  $CD5^{-/-}$  and  $CD5^{-/-}/mCD5$ -tg mice were equally (hyper)-responsive to TCR cross-linking, indicating that the sequences deleted from the  $mCD5$  protein are required for TCR signal inhibition (Fig. 7). Interestingly, neither  $CD4$ -SP thymocytes (Fig. 7) nor  $CD8$ -SP thymocytes or peripheral T cells (data not shown) from  $CD5^{-/-}$  and  $CD5^{-/-}/mCD5$ -tg mice were hyperresponsive to TCR cross-linking, indicating that DP thymocytes are selectively sensitive to the inhibitory effects of  $CD5$ . To determine whether the  $mCD5$  protein also was functionally inert with respect to its effect on thymocyte selection, we introduced the H-Y and ( $A^d$ ) DO10 TCR transgenes into  $CD5^{-/-}/CD5$ -tg and  $CD5^{-/-}/mCD5$ -tg mice. In both TCR-tg

backgrounds, we observed that the phenotype of  $CD5^{-/-}/mCD5$ -tg mice was nearly identical with that of  $CD5^{-/-}$  mice, whereas the phenotype of  $CD5^{-/-}/CD5$ -tg mice more closely resembled  $CD5^{+/+}$  or  $CD5$ -tg mice (Fig. 8) confirming that the  $CD5$  cytoplasmic domain is required for its inhibitory activity during thymocyte selection.



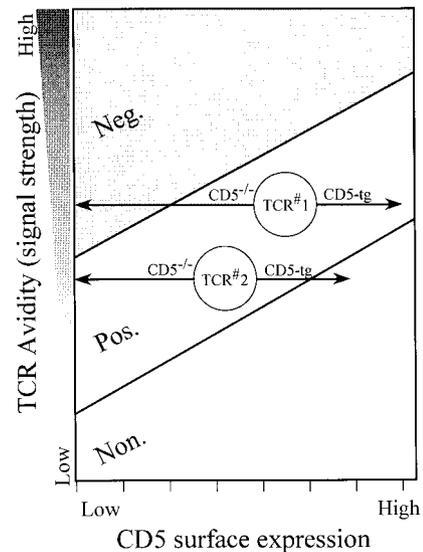
**FIGURE 7.** TCR-mediated calcium responses of thymocytes from  $CD5^{+/+}$ ,  $CD5^{-/-}$ ,  $CD5^{-/-}/CD5$ -tg, and  $CD5^{-/-}/mCD5$ -tg mice. Thymocytes were preloaded with the calcium dye indo-1. Data collection was initiated at time 0. At 30 s, 5.0  $\mu$ g (A) or 1.0  $\mu$ g (B) of anti-TCR $\beta$ -biotin was added and at 60 s 20  $\mu$ g streptavidin was added. Fluorescence indicating bound and unbound indo-1 was monitored for a total of 5 min. Thymocytes were also stained with anti- $CD4$  and anti- $CD8$ , so that data could be analyzed on gated subpopulations. A, Calcium response of  $CD4^+CD8^+$  thymocytes. B, Calcium response of  $CD4^+CD8^-$  thymocytes.  $\square$ ,  $CD5^{+/+}$ ;  $\triangle$ ,  $CD5^{-/-}$ ;  $\circ$ ,  $CD5^{-/-}/CD5$ -tg;  $\bullet$ ,  $CD5^{-/-}/mCD5$ -tg.



**FIGURE 8.** A, Positive selection in H-2D<sup>b</sup> female CD5<sup>+/+</sup>, CD5<sup>-/-</sup>, CD5<sup>-/-</sup>/CD5-tg, and CD5<sup>-/-</sup>/mCD5-tg mice expressing the H-Y TCR-transgene. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes with H-Y clonotype-specific Ab, T3.70 (the percentage of T3.70<sup>high</sup> cells is also shown), or CD5 surface expression on total thymocytes (mean CD5 fluorescence is shown in the boxes). B, Positive selection in A<sup>d</sup> CD5<sup>+/+</sup>, CD5<sup>-/-</sup>, CD5<sup>-/-</sup>/CD5-tg, and CD5<sup>-/-</sup>/mCD5-tg mice expressing the DO10 TCR-transgene. Two color plots show CD4/CD8 staining profiles. Numbers in quadrants indicate the percentage of cells within that quadrant. Histograms show staining of total thymocytes with DO10 clonotype-specific Ab, KJ126 (the percentage of KJ126<sup>high</sup> cells is also shown), or CD5 surface expression on total thymocytes (mean CD5 fluorescence is shown in the boxes).

## Discussion

In this report, we describe the results of experiments designed to elucidate the function of CD5 during thymocyte development and selection. Previous studies of CD5<sup>-/-</sup> mice show that CD5 negatively regulates signaling mediated by the T cell and B cell Ag receptors (6, 7). Although loss of CD5 expression has been shown to dramatically alter the outcome of thymocyte selection in a manner consistent with TCR signal enhancement, the effects of CD5 deletion on thymocyte selection are only apparent in TCR-tg mice, where the fate of a large cohort of cells that express the same TCR can be monitored (6, 16, 17). A possible explanation for this finding is that in non-TCR-tg mice, the relative impact of CD5 deletion varies from cell to cell such that in the entire population, this effect is largely obscured. Thus, in the absence of CD5, the fate of some DP thymocytes may shift from relatively weak positive selection



**FIGURE 9.** Summary of current experimental results. The model shown is based on the avidity model proposed in Ref. 1 except that the relationship between TCR avidity and CD5 surface expression is depicted (see Ref. 18). Shown are the predicted effects of CD5 deletion (CD5<sup>-/-</sup> mice) or incremental CD5 augmentation (CD5-tg mice) on selection of thymocytes that express relatively high or relatively low-avidity TCRs. Note that the avidity of the selecting TCR/ligand interaction dictates the level of endogenous (baseline) CD5 surface expression. In the current experiments, P14, AND, and (A<sup>bd</sup>) DO10 TCR transgenes most closely resemble TCR#1 whereas H-Y and (A<sup>d</sup>) DO10 TCR transgenes most closely resemble TCR#2.

toward more efficient positive selection and/or weak negative selection, whereas that of other DP thymocytes shifts from strong positive selection to strong negative selection. Indeed, analysis of the effect of CD5 deletion on positive selection of thymocytes that express the H-Y- or P14-tg TCRs suggests that deletion of CD5 results in exactly these outcomes, respectively (6). What has remained unclear from these studies is why CD5 deletion differentially effects the selection of thymocytes expressing TCRs with distinct specificities.

In a recent study, we reported that the level of CD5 surface expression on developing thymocytes and mature T cells parallels the avidity of the positively selecting TCR/MHC-ligand interaction (18). In view of these findings, we proposed that CD5 surface expression is titrated by the strength of the TCR signal (18). We also speculated that the requirement for CD5-mediated signal inhibition during thymocyte selection increases when the avidity of the positively selecting TCR/ligand interaction (and the TCR signal strength) is relatively high. Conversely, the requirement for CD5-mediated signal inhibition decreases when the avidity of the positively selecting TCR/ligand interaction (and the TCR signal strength) is relatively low. This model predicts that the impact on thymocyte selection of either CD5 deletion or CD5 overexpression will depend on the avidity of the selecting interaction, which in turn is reflected by the level of endogenous CD5 surface expression.

To test this model, we generated CD5-tg mice and examined the effect of augmented CD5 expression or CD5 deletion (using CD5<sup>-/-</sup> mice) on the selection of thymocytes that express the identical (transgenic) TCR. The results of these experiments (summarized in Fig. 9) were entirely consistent with the proposed model for CD5 function. In TCR-tg systems where the avidity of the selecting interaction is thought to be relatively high (P14, AND and (A<sup>bd</sup>) DO10) and, consequently, where the level of endogenous CD5 surface expression also was relatively high (Fig. 1A),

loss of CD5 converted positive selection to strong negative selection as assessed by the reduction in DP thymocytes, the reduction in mature clonotype-TCR<sup>high</sup> thymocytes and T cells, and the down-modulation of the TCR on DP thymocytes (Figs. 2–4). In contrast, in TCR-tg systems where the avidity of the positively selecting interaction is thought to be relatively low (H-Y and (A<sup>d</sup>) DO10) and the level of endogenous CD5 surface expression is lower (Fig. 1A), deletion of CD5 did not fully convert positive selection to negative selection, as normal numbers of clonotype TCR<sup>+</sup> SP thymocytes and T cells are still generated in these mice (Figs. 2A and 3A). The reduction in DP thymocytes in H-Y TCR-tg/CD5<sup>-/-</sup> and (A<sup>d</sup>) DO10 TCR-tg/CD5<sup>-/-</sup> mice suggests that even in these cases, large numbers of thymocytes are deleted in the absence of CD5. The reason why some thymocytes are deleted and others escape negative selection and are positively selected in H-Y TCR-tg/CD5<sup>-/-</sup> and (A<sup>d</sup>) DO10 TCR-tg/CD5<sup>-/-</sup> mice remains unclear. This could be attributed to other compensating factors similar to CD5 that function to adjust TCR signal intensity or to variable expression of signaling molecules in the pool of developing thymocytes, making some cells more or less susceptible to negative selection. These compensatory mechanisms are presumably sufficient to partially offset the loss of CD5 if the thymocytes express relatively low-avidity TCRs but not if they express high-avidity TCRs and are therefore closer to the signaling threshold for negative selection.

Augmentation of CD5 surface expression (by introduction of a CD5 transgene) only slightly impaired positive selection in P14 TCR-tg and AND TCR-tg mice but markedly inhibited positive selection in H-Y TCR-tg and (A<sup>d</sup>) DO10 TCR-tg mice (Figs. 2B and 3B). The selective decrease in clonotype-TCR<sup>high</sup> SP thymocytes and T cells but relatively unchanged total thymus cellularity in H-Y TCR-tg/CD5-tg and (A<sup>d</sup>) DO10 TCR-tg/CD5-tg mice suggests that increased expression of CD5 converted positive selection to “nonselection” in these mice (Fig. 9). It is important to note that the distinct effects of the CD5 transgene on positive selection in H-Y TCR-tg vs P14 TCR-tg mice or AND TCR-tg vs (A<sup>d</sup>) DO10 TCR-tg mice cannot be attributed to variable CD5 transgene expression, as surface expression of CD5 on thymocytes was increased by the same increment (Figs. 2 and 3). Thus, thymocytes that normally express low levels of CD5 are much more susceptible to a forced increase in CD5 surface expression than are thymocytes that normally express relatively high levels of CD5. This could indicate that the inhibitory effect of CD5 is already at or near maximal in P14 TCR-tg and AND TCR-tg mice such that the increase in CD5 surface expression has little or no additional effect on TCR signaling. Alternatively, the CD5 transgene could attenuate TCR signaling to the same degree in all TCR-tg/CD5-tg mice; however, the decrease in TCR signal intensity is sufficient to convert positive selection to nonselection in the case of thymocytes expressing lower-avidity TCRs (H-Y and (A<sup>d</sup>) DO10 TCR-tg mice), whereas a similar decrease in TCR signal intensity is not sufficient to alter cell fate in the case of cells expressing higher-avidity TCRs (P14 and AND TCR-tg mice; Fig. 9).

A difficulty that arises when interpreting these and other studies concerning thymocyte selection is that the relative affinities of the transgenic TCRs for their selecting ligands and the concentration of these ligands in the thymus are currently unknown. In the case of the MHC class I-restricted TCRs, several observations lead us to infer that the relative avidity of the positively selecting interaction is P14-TCR > H-Y-TCR. First, the “efficiency” of positive selection, reflected by the percentage of CD8-SP T cells that express high levels of the clonotypic TCR, and the ratio of CD8 to CD4 SP T cells in the periphery is greater in P14-TCR-tg mice than in H-Y-TCR-tg mice (Fig. 2). Second, reduction of the TCR

signaling potential by substitution of endogenous  $\zeta$ -chain (which contains three ITAMs) with a signaling-deficient protein ( $\zeta$ -0 ITAM) markedly inhibits positive selection in H-Y TCR-tg mice but not in P14 TCR-tg mice (29–31). The relative avidities of the positively selecting interactions in the MHC class II-restricted TCR-tg systems are more difficult to discern with the first set of criteria. However, the results of experiments similar to those described above in which signaling deficient  $\zeta$ -chain variant transgenes were substituted for endogenous  $\zeta$  suggest that AND > (A<sup>d</sup>) DO10, as positive selection is less dependent on the presence of  $\zeta$ -chain ITAMs in AND TCR-tg mice (29, 31). We also compared the effect of CD5 deletion or introduction of the CD5 transgene on selection of thymocytes that express the same MHC class II-restricted transgenic TCR (DO10) under conditions that have been shown to alter the avidity of the positively selecting TCR/MHC-ligand interaction. When the effects of CD5 deletion or forced CD5 overexpression on thymocyte selection were examined in (A<sup>bd</sup>) DO10 TCR-tg mice, the outcome was consistent with that predicted for a relatively high-avidity selecting interaction (i.e., resembling the results obtained with P14 TCR-tg and AND TCR-tg mice rather than (A<sup>d</sup>) DO10 TCR-tg or H-Y TCR-tg mice; Figs. 2–4).

To determine whether the CD5 cytoplasmic domain (and by extension, CD5-mediated signaling) is required for its inhibitory effect, we generated transgenic mice that express a mutant form of CD5 lacking most of the cytoplasmic tail (mCD5-tg). The mCD5 protein was expressed on the cell surface but, unlike the intact CD5 transgene, failed to reconstitute inhibitory function in CD5<sup>-/-</sup> mice. These results demonstrate that the sequences deleted in mCD5 (which include the imperfect ITAM and the distal tyrosine residue, Y<sup>463</sup>) are required for its inhibitory activity in thymocytes. Deletion mutants structurally similar to mCD5 also were found to lack inhibitory activity when expressed in mature T cells, T cell hybridomas, or B lymphoma cell lines (17, 32, 33). However, results reported in another study performed with T lymphoma lines indicate that Y<sup>378</sup> is required for CD5 inhibitory activity (34). Y<sup>378</sup> is contained within a membrane-proximal sequence that matches the consensus for an ITIM (10), and this motif has been shown to bind to the protein tyrosine phosphatase SHP-1 (14, 34). Our results do not rule out a role for the putative ITIM in mediating CD5 inhibition, as the deletion in mCD5 could render the ITIM sequences nonfunctional, perhaps because of suboptimal tyrosine phosphorylation (Fig. 5). Thus, the precise mechanism of CD5 inhibition of TCR signaling *in vivo* remains to be established. Comparison of the proteins that associate with CD5 and mCD5 before and after activation should assist in identifying the signaling components responsible for CD5 inhibitory function in thymocytes.

Our results indicate that inhibition of TCR signaling by CD5 does not require its coligation with the TCR (Fig. 7), suggesting that CD5 may spontaneously coaggregate with the TCR after TCR cross-linking. The present data also reveal that DP thymocytes are especially sensitive to the inhibitory effects of CD5 relative to mature SP thymocytes, even when SP thymocytes are suboptimally stimulated by TCR cross-linking (Fig. 7). Indeed, an inhibitory effect of CD5 has been difficult to demonstrate in mature T cells (6, 34–37). However, SP T cells are not refractory to CD5 inhibition, as transfection of full-length CD5 into mature T cells from CD5<sup>-/-</sup> mice inhibits Ag-induced IL-2 production (17). These results are not necessarily contradictory. Preselected thymocytes might be especially sensitive to fluctuations in CD5 surface expression. Because these cells have not been subjected to the process of thymocyte selection, their TCR signaling response directly reflects the input of the TCR plus costimulatory and inhibitory molecules. In contrast, postselected cells (SP thymocytes or

peripheral T cells) presumably survive selection only if the integrated signal delivered by the TCR plus costimulatory and inhibitory molecules is appropriate to allow them to mature to the SP stage. Thus, in CD5<sup>-/-</sup> mice, only those thymocytes that can generate the appropriate signaling response for positive selection in the absence of CD5 emerge as SP T cells.

A potential role for negative regulators of TCR signaling such as CD5 could be to influence the repertoire of TCRs that undergo positive selection in the thymus. The constitutive low-level surface expression of CD5 on all DP thymocytes could function to prevent thymocytes expressing TCRs that fail to bind to self-MHC plus self-ligand or do so with extremely low affinity from inadvertently receiving survival signals through nonspecific cell surface interactions. Most T cells expressing these TCRs would presumably not be activated by foreign Ag presented by self-MHC and would therefore not be useful to the mature T cell repertoire. In contrast, the inducible regulation of CD5 surface expression in response to TCR signal strength could enable thymocytes that express TCRs that bind with relatively high affinity to self-MHC plus self-ligand to transduce signals in the range appropriate for positive selection. As shown here and in previous studies (6, 17), thymocytes expressing these TCRs undergo negative selection in the absence of CD5, presumably because the intensity of the signals transduced by these TCRs is too strong for positive selection. Biasing the repertoire toward relatively high-avidity TCRs may be important for mature T cell survival and/or subsequent activation by Ag. Thus, "fine tuning" of TCR signaling by CD5 may help to optimize the repertoire of TCRs expressed on mature T cells.

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