Requirement for p56^{lck} Tyrosine Kinase Activation in T Cell Receptor–mediated Thymic Selection

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Summary

The nonreceptor protein tyrosine kinase p56^{lck} (Lck) serves as a fundamental regulator of thymocyte development by delivering signals from the pre-T cell receptor (pre-TCR) that permit subsequent maturation. However, considerable evidence supports the view that Lck also participates in signal transduction from the mature TCR. We have tested this conjecture by expressing a dominant-negative form of Lck under the control of a promoter element (the distal *lck* promoter) that directs high expression in CD4$^+$CD8$^+$ thymocytes, mature thymocytes, and peripheral T cells, thereby avoiding complications that result from the well-documented ability of dominant-negative Lck to block very early events in thymocyte maturation. Here we report that expression of the catalytically inactive Lck protein at twice normal concentrations inhibits thymocyte positive selection by as much as 80%, while leaving other aspects of T cell maturation intact. This effect was studied in more detail in mice simultaneously bearing the male-specific H-Yα/β TCR transgene and ovalbumin-specific DO10α/β TCR transgene, where even equimolar expression of the dominant-negative Lck protein substantially vitiated the positive selection process. Although deletion of H-Yα/β thymocytes proceeded normally in male mice despite the presence of catalytically inactive Lck, modest inhibition of superantigen-mediated deletion was in some cases observed. These data further implicate Lck in the propagation of all TCR-derived signals, and indicate that even very modest deficiencies in the representation of functional Lck molecules could, in humans, profoundly alter the character of the peripheral TCR repertoire.

The fate of individual T cells developing in the thymus is determined by the specificity of the TCRs they express. The maturation of thymocytes expressing TCRs with potential reactivity with self-ligands is aborted by a process referred to as negative selection (1–5), whereas the maturation of thymocytes expressing TCRs that are potentially reactive with foreign antigens presented by self-MHC–encoded molecules is promoted by a process termed positive selection (6–9). Since intrathymic selection events are clonally restricted and TCR specific, they are thought to be a consequence of TCR-mediated signals stimulated by self-ligands presented on thymic stromal cells.

Previous studies demonstrate that positive selection involves at least two distinct stages (10–14). The development of thymocytes from CD4$^+$CD8$^+$ (DP)[1] to CD4^{high} CD8^{low} or CD4^{low}CD8^{high} stages occurs independently of class I and II molecules, and hence is possibly mediated by CD4- and CD8-independent signals. Further development to the mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ single positive (SP) thymocyte stages is mediated by signals dependent on CD4 and CD8 molecules, a conclusion supported by the observation that no mature CD4 or CD8 SP thymocytes are generated in MHC class II or I knockout mice, respectively (10–14). Although the molecular mechanisms governing each step of the positive selection process have not been fully resolved, there are emerging views from recent published reports. When the TCRs on DP thymocytes are engaged with anti-TCR mAb in vitro, transcription of recombinant-activating gene (RAG) 1 and RAG-2 in DP

[1] *Abbreviations used in this paper:* B6, C57BL/6; DLGKR mouse, a dominant-negative *lck* transgenic mouse; DN, double negative; DO10Tg, anti-OVA TCR-α/β transgenic; DP, double positive; FCM, flow cytometry; Fyn, p59^{fyn}; hGH, human growth hormone; HSA, heat-stable antigen; H-YTg, anti-H-Y TCR-α/β transgenic; Lck, p56^{lck}; LM, littermate; PI, propidium iodide; RAG-1, recombination-activating gene 1; SP, single positive; Tg, transgenic; ZAP-70, zeta-associated protein.

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thymocytes is turned off (15, 16), expression of CD4 and CD8 is downregulated (17, 18), and surface expression of CD69 is induced (17, 19, 20). These changes occur in the first step of thymic positive selection within the DP thymocyte population, since the first distinct population observed during positive selection is comprised of $CD69^+$ $CD4^{low}CD8^{low}$ thymocytes (17). More recently, qualitative differences in signal transduction controlling positive and negative selection have been reported. Activation of $p21^{ras}$ and mitogen-activated protein kinase kinase (MAPKK) appears to be required for thymic positive but not negative selection (21, 22). Similarly, the activation of calcineurin, a calcium- and calmodulin-dependent phosphatase, is essential for the first step of positive selection, but calcineurin activation is not required for negative selection in the thymus (23, 24).

CD4 and CD8 glycoproteins are thought to participate in TCR-mediated signal transduction by binding to the same class II or I MHC molecules that are engaged by the TCR (25, 26). The cytoplasmic domains of both CD4 and CD8 interact with the protein tyrosine kinase $p56^{lck}$ (Lck) (27–30), an essential molecule for thymocyte maturation (31, 32) and for signaling in some transformed T cell lines (33–35). Although the association of Lck with CD4 is important for effective in vitro T cell responses to antigen (36), this function is not necessary for either the generation of CD4 T cells in the thymus or for helper T cell development (37). Similarly, Chan et al. (38) reported that CD8/Lck association was not essential for CD8 T cell development in a TCR transgenic (Tg) model.

In this study, we have examined the requirement for Lck activity in thymic positive and negative selection using Tg mice created to express an enzymatically inactive Lck protein, bearing an arginine for lysine substitution at position 273, under the control of the distal promoter of the *lck* gene (designated DLGKR). In DLGKR Tg mice, the maturation of thymocytes to the DP stage is normal, and therefore, the effect of enzymatically inactive Lck on thymic selection can be investigated. Our results demonstrate that thymic positive selection requires Lck activity.

Materials and Methods

Assembly of DLGKR Expression Vector. Previous work has shown that elements within the *lck* gene contribute to transcriptional regulation and copy number–dependent expression of transgenes (39). To preserve elements of this regulation, we used a piece of the *lck* gene that had previously been modified in three ways (32). First, lysine codon 273 was changed to an arginine codon to encode a catalytically inactive kinase. Second, the *lck* 3′-untranslated region was substituted with the 3′-untranslated region from the human growth hormone (hGH) gene, to provide a tag for detection of transgene integration and transgene-specific transcripts. Finally, several out-of-frame ATG codons in the 5′-untranslated region were deleted. These codons had previously been shown to interfere with translation of the *lck* transcript (40). A 9.6-kb fragment, including the entire *lck* coding region and the hGH 3′-untranslated region, was removed from the *lck*R273 construct (32) by partial digestion with BamHI and complete digestion with NotI. A 7-kb BamHI/NotI fragment containing the *lck* distal promoter in pUC19 (41) was then ligated to provide the expression vector designated pDLGKR with the *lck*R273 genomic fragment juxtaposed to the *lck* distal promoter. The diagrammatic representation of the DLGKR transgene is depicted in Fig. 1 *A*.

Animals. C57BL/6 (B6), CBA/J, and BALB/c mice were purchased from Clea Inc. (Tokyo, Japan). The dominant-negative *lck* Tg mice (DLGKR mice) were produced by injection of a 13.6-kb NotI fragment from pDLGKR into $(DBA \times B6)F_2$ embryos. Transgene-positive founders were backsrossed to B6 mice to establish lines. Transgene expression was determined by analyzing total cellular RNA from thymocytes and splenocytes of DLGKR mice (Fig. 1 *B*). Anti-H-Y TCR-α/β Tg (H-YTg) mice (5) were established by Drs. H. von Boehmer and M. Steinmetz (Basel Institute for Immunology, Basel, Switzerland), and provided by Dr. Alfred Singer (National Cancer Institute, National Institutes of Health [NIH] Bethesda, MD). Anti-OVA TCR-α/β Tg (DO10Tg) mice (42) were kindly provided by Dr. Dennis Loh (Nippon Roche Research Center, Kanagawa, Japan). All mice used in this study were maintained under specific pathogen-free conditions.

RNA Analysis by Northern Blotting. Total cellular RNA was isolated from unfractionated thymocytes and splenocytes, according to a method described by Chomoczynski and Sacchi (43). 10 μg of RNA for each sample was fractionated on 1% formaldehyde gels and transferred onto nylon membranes (Hybond-N; Amersham International, Amersham, Bucks, UK). The blot was subsequently hybridized with ^{32}P-labeled full-length *lck* cDNA, visualized by autoradiography, and analyzed by PhosphorImager. Because the total abundance of *lck* transcript in thymocytes is much higher than that in splenocytes, the duration of exposure was varied to allow optimal visualization of the distinct signals representing the endogenous and transgene-derived transcripts.

Reagents. The reagents used in this study were as follows: FITC-conjugated anti-TCR-α/β-FITC (H57-597-FITC) (44), anti-TCR-α/β-biotin (H57-597-biotin), anti–heat-stable antigen (HSA)–FITC (J11d-FITC) (45), anti-H-Y Tg TCR-α-FITC (T3.70-FITC) (46), anti-Qa-2-biotin (1-9-9-biotin) (47), and anti-CD8-APC (53-6.72-APC) (48) were prepared in our laboratory. PE-conjugated anti-CD4 mAb (GK1.5-PE) was purchased from Becton Dickinson Immunocytometry Systems ([BDIS], Mountain View, CA). Anti-TCRVβ7-FITC (TR310-FITC) and anti-CD69-FITC (H1.2F3-FITC) were purchased from PharMingen (La Jolla, CA). Anti-DO10 Tg TCR-α (KJ-1) (49), anti-TCRVβ6 (44-22-1) (50), anti-Vβ11 (RR3-15) (51), anti-Vβ5 (MR9-4) (52), anti-TCRVβ8.2 (F23.2) (53), and anti-FcRγI mAbs (2.4G2) (54) were used as culture supernatants. PE-conjugated streptoavidin (PE-avidin) and Texas red-avidin were purchased from GIBCO-BRL, Gaithersburg, MD). Polyclonal goat anti–mouse IgG-FITC was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Polyclonal goat anti–rat IgG-FITC was purchased from Cappel (Durham, NC).

Immunofluorescent Staining and Flow Cytometry Analysis. Freshly prepared thymocytes were suspended in PBS supplemented with 2% FCS and 0.1% sodium azide. In general, 10^6 cells were incubated on ice for 30 min with appropriate staining reagents as described (55). For direct staining, cells were first incubated with 2.4G2 to prevent nonspecific binding of mAbs via FcR interactions. For indirect flow cytometry (FCM) analysis, thymocytes were first incubated with culture supernatant of anti-TCRVβ mAbs followed by goat anti–rat IgG-FITC or goat anti–mouse IgG-FITC. The stained cells were washed extensively and then anti-CD4-PE and anti-CD8-APC were added after blocking of

residual binding sites of FITC-labeled anti-IgGs. In multicolor FCM analyses, electronic compensation was done by using cell mixtures of positive and negative cell populations in each fluorescence emission. FCM analysis was performed on FACSvantage® (BDIS), and fluorescence data were collected as a list mode on 40,000 viable cells as determined by light scatter parameters and propidium iodide (PI) exclusion. Where indicated, 400,000 cells were collected. FACSvantage® and CELLQuest® software programs (BDIS) were used for collecting and analyzing data.

Purification of $CD4^+CD8^+$ DP Thymocytes. DP thymocytes were isolated by adherence to plates coated with anti-CD8 mAb (83-12-5), and were >96% $CD4^+CD8^+$ as described (56).

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting with antiphosphotyrosine mAb (4G10; Upstate Biotechnology, Inc., Lake Placid, NY), anti-Lck antiserum (#688, specific for a part of the unique sequence of Lck, RNGSEVRDPLVTYEGSLPPASPLQDN; a gift from Dr. Larry Samelson, NIH), and anti-TCR-ζ antiserum (#551; a gift from Dr. Alfred Singer, NIH) were performed as previously described (55, 56). In brief, thymocytes were solubilized in 1% digitonin lysis buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM sodium vanadate, and 1 mM EDTA). Cell lysates were immunoprecipitated with protein G–Sepharose preabsorbed with anti-CD4 mAb (GK1.5), anti-CD8 mAb (53-6.72), or anti-Lck antiserum. The immunoprecipitates were applied to 10% SDS-PAGE under reducing conditions and then subjected to electrotransfer to polyvinylidene difluoride nylon membranes. The membranes were incubated first with 3% FCS containing PBS for 1 h at room temperature, and then with 1:200-diluted anti-Lck antiserum or 1 μg/ml of antiphosphotyrosine mAb (4G.10) in PBS containing 0.4% Tween-20. For immunoblotting with 4G.10 mAb, the membrane was washed and then incubated with horseradish peroxidase–conjugated protein A (Cappel). A chemiluminescence detection system (ECL; Amersham International) was used for 4G.10 immunoblottings. Protein A-^{125}I was used for immunoblottings with anti-Lck antiserum. The band intensities were measured by a densitometer and an arbitrary densitometric unit was assigned to each band.

In Vitro Immune Complex Kinase Assay. Immune complex kinase assays were performed as described (57). In brief, cells were solubilized at a concentration of 10^8/ml in lysis buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, and 1 mM sodium vanadate) containing digitonin at 1%. Cell lysates were immunoprecipitated with anti-CD4 mAb (GK1.5), anti-CD8 mAb (53.6-72), or anti-Lck antiserum (#688) preabsorbed to protein G–Sepharose. The specificity of bands running at the expected size of Lck from the immunoprecipitates with anti-Lck antisera was confirmed by adding the appropriate antigenic peptides during immunoprecipitation and monitoring the disappearance of the relevant band. After incubation, beads were washed in lysis buffer lacking EDTA and sodium vanadate, and then incubated with 15 μCi of γ-[^{32}P]ATP (5,000 Ci/mmol; Amersham International) for 5 or 15 min on ice in kinase buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 μM non–radiolabeled ATP). Kinase reactions were quenched with 50 ml of 2× Laemmli sample buffer with 4 mM EDTA and resolved on 10% gels.

Results

Gene Dose-dependent Reduction in the Generation of Mature SP Thymocytes in DLGKR Mice. The requirement for Lck tyrosine kinase activity in thymic positive and negative selection was examined in a Tg mouse strain (DLGKR) overexpressing catalytically inactive LckR273 under the control of the distal *lck* promoter. In contrast to the proximal *lck* promoter, the distal *lck* promoter, when used in Tg mice, is 5–10 times more active in mature thymocytes and peripheral T cells than in $CD4^-CD8^-$ cells (41). We reasoned, therefore, that the DLGKR mouse might provide a good model system for analyzing the role of Lck in thymic selection. The characteristics of DLGKR thymocytes were examined by comparing the FCM profiles of normal, Tg heterozygous ($DLGKR^{-/+}$) and homozygous ($DLGKR^{+/+}$) DLGKR mice. Yields of thymocytes among these different mice were essentially similar (data not shown). CD4/CD8 profiles (Fig. 2, *top*) revealed normal numbers of DP thymocytes in both Tg heterozygous and homozygous DLGKR mice as compared with normal littermates (LM), however, a gene dose-dependent reduction of CD4 SP cells was noted. These characteristic profiles were obtained from seven independent experiments. The frequency of TCR-α/β^{high} CD4 and CD8 SP mature thymocytes was determined by three-color FCM analysis with anti-TCR, anti-CD4, and anti-CD8 reagents, and a similar type of decrease was detected in both SP thymocyte subpopulations of DLGKR mice.

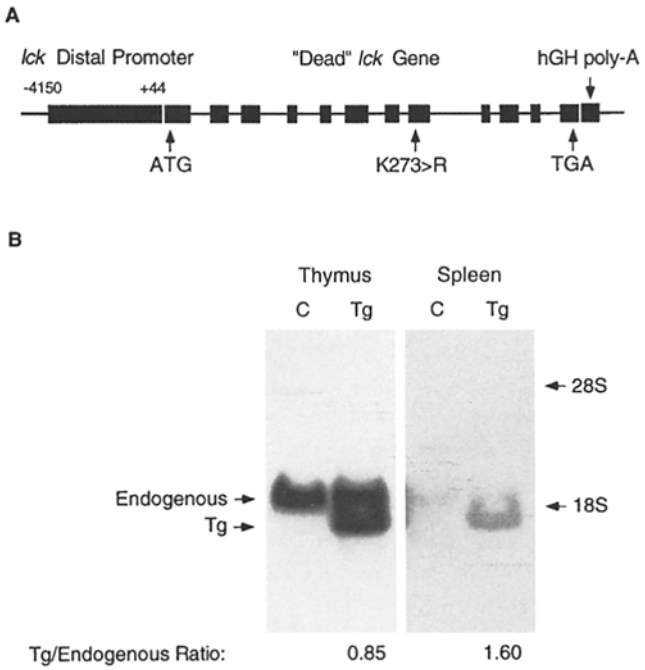

Figure 1. Diagrammatic representation of the DLGKR transgene and its pattern of expression. (*A*) The mouse *lck* gene (exon 1–12) with a Lys-to-Arg mutation at codon 273 was engineered into a vector that contains the *lck* distal promoter. Exon 12 is fused to the poly(A) signal from the 3′-untranslated region of the hGH gene. See Materials and Methods for a detailed description. (*B*) Transgene expression was determined by analyzing total cellular RNA from thymocytes and splenocytes of control (*C*) and Tg animals. A ^{32}P-labeled *lck* cDNA probe detects both the endogenous and transgene-derived transcripts. Exposure time for the thymocyte panel was 8 h and for the splenocyte panel, 24 h. The relative ratio of transcript abundance was determined by PhosphorImager analysis.

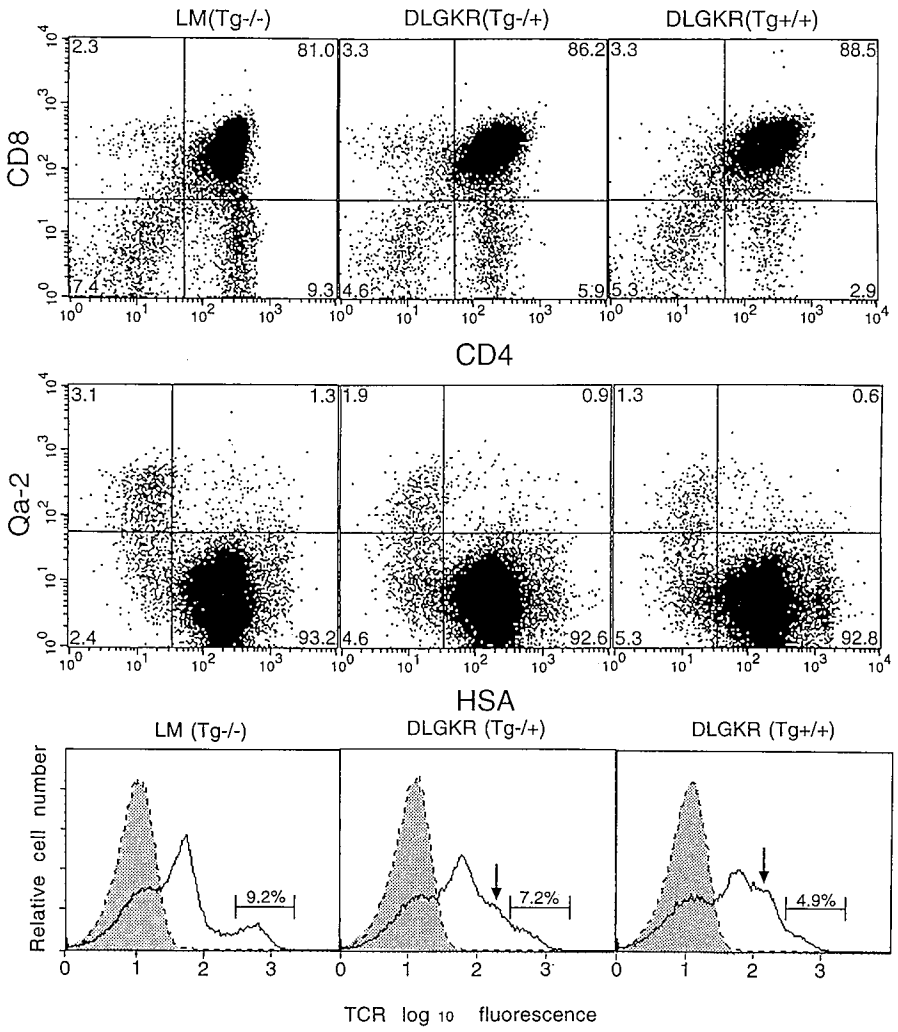

Figure 2. Phenotypic characterization of thymocytes of DLGKR mice. Thymocytes were prepared from 8-wk-old Tg^- LM, DLGKR Tg heterozygous ($Tg^{-/+}$), and DLGKR Tg homozygous ($Tg^{+/+}$) mice, and stained with anti-TCR-α/β-FITC, anti-CD4-PE, and anti-CD8-APC (*top*), anti-HSA-FITC and anti-Qa-2-biotin followed by avidin-PE (*middle*), or anti-TCR-α/β-FITC (*bottom*). Representative profiles of CD4/CD8, HSA/Qa-2, and TCR-α/β are demonstrated. The percentages of cells present in each area are indicated. Arrows denote the cells with increased TCR expression on TCR^{dull} positive thymocytes (*bottom*). The percentages of TCR^{high} cells in CD4 SP thymocyte subpopulation calculated from the data shown (*top*) were 8.49% in LM ($Tg^{-/-}$), 7.12% in DLGKR ($Tg^{-/+}$), and 3.45% in DLGKR ($Tg^{+/+}$) mice. The percentages of TCR^{high} cells in CD8 SP thymocyte subpopulation were 2.10% in LM ($Tg^{-/-}$), 1.90% in DLGKR ($Tg^{-/+}$), and 1.50% in DLGKR ($Tg^{+/+}$) mice.

To clarify further the differentiation status of SP thymocytes in DLGKR mice, additional T cell differentiation markers were analyzed (Fig. 2, *middle* and *bottom*). Among SP thymocytes, functionally mature thymocytes manifest a $HSA^-Qa\text{-}2^+$ phenotype (47). The number of $HSA^-Qa\text{-}2^+$ cells in DLGKR mice decreased in a gene dose-dependent manner (Fig. 2, *middle*). Similarly, a single-parameter histogram of TCR-α/β revealed decreased numbers of TCR-α/β^{high} mature thymocytes in DLGKR mice (Fig. 2, *bottom*). In addition, increased TCR expression was detected in DLGKR DP thymocytes (arrows, Fig. 2, *bottom*; data not shown). This characteristic phenotype of DP thymocytes has been observed in anti-CD4 mAb-treated (58), MHC class II–negative (59), and CD4 Tg mice (57), and in in vitro 37°C cultured DP thymocytes (55), and is consistent with the view that Lck activity reduces the fidelity of TCR assembly (57).

Cell Surface Expression of CD69 in DLGKR Mice. CD69, an early activation antigen expressed on ~10% of normal thymocytes, was shown to be a marker of thymocytes undergoing TCR-mediated thymic selection (17, 19, 20). The surface expression of CD69 in the thymus is thought to be a consequence of intracellular signaling events initiated by TCR engagement of ligands on thymic stromal cells. All $CD69^+$ thymocytes express TCR-α/β and they include a fraction of DP thymocytes and about one half of the CD4 and CD8 SP thymocytes (17). Consequently, we examined CD69 expression on fresh thymocytes of normal, DLGKR heterozygous, and DLGKR homozygous mice. The FCM analysis of fresh thymocytes stained with an anti-CD69 mAb is shown in Fig. 3, *top*. A decrease in the number of $CD69^+$ cells was seen in DLGKR mice which appeared to be dependent on gene dosage. Thus, a change in CD69 expression is another consequence of the expression of catalytically inactive Lck.

We have previously demonstrated that DP thymocytes express CD69 within a few hours after in vitro stimulation with immobilized anti-TCR mAb (17). Thus, we evaluated the ability of DLGKR thymocytes to express CD69 upon stimulation with anti-TCR mAb. Thymocytes from normal, DLGKR heterozygous, and DLGKR homozygous mice were stimulated for 14 h at 37°C in vitro with anti-TCR mAb (H57-597) that had been precoated on plates at different doses (1–30 μg/ml). The cultured thymocytes

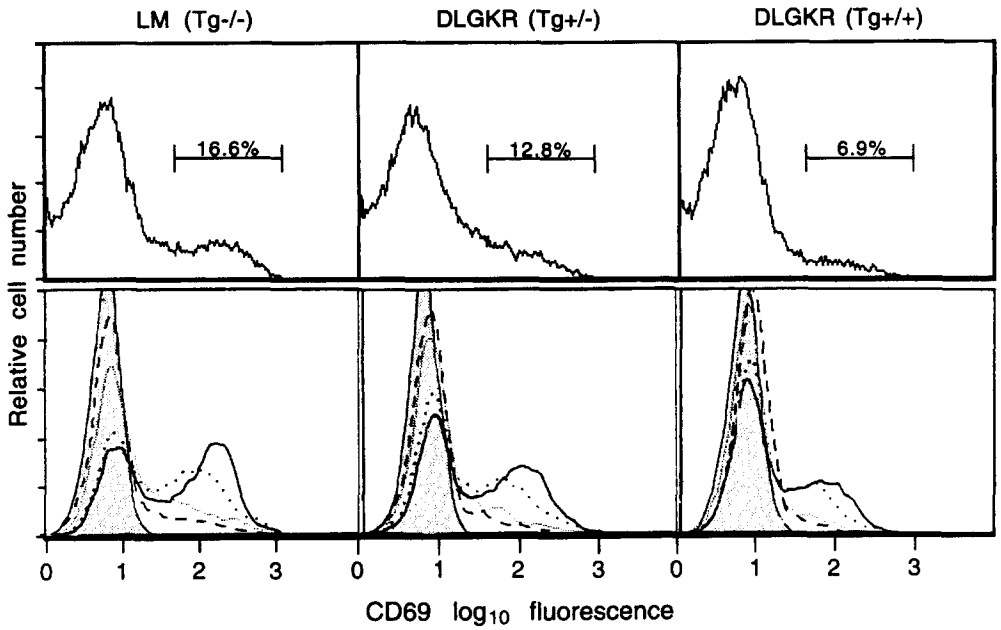

Figure 3. CD69 expression on freshly prepared thymocytes and in vitro anti-TCR–stimulated thymocytes from DLGKR mice. (*Top*) Cell surface expression of CD69 on thymocytes from Tg^- LM, Tg heterozygous ($Tg^{-/+}$), and homozygous ($Tg^{+/+}$) DLGKR mice was examined by FCM analysis with anti-CD69-FITC. The percent representation of $CD69^+$ cells is indicated. (*Bottom*) Thymocytes were stimulated on plates coated with anti-TCR mAb (H57-597) at doses indicated at 37°C for 14 h. The cells were harvested and stained with anti-CD69-FITC: FCM analysis was then performed with PI for dead cell exclusion. The CD69 profiles are shown with background stainings (*shaded areas*). The percentage of $CD69^+$ cells in each line was as follows: 56.4, 48.4, 23.5, and 12.1% (in normal LM mice); 46.8, 35.2, 14.0, and 8.0% (in heterozygous mice); and 32.1, 23.8, 8.4, and 4.8% (in homozygous mice) for anti-TCR stimulations at concentrations of (——) 30, (· · ·) 10, (····) 3, and (– – –) 1 μg/ml, respectively.

were harvested, washed, and then stained with anti-CD69-FITC. Dead cells were excluded from the FCM analysis by the use of PI. The CD69 profiles with control background stainings are shown in Fig. 3, *bottom*. Normal thymocytes stimulated with immobilized anti-TCR mAb express CD69 on their surfaces in a dose-dependent manner. As compared with the normal LM ($Tg^{-/-}$), CD69 induction was found to be less efficient in heterozygous ($Tg^{+/-}$), and substantially reduced in homozygous ($Tg^{+/+}$), DLGKR mice. These results suggest that Lck contributes to the TCR-derived signaling pathway that regulates CD69 expression.

The Amount of Lck Protein in DLGKR Thymocytes. We determined the amount of Lck protein in thymocytes from control LM and heterozygous DLGKR Tg mice. The Lck molecules were immunoprecipitated with anti-CD4 mAb, anti-CD8 mAb, or anti-Lck antiserum, and then the immunoprecipitates were analyzed by immunoblotting with anti-Lck antiserum as described in Materials and Methods. The anti-Lck antiserum reacts with both endogenous normal and transgene-derived mutant Lck molecules. As shown in Fig. 4 *A*, the total amount of Lck protein as well as the amount of Lck coprecipitated with CD4 and CD8 molecules was increased about twofold in DLGKR mice. A similar level of increase was also observed in anti-Lck immunoblottings by using total cell lysates (data not shown).

Specific Reduction of Tyrosine Kinase Activity of Lck in DLGKR Thymocytes. To assess the extent to which catalytic activity was preserved among Lck molecules in DLGKR mice, the tyrosine kinase activity of Lck in thymocytes of DLGKR mice was directly assessed. Lck associated with CD4 or CD8, and the total cellular Lck in the digitonin lysates from normal and heterozygous DLGKR thymocytes were immunoprecipitated with anti-CD4, anti-CD8, or anti-Lck reagents, respectively. As shown in Fig. 4 *B*, the kinase activity of Lck associated with CD4 or CD8 in DLGKR thymocytes was substantially decreased, as measured by autophosphorylation and by transphosphorylation of an exogenous substrate, enolase. In addition, tyrosine kinase activity per Lck protein in anti-Lck immunoprecipitates was also decreased by at least twofold. Similar results were obtained in six independent experiments, including an experiment with titration of cell lysates used in immunoprecipitations (data not shown).

Decreased Activation of Lck upon CD4 Cross-linking in DLGKR Thymocytes. The tyrosine kinase activity of CD4-associated Lck is increased when CD4 molecules are cross-linked (60). Consequently, we assessed the activity of CD4-associated Lck after cross-linking of CD4 in DLGKR thymocytes. Since Lck in freshly prepared thymocytes were heavily phosphorylated by continuous CD4 engagement in vivo (52, 54), thymocytes from heterozygous DLGKR mice were first cultured at 37°C overnight. The cells were then stimulated with IgM anti-CD4 mAb (RL-172) for 5–10 min as described (57). Lck kinase activity associated with CD4 was assessed by immunoprecipitation with anti-CD4 (GK1.5) using an in vitro immune complex kinase assay. Increased kinase activity at the 5-min time point in control non-Tg thymocytes was observed, however, this induction was reduced and delayed in DLGKR Tg thymocytes (Fig. 4 *C*). This result almost certainly reflects competition by the overexpressed inactive Lck protein for CD4 molecules.

Tyrosine Phosphorylation of TCR-ζ in DP Thymocytes from DLGKR Mice. Tyrosine residues of TCR-ζ in freshly prepared DP thymocytes are phosphorylated (56) and a

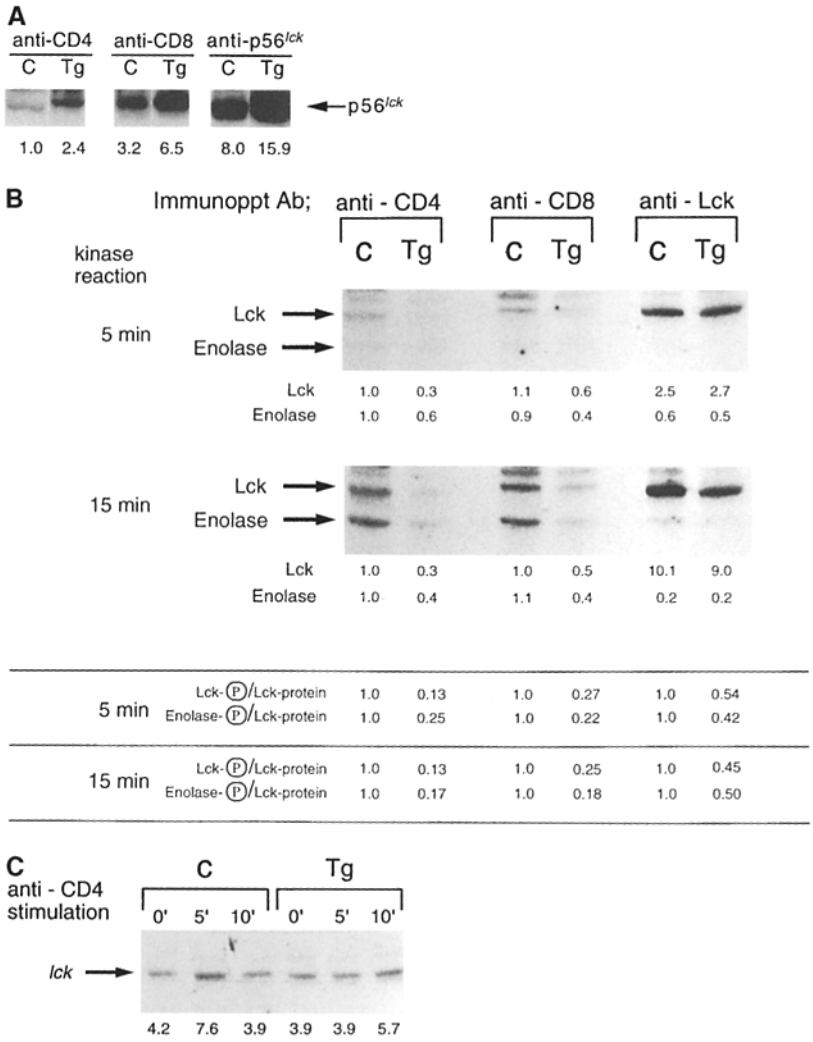

Figure 4. The amount of protein and tyrosine kinase activity of Lck in DLGKR thymocytes. Digitonin cell lysates were prepared from normal and heterozygous DLGKR thymocytes. The Lck associated with CD4 or CD8 and total cellular Lck were immunoprecipitated with anti-CD4, anti-CD8 mAb, or anti-Lck antiserum, respectively. The amount of Lck protein was visualized by immunoblotting with anti-Lck antiserum (*A*). Tyrosine kinase activity in each precipitate was determined by in vitro immune complex kinase assay (*B*). In addition to autophosphorylation of Lck, enolase was used as an indicator of transphosphorylation activity. The phosphorylated proteins migrating at the expected size of Lck and enolase are indicated. Arbitrary densitometric units are indicated under each band. The values normalized for the amount of Lck protein present in each precipitate are also demonstrated. In *C*, thymocytes were first cultured at 37°C overnight and then stimulated with IgM anti-CD4 mAb (RL-172) for another 5–10 min. Lck kinase activity associated with CD4 was assessed by immunoprecipitation with anti-CD4 (GK1.5) and after in vitro immune complex kinase assay. Arbitrary densitometric units are indicated under each band.

substantial portion of the phosphorylation on TCR-ζ detected in fresh DP thymocytes is mediated by CD4-associated Lck in vivo (57). It would, therefore, be anticipated that reduced ζ phosphorylation would occur in the DLGKR mice. We evaluated the phosphorylation status of TCR-ζ in fresh DP thymocytes as well as the ability of CD4-associated Lck to rephosphorylate TCR-ζ in DLGKR DP thymocytes. DP thymocytes were purified from heterozygous DLGKR mice, and tyrosine phosphorylation on TCR-ζ was assessed by immunoprecipitation with anti-TCR-β mAb (H57-597) and immunoblotting with antiphosphotyrosine mAb (4G.10). A 50% decrease in tyrosine phosphorylation on TCR-ζ was detected in freshly prepared DLGKR DP thymocytes (Fig. 5 *A*), although the amount of TCR-ζ protein in the same precipitates, assessed by immunoblotting with anti-TCR-ζ antiserum (#551), was equivalent (Fig. 5 *B*).

To evaluate the capacity of CD4 cross-linking to stimulate TCR-ζ phosphorylation, DP thymocytes were cultured at 4 or 37°C overnight, and then stimulated for 30 min with anti-CD4 mAb in the presence of FcR^+ cells (56). As can be seen in Fig. 4 *C*, rephosphorylation of TCR-ζ after CD4 cross-linking was significantly reduced in DLGKR DP thymocytes. These results provide further documentation of the reduced Lck kinase activity associated with CD4 in DLGKR DP thymocytes.

Effect of Overexpression of Enzymatically Inactive Lck on Positive and Negative Selection of H-Y– and OVA-specific TCR-α/β Tg Thymocytes. Another system that allows for the evaluation of Lck involvement in the generation of SP thymocytes is the use of TCR Tg mice. DLGKR mice were crossed with TCR Tg mice that express TCR derived from an H-Y–specific, $H\text{-}2D^b$-restricted CTL clone whose ligand is the male antigen H-Y in the context of $H\text{-}2D^b$. In the $H\text{-}2^b$ female H-Y Tg thymus, Tg TCR-expressing thymocytes are subject to positive selection, whereas the transgene-expressing thymocytes are subject to negative selection in the male thymus because of the expression of both D^b and H-Y antigens (5, 8). The representative results of the yield of thymocytes and the CD4/CD8 profiles of female and male H-Y/DLGKR double-Tg mice (heterozygous for DLGKR) are shown in Fig. 6. The generation of $CD4^-CD8^+$ thymocytes in female mice bearing the H-Y TCR-α/β transgene was substantially reduced by the pres-

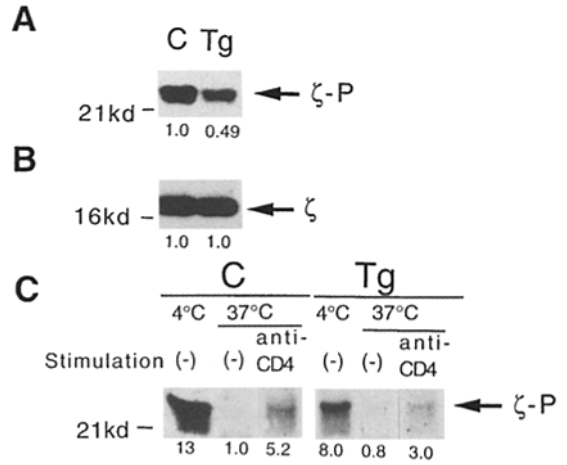

Figure 5. TCR-ζ phosphorylation in DP thymocytes from DLGKR mice. (*A*) Purified DP thymocytes from heterozygous DLGKR mice were lysed with digitonin in the presence of phosphatase inhibitors. TCR-ζ was immunoprecipitated with anti-TCR-α/β mAb, and the amount of tyrosine phosphorylation was determined by immunoblotting with anti-phosphotyrosine mAb 4G10. (*B*) The amount of TCR-ζ protein on the same membrane was determined by using anti-TCR-ζ antiserum (#551). (*C*) Purified DP thymocytes were first cultured at 4 or 37°C overnight, and then stimulated at 37°C for 30 min with anti-CD4 mAb (GK1.5) in the presence of FcR^+ LK35.2 cells. The phosphorylation status of TCR-ζ was determined by the same methods as in (*A*). Arbitrary densitometric units are indicated under each band.

ence of catalytically inactive Lck, whereas the generation of DP and double negative (DN) thymocytes was not influenced in either ungated or gated (Tg TCR-α^+) population. In contrast, CD4/CD8 profiles of male H-Y/DLGKR double-Tg (DLGKR $Tg^{-/+}$) animals showed little difference in the extent of thymocyte deletion, indicating that negative selection of H-Y T cells is not significantly affected in the DLKGR thymus.

Next, DLGKR mice (H-2^b background) were crossed with TCR Tg mice (H-2^d background) that express TCR derived from an OVA-specific I-A^d–restricted T cell hybridoma (DO11-10). In the H-2^d–positive DO10 Tg animals, Tg TCR-expressing thymocytes are subject to positive selection (42). In Fig. 7, representative CD4/CD8 profiles of DO10/DLGKR double-Tg mice (heterozygous for DLGKR) are demonstrated with yields of thymocytes. The generation of $CD4^+CD8^-$ thymocytes bearing DO10 Tg TCR was dramatically reduced by the presence of catalytically inactive Lck, whereas the generation of DP and DN thymocytes was not influenced. The results obtained from DO10 Tg^- LM control mice (H-$2^{b/d}$) provided a confirmation of the results demonstrated in Fig. 2.

Effect of DLGKR on Endogenous Superantigen-mediated Thymocyte Deletion. Since negative and positive selection both result from TCR-mediated signals that should require Lck, we studied the effects of DLGKR expression on thymocyte deletion in more detail, making use of the intrathy-

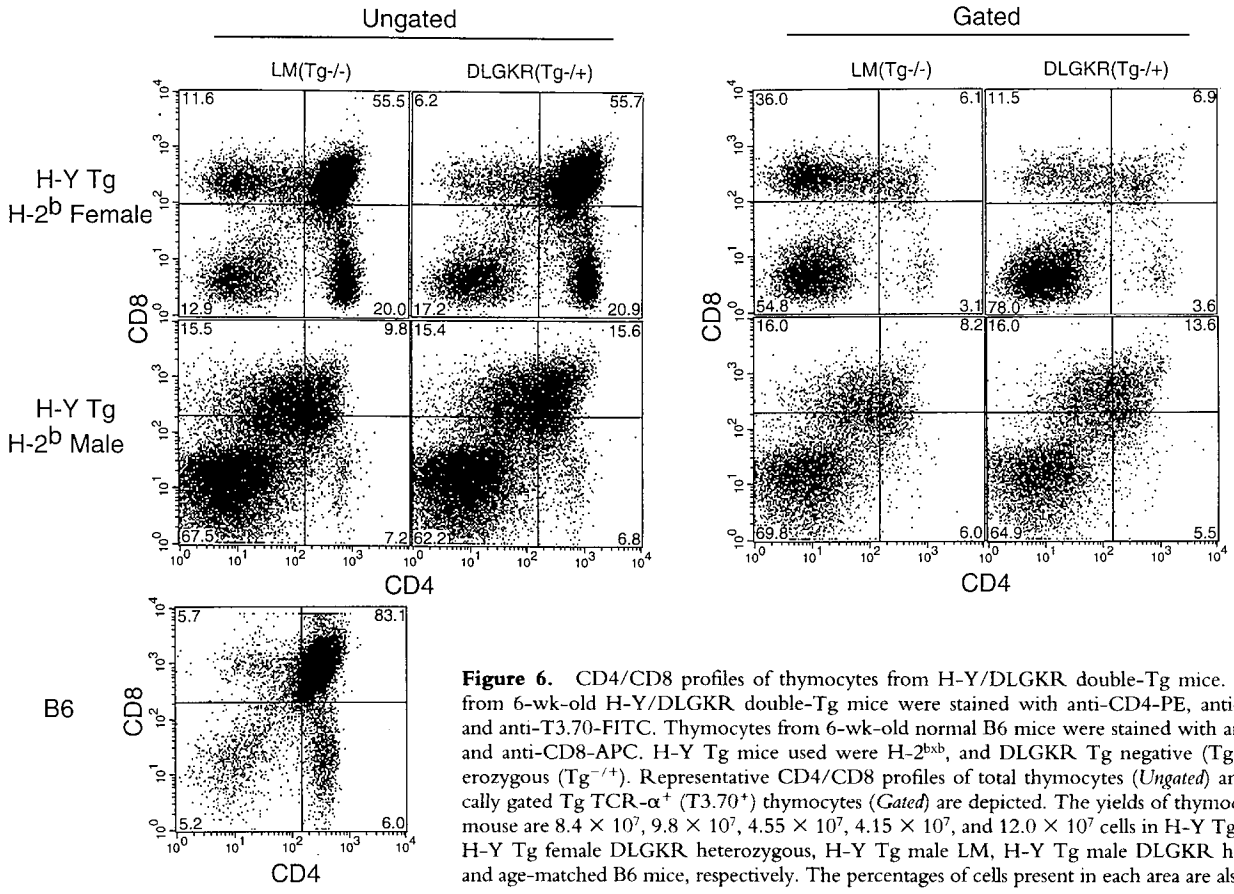

Figure 6. CD4/CD8 profiles of thymocytes from H-Y/DLGKR double-Tg mice. Thymocytes from 6-wk-old H-Y/DLGKR double-Tg mice were stained with anti-CD4-PE, anti-CD8-APC, and anti-T3.70-FITC. Thymocytes from 6-wk-old normal B6 mice were stained with anti-CD4-PE and anti-CD8-APC. H-Y Tg mice used were H-2^{bxb}, and DLGKR Tg negative ($Tg^{-/-}$) or heterozygous ($Tg^{-/+}$). Representative CD4/CD8 profiles of total thymocytes (*Ungated*) and electronically gated Tg TCR-α^+ ($T3.70^+$) thymocytes (*Gated*) are depicted. The yields of thymocytes in each mouse are 8.4×10^7, 9.8×10^7, 4.55×10^7, 4.15×10^7, and 12.0×10^7 cells in H-Y Tg female LM, H-Y Tg female DLGKR heterozygous, H-Y Tg male LM, H-Y Tg male DLGKR heterozygous, and age-matched B6 mice, respectively. The percentages of cells present in each area are also indicated.

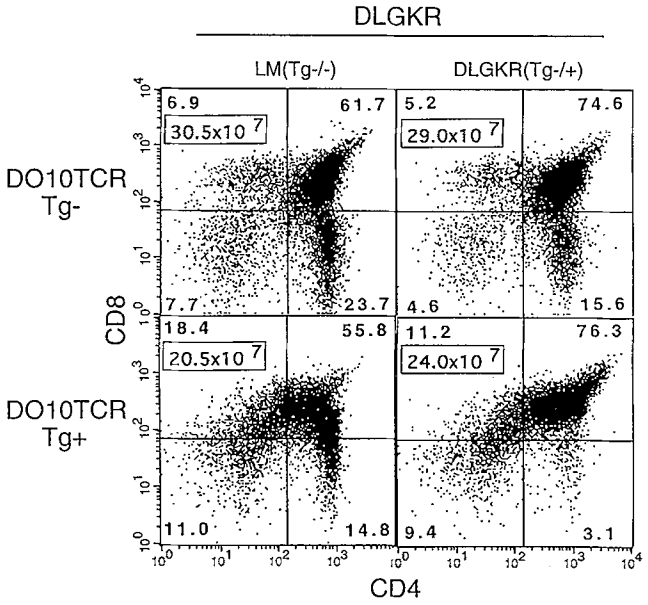

Figure 7. CD4/CD8 profiles of thymocytes from DO10/DLGKR double-Tg mice. Thymocytes from 6-wk-old DO10/DLGKR double-Tg mice were stained with culture supernatant of KJ-1 mAb followed by goat anti-mouse Ig-FITC, and anti-CD4-PE and anti-CD8-APC. Representative CD4/CD8 profiles of ungated thymocytes of DO10 Tg^- LM (*top*) and electronically gated Tg TCR-α^+ ($KJ1^+$) thymocytes of DO10TCR Tg (*bottom*) are depicted. The yield of thymocytes in each mouse is shown as a boxed number. The percentages of cells present in each area are also indicated.

mic clonal deletion of Vβ6-, Vβ11-, Vβ7-, and Vβ5-bearing cells in mice expressing retroviral superantigens, e.g., (CBA/J × B6)F_1 animals (Mls-1^a, I-E^+) (4). The presence of the retroviral superantigens Mls-1^a and Mls-2^a in these mice, along with expression of MHC class II I-E molecules, leads to deletion of Vβ6-, Vβ7-, and Vβ11-bearing cells (61, 62). The presence of Mtv-9 gene products in B6 mice leads to deletion of Vβ5-bearing T cells in I-E^+ mice (63).

We crossed heterozygous DLGKR mice of B6 background to normal CBA/J mice. Three-color FCM analysis in thymocytes was performed with anti-CD4, anti-CD8, and mAbs specific for TCRCβ, TCRVβ6, Vβ11, Vβ7, Vβ5, or Vβ8.2. As shown in Table 1, the generation of CD4 SP thymocytes was significantly inhibited in (CBA/J × B6)F_1 DLGKR Tg^+ mice (leftmost column). This result is consistent with the results obtained from the analysis of B6 background mice expressing a single copy of the DLGKR transgene (Fig. 2). In the (CBA/J × B6)F_1 non-Tg mice, only a small number of Vβ6, Vβ11, and Vβ5 cells was detected in the CD4 SP thymocyte subpopulation, as expected (4). No detectable rescue of CD4 SP thymocytes expressing Vβ6, Vβ5, or Vβ11 TCRs was observed in Tg^+ (CBA/J × B6)F_1 animals. Percentages of Vβ8.2 cells, a nondeleting control Vβ, were not significantly different between DLGKR Tg and non-Tg mice.

In the CD8 SP thymocyte subpopulation, statistically significant rescue of Vβ6-bearing cells was observed in DLGKR Tg^+ mice in both experiments 1 and 2 (boldface type). In contrast, deletion of Vβ11-, Vβ7-, and Vβ5-bearing cells in the CD8 SP thymocyte subpopulation was less efficient than that seen in CD4 SP thymocytes, and no significant rescue was observed in DLGKR Tg^+ mice. Thus, most of the endogenous superantigen-mediated clonal deletion of particular Vβ-bearing cells was not affected in DLGKR mice. However, deletion of Vβ6-bearing cells in the CD8 SP thymocyte subpopulation was attenuated, suggesting that Lck may participate in signal transduction leading to negative selection, at least in some cases.

Discussion

The lymphocyte-specific protein tyrosine kinase, Lck, first identified by virtue of its illegitimate activation in some lymphoid malignancies (64), plays pivotal roles in antigen receptor signaling. Prior studies have shown that Lck is physically and functionally associated with the CD4 and CD8 coreceptors, the β chain of the IL-2 receptor, and with numerous other lymphocyte surface proteins (65). The importance of Lck function was dramatically demonstrated by the isolation of the JCaM1 cell line, in which a mutation in the *lck* gene compromises its expression, yielding lymphoblasts incapable of signaling via their antigen receptors (35). Similarly, mice bearing a targeted disruption of the *lck* gene have very small thymuses and generate no functional T lymphocytes (31). This results from the absence of Lck catalytic function, since Tg animals expressing high levels of catalytically inactive LckR273 protein manifest similar or even more severe defects (32).

Careful analysis has defined important aspects of the mechanism whereby Lck entrains early thymocyte development (65). Immature T-lineage progenitors colonize the thymus throughout early adult life, and undergo a series of proliferative and differentiative steps to yield mature, functional T lymphocytes. For conventional T cells, production of a functional β chain polypeptide through β locus gene rearrangement permits assembly of a pre-TCR, composed at a minimum of the CD3γ, δ, and ε chains, a monomorphic pre-Tα chain, and the β chain itself, which directly promotes the development of DP cells, through a series of replicative steps from immature DN progenitors (66, 67). Delivery of this pre-TCR signal clearly proceeds under the aegis of Lck, since transgenes encoding an activated version of Lck direct development of DP cells even in RAG-$1^{-/-}$ mice (68).

Although these studies have proved revealing with regard to pre-TCR signaling, they leave open the question of whether the catalytic activity of Lck is essential for signaling from the TCR itself. Inhibition of Lck function arrests thymocyte development at the DN stage, and hence mature $CD3^+$ cells do not mature. We therefore used the *lck* distal promoter, which is expressed at high levels in DP thymocytes, mature SP thymocytes, and peripheral T cells (41), to direct the expression of the same catalytically inactive form of Lck that had been previously shown to competitively inhibit normal Lck function in immature thy-

Table 1. *Effect of DLGKR Tg on Endogenous Superantigen-induced Thymocyte Deletion*

Expt. 1	Mouse strain	Percent CD4 SP thymocytes	Percent TCRVβ positive cells in CD4 SP thymocytes				
			Vβ8.2	Vβ6	Vβ11	Vβ7	Vβ5
	B6	13.70 ± 1.11	11.82 ± 0.09	8.22 ± 0.15	1.72 ± 0.15	2.89 ± 0.11	1.84 ± 0.31
	(CBA/J × B6)F_1Tg^-	13.56 ± 0.75	18.79 ± 0.09	0.59 ± 0.07	0.61 ± 0.18	2.66 ± 0.29	0.21 ± 0.05
	(CBA/J × B6)F_1Tg^+	9.70 ± 0.45	18.73 ± 0.27	0.58 ± 0.05	0.67 ± 0.12	3.29 ± 0.40	0.21 ± 0.03
	Mouse strain	Percent CD8 SP thymocytes	Percent TCRVβ positive cells in CD8 SP thymocytes				
			Vβ8.2	Vβ6	Vβ11	Vβ7	Vβ5
	B6	4.32 ± 0.52	8.44 ± 0.54	6.29 ± 0.05	4.60 ± 1.17	6.45 ± 0.30	12.89 ± 1.32
	(CBA/J × B6)F_1Tg^-	3.46 ± 0.32	14.12 ± 0.28	**0.15 ± 0.05**	3.25 ± 0.46	1.78 ± 0.29	1.49 ± 0.59
	(CBA/J × B6)F_1Tg^+	3.07 ± 0.21	14.64 ± 0.35	**0.74 ± 0.06**	2.44 ± 0.08	2.64 ± 0.28	1.22 ± 0.25
Expt. 2	Mouse strain	Percent CD4 SP thymocytes	Percent TCRVβ positive cells in CD4 SP thymocytes				
			Vβ8.2	Vβ6			
	B6	11.85 ± 1.39	15.16 ± 3.32	7.67 ± 0.10			
	(CBA/J × B6)F_1Tg^-	11.26 ± 1.64	17.34 ± 1.54	0.59 ± 0.27			
	(CBA/J × B6)F_1Tg^+	7.74 ± 0.87	17.44 ± 0.85	0.75 ± 0.24			
	Mouse strain	Percent CD8 SP thymocytes	Percent TCRVβ positive cells in CD4 SP thymocytes				
			Vβ8.2	Vβ6			
	B6	3.45 ± 0.66	8.78 ± 0.51	5.77 ± 0.39			
	(CBA/J × B6)F_1Tg^-	4.37 ± 1.06	12.45 ± 1.33	**0.21 ± 0.09**			
	(CBA/J × B6)F_1Tg^+	3.37 ± 0.43	12.14 ± 1.88	**0.47 ± 0.12**			

Thymocytes from normal B6 and heterozygous DLGKR mice with (CBA/J × B6)F_1 background were stained with anti-CD4-PE, anti-CD8-APC, and mAbs specific for TCRVβ6 (44-22-1), TCRVβ11 (RR3-15), TCRVβ5 (MR9-4), and TCRVβ8.2 (F23.2). Goat anti-rat Ig-FITC was used for anti-TCRVβ6, anti-TCRVβ11, and anti-TCRVβ5 stainings. Goat anti-mouse Ig-FITC was used for anti-TCRVβ8.2 staining. TCRVβ7 staining was done with FITC-labeled TR310 mAb. Percentages were calculated from 400,000 viable cells. Percentages of Vβ6-, Vβ11-, Vβ7-, Vβ5-, and Vβ8.2-bearing cells in CD4 and CD8 SP thymocytes are demonstrated with standard deviations. In expt. 1, three B6 mice, four (CBA/J × B6)F_1 Tg^- LM mice, and five (CBA/J × B6)F_1 Tg^+ mice were analyzed at 6 wk of age. In Expt. 2, three B6 mice, five (CBA/J × B6)F_1 Tg^- LM mice, and four (CBA/J × B6)F_1 Tg^+ mice were analyzed at 8 wk of age. Statistically significant differences ($P < 0.001$ in Expt. 1 and $P < 0.05$ in Expt. 2) appear in boldface type.

mocyte (32). Here we use these animals to demonstrate that the catalytic activity of Lck contributes to TCR-driven selection of mature T-lineage cells.

Our studies made use of a single line of DLGKR animals in which expression of LckR273 protein in thymocytes was approximately equivalent to that of the endogenous, wild-type protein (Fig. 4 *A*). This level of transgene expression resulted in an ~50% compromise in coreceptor-associated phosphotransferase activity, demonstrating that the LckR273 protein does indeed compete with its wild-type counterpart (Fig. 4 *B*). Although transgene-derived protein was expressed at only modest levels, positive selection, defined either as the representation of $CD69^+$, $CD3^{hi}$ cells, or using the H-Y TCR-α/β and DO10 TCR-α/β transgene, was inhibited by 30% in heterozygotes, and by 60% in mice bearing two copies of DLGKR (Figs. 2, 3, 6, and 7). These results, representing two points of a dose–response curve, do not by themselves provide any insight into the nature of the signaling process that the DLGKR transgene interdicts. However, as in the case of the proximal promoter-driven LGKR transgene, where equimolar expression of wild-type (endogenous) and catalytically inactive protein produced a 50% reduction in thymus cellularity (32), our ability to detect a DLGKR effect permits the inference that functional Lck protein must ordinarily exist in a stable, titratable complex.

Does Lck Deliver Signals from the TCR Itself? As much as 50% of Lck protein associates constitutively with the CD4 and CD8 coreceptors in DP cells (60). Moreover, coreceptor expression is required for the differentiation of most

functional T cells (69, 70). Hence it is possible that the titratable complex for which the LckR273 protein competes involves binding to CD4 and CD8. However, several considerations confound this interpretation. First, reconstitution of $CD4^{-/-}$ mice with CD4 transgenes encoding a truncated form of CD4 that lacks interaction sites for Lck permitted recovery of CD4 SP cells (71). Similarly, the Lck–CD8α interaction appears dispensable for differentiation of class I–restricted T cells when examined in an analogous system (37). Xu and Littman (36) have also shown that although Lck is required for CD4-dependent TCR signaling in a hybridoma cell line, this effect does not depend upon the kinase activity of Lck, but rather on its protein interaction (notably SH3) domains (36). Hence, the inhibition of positive selection that we observe in DLGKR mice may not depend upon its interaction with coreceptors. Indeed, the TCR-derived signals that require Lck in the Jurkat cell line do not involve either CD4 or CD8 (35). Similarly, the interdiction of pre-TCR signals by LckR273 protein, and the ability of activated Lck to mimic these signals, are both coreceptor independent (32, 72). From this perspective, it seems likely that the DLGKR-derived protein competes with its endogenous counterpart for interaction with the TCR complex itself, and thereby blocks downstream activation events.

Distinguishing Positive and Negative Selection. Biochemical studies suggest that Lck acts very early during the normal TCR signaling cascade, in part by phosphorylating immunoreceptor tyrosine-based activation motif (ITAM) residues in the CD3γ, δ, ε, and TCR-ζ proteins, and by phosphorylating (and thereby activating) the zeta-associated protein (ZAP) 70 kinase (73), with which it interacts (74). Indeed, TCR-ζ phosphorylation is reduced in DLGKR mice (Fig. 5). Hence the presence of the LckR273 inhibitory protein should serve to attenuate ZAP-70 activation. Prior studies in humans and mice show that ZAP-70 controls the maturation of SP cells from DP progenitors (75, 76), so it is perhaps unsurprising that DLGKR exerts its effects at the same point. However, both positive and negative selection are substantially interdicted in mice lacking ZAP-70 (75). Can Lck be required only for TCR-derived signals that mediate positive selection? We suspect not, particularly since some superantigen-mediated deletion events are partially reversed by DLGKR transgene (Table 1). However, a more comprehensive analysis will be required to assess the quantitative influences of Lck on negative selection.

Lck Behaves Uniquely. By directing the expression of catalytically inactive Lck protein to cells that have already reached the DP stage, we have learned that the kinase function of Lck contributes to TCR-derived signals that regulate positive selection. Although both Lck and $p59^{fyn}$ (Fyn) have been shown to participate in the TCR-associated signaling cascade, Lck plays a pivotal role in these developmental events. Signaling from the TCR is indeed compromised in $fyn^{-/-}$ mice (77), however this impediment arises only in SP cells, perhaps because Fyn is expressed at only very low levels in the DP compartment (78). More to the point, high level expression of catalytically inactive Fyn does not inhibit thymocyte development, which also proceeds normally in $fyn^{-/-}$ mice (as above). In this context, although Fyn protein was expressed at normal levels and retained normal activity in DLGKR mice (data not shown), it clearly could not substitute for Lck. These observations are again reminiscent of those made in studying pre-TCR signaling, where Lck expression is uniquely required for normal development.

Our analysis of the DLGKR mice suggests that Lck activity will prove essential for all TCR-derived signaling events. In addition, the profound effects resulting from modest expression of this transgene encourage the view that variations in Lck expression in the human population may influence repertoire selection, and hence may affect susceptibility to autoimmune and infectious diseases.

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