Autonomous Maturation of α/β T Lineage Cells in the Absence of COOH-terminal Src Kinase (Csk)

By Christian Schmedt and Alexander Tarakhovsky

From the Laboratory for Lymphocyte Signaling, The Rockefeller University, New York, New York 10021; and the Laboratory for Lymphocyte Signaling, Institute for Genetics, University of Cologne, 50931 Cologne, Germany

Abstract

The deletion of COOH-terminal Src kinase (Csk), a negative regulator of Src family protein tyrosine kinases (PTKs), in immature thymocytes results in the development of α/β T lineage cells in T cell receptor (TCR) β-deficient or recombination activating gene (*rag*)-*1*–deficient mice. The function of Csk as a repressor of Lck and Fyn activity suggests activation of these PTKs is solely responsible for the phenotype observed in *csk*-deficient T lineage cells. We provide genetic evidence for this notion as α/β T cell development is blocked in $lck^{-/-}fyn^{-/-}$ *csk*-deficient mice. It remains unclear whether activation of Lck and Fyn in the absence of Csk uncouples α/β T cell development entirely from engagement of surface-expressed receptors. We show that in mice expressing the α/β TCR on *csk*-deficient thymocytes, positive selection is biased towards the CD4 lineage and does not require the presence of major histocompatibility complex (MHC) class I and II. Furthermore, the introduction of an MHC class I–restricted transgenic TCR into a *csk*-deficient background results in the development of mainly CD4 T cells carrying the transgenic TCR both in selecting and nonselecting MHC background. Thus, TCR–MHC interactions have no impact on positive selection and commitment to the CD4 lineage in the absence of Csk. However, TCR-mediated negative selection of *csk*-deficient, TCR transgenic cells is normal. These data suggest a differential involvement of the Csk-mediated regulation of Src family PTKs in positive and negative selection of developing thymocytes.

Key words: thymocyte development • thymic selection • conditional gene targeting • T cell receptor • Src family kinases

Introduction

The normal development of α/β T cells is controlled by signals originating from the pre-TCR, TCR, and coreceptors CD4 and CD8. Pre-TCR–derived signals are necessary for the progression from $CD4^-CD8^-$ double-negative (DN)[1] to $CD4^+CD8^+$ double-positive (DP) thymocytes (for a review, see reference 1). Signaling from the TCR and coreceptors are required for the complex maturation of DP thymocytes into $CD4^+$ or $CD8^+$ single-positive (SP) cells (for reviews, see references 2 and 3). TCRs and CD4 or CD8 coreceptors on DP thymocytes interact with MHC/peptide complexes present on thymic stromal cells. If this interaction is of sufficient avidity, the DP cells receive a survival signal and are positively selected to proceed in development to the SP stage. In the case of too strong MHC/peptide–TCR interaction, the cells are negatively selected and die by apoptosis, similar to those DP cells which receive no survival signal for lack of MHC/peptide–TCR interaction (for a review, see reference 3). Positive selection is accompanied by the commitment to either the CD4 or CD8 lineage of T cells. The outcome of lineage commitment and positive selection is coordinated, as usually CD4 SP thymocytes are self-MHC class II restricted, whereas CD8 SP thymocytes are restricted to self-MHC class I molecules. Consequently, mice, which cannot express MHC class I, MHC class II, or both MHC class I and class II molecules, lack CD8 T cells, CD4 T cells, or both CD4 plus CD8 T cells, respectively (4–9).

The first biochemical changes detectable upon TCR engagement are the activation of the Src family protein tyrosine kinases (PTKs) Lck and Fyn, which physically associate with CD4/CD8 coreceptors and the TCR, respectively (10–12). The combined deletion of *lck* and *fyn* genes, as well as the overexpression of a dominant negative *lck*

Address correspondence to C. Schmedt, Laboratory for Lymphocyte Signaling, The Rockefeller University, Box 301, 1230 York Ave., New York, NY 10021. Phone: 212-327-8265; Fax: 212-327-8258; E-mail: schmedc@mail.rockefeller.edu

[1]*Abbreviations used in this paper:* Csk, COOH-terminal Src kinase; DN, double-negative; DP, double-positive; PTK, protein tyrosine kinase; *rag*, recombination activating gene; SP, single-positive.

transgene in mice result in the arrest of thymocyte maturation at the DN stage of development (13–15). Conversely, expression of constitutively active Lck or Fyn drives recombination activating gene (*rag*)-deficient DN thymocytes to mature to the DP stage of development, and activated Lck is implicated in mediating allelic exclusion at the TCR β locus (14, 16, 17). Hence, Lck and Fyn are involved at least in the early steps of α/β T cell developmental leading to the generation of DP thymocytes. It is unknown whether the activation of Lck and Fyn in the presence of TCR and coreceptors is sufficient to generate all signals required for the complete developmental program of thymocyte maturation.

To investigate if Src family PTKs are involved in all steps of α/β T cell development, we took the approach of conditional inactivation of the negative regulator of Src family PTKs (COOH-terminal Src kinase [Csk]) during α/β T cell development. We previously showed that α/β T cells can develop from csk-deficient DN thymocytes in the absence of pre-TCR and α/β TCR (18). This TCR-independent T cell maturation passes through all stages of thymocyte development and is accompanied by an increase in the specific activity of Lck and Fyn in *csk*-deficient thymocytes. From our previous analysis, it remained unclear whether γ/δ TCR–derived signals contribute to the α/β TCR–independent generation of T cells. Furthermore, Csk substrates other than Lck and Fyn could be responsible for the autonomous T cells maturation. Finally, it is unknown whether the deletion of *csk* in thymocytes that express a TCR causes an uncoupling of TCR engagement from thymocyte maturation, or if TCR-derived signals dominate over the activation of Lck and Fyn caused by the absence of Csk.

Here we provide evidence that α/β T cell development in the absence of Csk is also independent of γ/δ TCR expression and that no other substrate of Csk is sufficient to drive T cell development in the absence of Lck and Fyn. Furthermore, we show that the positive selection of *csk*-deficient thymocytes is largely uncoupled from TCR-derived input on the developmental fate, whereas TCR-mediated negative selection appears normal in the absence of Csk.

Materials and Methods

Mice. All mice used in these experiments were maintained in a conventional animal facility and analyzed at 6–8 wk of age. Maintenance and analysis of mice were done according to the German animal protection law. The generation of $csk^{fl/fl}$ mice has been described (18). $Csk^{fl/fl}$*lck-cre*, $csk^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* $csk^{fl/fl}$ β2m$^{-/-}$*A*α$^{-/-}$*lck-cre*, and $csk^{fl/fl}$*lck-creHY-TCRtg* mice are on mixed genetic background of 129, CB.20, and C57Bl/6. $csk^{fl/fl}$*lck-creHY-TCRtg* mice were bred onto H-2^b or H-2^d MHC background. *MX-cre* mice were provided by R. Kühn and K. Rajewsky (University of Cologne, Cologne, Germany). *Lck-cre* mice were provided by J. Marth (University of California San Diego, San Diego, CA). *A*α$^{-/-}$ and β2m$^{-/-}$ mice on C57Bl/6 genetic background were provided by H. Bluethmann and H.J. Fehling (Roche, Basel, Switzerland), respectively. *rag-1*$^{-/-}$ mice on C57Bl/6 genetic background were obtained from The Jackson Laboratory. *Lck*$^{-/-}$ mice on C57Bl/6 genetic background and *fyn*$^{-/-}$ mice were provided by T. Mak (Amgen, Toronto, Canada) and R. Perlmutter (Merck, Rahway, NJ), respectively.

Flow Cytometric Analysis. Mice were killed with CO_2 and lymphoid organs removed. Single cell suspensions were prepared by gentle tearing of spleens, lymph nodes, or thymi in RPMI 1640 (GIBCO BRL) containing 10% FCS (Roche). When necessary, erythrocytes were lysed by incubating cells in red blood cell lysis buffer (0.75% NH_4Cl, 100 mM Tris/HCl, pH 7.65) for 2–4 min at room temperature. Lysis was stopped by adding 10 ml of compete RPMI.

Fluorescence staining was performed as described (19). In brief, 1–2 × 10^6 cells were washed in PBS/1% BSA/0.01% sodium azide (PBA) and incubated with biotinylated antibodies at saturating concentrations in PBA for 20 min at 4°C. After washing with PBA, cells were incubated in PBA containing fluorochrome-conjugated antibodies and streptavidin for 20 min at 4°C. After a final washing step, cells were resuspended in 100–300 μl PBA and analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson). For the exclusion of dead cells during analysis, propidium iodide (PI; 0.2 μg/ml; Molecular Probes) or TO-PRO-3 (1 nM; Molecular Probes) was added to the cells before acquisition. The following reagents were purchased from BD PharMingen: CD3ε (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD24/HSA (M1/69), CD25 (7D4), CD69 (H1.2F3), CD90/Thy1.2 (53-2.1), TCR β (H57-597), and streptavidin-cychrome. Streptavidin-Cy7–PE was purchased from Medac. Biotinylated T3.70 and FITC-coupled F23.1 antibodies were gifts from H. von Boehmer (Dana-Farber, Boston, MA). Antibodies against B220 and Mac-1 were produced at the Institute for Genetics (Cologne, Germany).

Cell Sorting. CD4 T cells were purified by MACS depletion with anti-CD8, anti-B220, and anti–Mac-1 antibodies according to the manufacturer's instructions (Miltenyi Biotec), followed by FACS®-assisted enrichment of $CD4^{hi}$ and $CD4^{lo}$ cells on a FACStar™ (Becton Dickinson) with anti-CD4 and anti-CD8 antibodies. The purity of sorted cells was >95%.

Southern Blotting. Genomic DNA was isolated from 10^6–10^7 cells in suspension following the protocol by Laird et al. (20), followed by phenol/chloroform extraction. Southern blot analysis was done by standard procedures (21). Genomic DNA was digested with EcoRI, size fractionated on 0.7% agarose gels, and blotted onto nitrocellulose membranes (Geenscreen; NEN Life Science Products) by capillary transfer. *Csk*-specific fragments were detected with the radiolabeled probe C (18).

Results

TCR-independent Development of T Lineage Cells in the Absence of Csk. We have previously shown the TCR-independent development of *csk*-deficient α/β T lineage cells in $csk^{fl/fl}$TCRβ$^{-/-}$*MX-cre* mice. As these mice can express γ/δ TCR, we wanted to exclude a possible involvement of γ/δ TCR–derived signaling in the observed phenotype. Mice were generated to carry homozygous null mutation for *rag-1* in combination with two *loxP*-flanked *csk* alleles and the *MX-cre* transgene ($csk^{fl/fl}$*rag-1*$^{-/-}$*MX-cre*; references 18, 22, and 23). The *MX-cre* transgene is interferon inducible, but shows basal activity which results in the expression of Cre in a small fraction of all thymocyte populations including DN cells, as determined by Southern blot analysis of Cre-mediated deletion of a single *loxP*-flanked *csk* allele in these

cells (18). Thymi of *Csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice without injection of interferon are ~10-fold larger than thymi of *rag-1*$^{-/-}$ mice and close to the size of control thymi of *csk*$^{fl/fl}$ *rag-1*$^{+/-}$ mice (Fig. 1 A). Southern blot analysis of total thymocyte genomic DNA provides evidence that the thymus of *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice consists of exclusively *csk*-deficient cells (Fig. 1 B). The analysis of *csk*-deficient thymocytes reveals the presence of DP and CD4-SP cells. The fraction of DP thymocytes is slightly decreased and the percentage of CD4-SP cells is increased compared with *csk*$^{fl/fl}$-*rag-1*$^{+/-}$ control mice. The analysis of thymocyte maturation markers on DP and CD4-SP cells of *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice shows increases in CD69 and CD5 surface expression on CD4-SP cells as signs of positive selection and also decreased CD24/HSA surface expression on CD4-SP cells, which indicates further thymocyte maturation (Fig. 1 C). The fraction of CD4-SP cells as well as CD69^{+}HSA^{lo} cells is increased in *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice compared with *csk*$^{fl/fl}$*rag-1*$^{+/-}$ control mice (Fig. 1, A and D). CD8-SP cells are largely absent in *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice. Surface expression levels of CD4 on DP and CD4-SP thymocytes are reduced in *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice, compared with similar cells of control mice (Fig. 1 A). These *csk*-deficient cells leave the thymus to form a sizeable peripheral compartment of CD4^{lo} cells in the spleen and lymph nodes (Fig. 1 D, and data not shown). Surface expression of CD90/Thy1 and CD5 suggests the T lineage identity of these CD4^{lo} cells (Fig. 1 D, and data not shown).

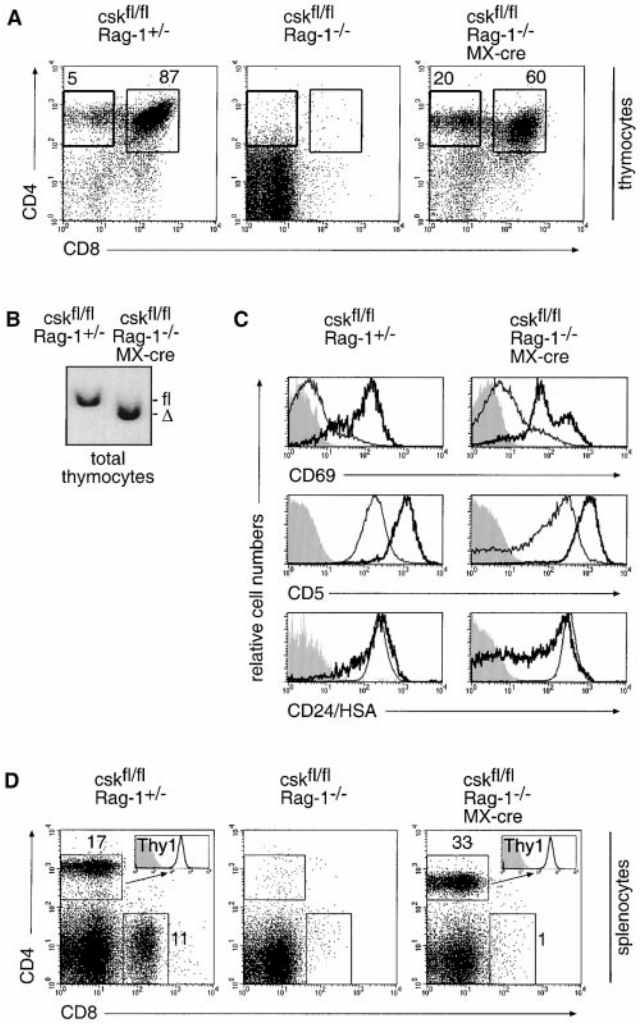

Figure 1. Positive selection and a peripheral pool of TCR-deficient T lineage cells in the absence of Csk. (A) FACS® analysis of surface expression of CD4, CD8, on thymocytes isolated from control and *csk*$^{fl/fl}$ *rag-1*$^{-/-}$*MX-cre* mice, as indicated. Numbers show percentages of live-gated cells. Total thymocyte numbers were 17×10^7 for *csk*$^{fl/fl}$*rag-1*$^{+/-}$, 0.06×10^7 for *rag-1*$^{-/-}$, and 6.3×10^7 for *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice. Representative data of three independent experiments are shown. (B) Southern blot analysis of genomic DNA isolated from total thymocytes of control and *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice, as indicated. For Southern blotting, DNA was digested with *Eco*RI and hybridized with a probe, which allows to distinguish wild-type, loxP-flanked (fl), and deleted (Δ) csk alleles. (C) Histograms show the surface expression of thymocyte maturation markers based on three-color FACS® analysis, as indicated. DP (thin line) and CD4-SP (thick line) cells were gated as shown in A. Staining controls are shown as shaded area in all histograms. (D) FACS® analysis of surface expression of CD4, CD8, and CD90/Thy-1 on splenocytes isolated from control and *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice, as indicated. Numbers show percentages of live-gated cells. The total number of CD4 T cells were 11.3×10^7 and 2.8×10^7 for *csk*$^{fl/fl}$*rag-1*$^{+/-}$ and *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice, respectively.

Development of TCR-bearing T Cells in the Absence of Csk. Considering that T lineage development from *csk*-deficient thymocytes is possible without any TCR, the question arises whether the TCR can at all influence the fate of *csk*-deficient thymocytes. To address this issue, T lineage development in *csk*$^{fl/fl}$*lck-cre* mice was analyzed. In these mice, the *cre*-transgene is under the control of the proximal *lck* promoter. As expected from earlier studies (24, 25), this promoter limits Cre expression and *csk* deletion to DN and DP thymocytes (data not shown). The numbers of thymocytes and splenic T cells in *csk*$^{fl/fl}$*lck-cre* mice are comparable to *csk*$^{fl/fl}$ control mice (Table I). Analysis of thymocyte subpopulations in *csk*$^{fl/fl}$*lck-cre* mice reveals that the percentages of DP and CD8-SP cells are slightly reduced, whereas CD4-SP and DN fractions are about twofold increased compared with thymi of *csk*$^{fl/fl}$ control mice (Table I, and Fig. 2 A, top). Changes in the expression of thymocyte maturation markers CD69, CD5, and CD24/HSA are similar to those described for *csk*$^{fl/fl}$rag-*1*$^{-/-}$*MX-cre* mice (data not shown). A skewing of the CD4/CD8 cell ratio is also apparent in peripheral T cells of *csk*$^{fl/fl}$*lck-cre* mice, which have strongly reduced numbers of CD8 T cells (Fig. 2 A, bottom, and data not shown).

The analysis of TCR surface expression on α/β T lineage cells in *csk*$^{fl/fl}$*lck-cre* mice reveals a small population of CD4^{lo}TCR^{-} cells present in *csk*$^{fl/fl}$*lck-cre* mice (Table I and Fig. 2 B). These cells are likely to be descendents of DN thymocytes that acquired the *csk* deletion before a successful rearrangement of their TCR β genes. Overexpression of an Lck transgene expressed in DN thymocytes has been reported to mediate allelic exclusion at the TCR β locus (17). The activation of Lck as a consequence of *csk* deletion in DN thymocytes is expected to act similarly and therefore suppress TCR β gene rearrangements. Hence, similar to *csk*$^{fl/fl}$*TCR*β$^{-/-}$*MX-cre* and *csk*$^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice, TCR-deficient cells develop in *csk*$^{fl/fl}$*lck-cre* mice.

Table I. *Cell Numbers and Distribution of α/β T Lineage Cells in Control and csk-deficient Mice*

	Thymocytes	DN	DP	CD4-SP	CD8-SP	Splenic T
	$\times 10^6$	%	%	%	%	$\times 10^6$
Control	307.8 ± 85.3	2.2 ± 0.3	86.8 ± 2.6	9.6 ± 2.1	1.5 ± 0.5	36.8 ± 13.3
	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 3$)
csk-deficient	312.6 ± 129.9	3.6 ± 1.0	77.4 ± 5.7	18.3 ± 5.7	0.7 ± 0.1	36.7 ± 13.7
	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 3$)

Cell numbers and percentages of lymphocyte populations of control ($csk^{fl/fl}$ or $csk^{fl/\Delta}$) and *csk*-deficient ($csk^{fl/fl}$*lck-cre* or $csk^{fl/\Delta}$ *lck-cre*) mice. No difference was found comparing $csk^{fl/fl}$ and $csk^{fl/\Delta}$ mice.

CD4 expression is reduced on thymocytes and peripheral CD4 T cells of $csk^{fl/fl}$*lck-cre* mice, similar to $csk^{fl/fl}$*rag-1*$^{-/-}$ *MX-cre* mice (Fig. 2 A). Also, CD8 expression levels are reduced on the remaining peripheral CD8 T cells (Fig. 2 A). Surface expression levels of TCR on the majority of CD4-SP thymocytes and CD4 T cells is three- to fourfold reduced compared with $csk^{fl/fl}$ control cells (Fig. 2 B). The Southern blot analysis of genomic DNA isolated from total thymocytes or FACS®-sorted $CD4^{hi}$ and $CD4^{lo}$ peripheral T cells shows a clear correlation between low CD4 and TCR expression and deleted *csk* loci (Fig. 2 C).

MHC-independent Positive Selection and Development of α/β T Lineage Cells in the Absence of Csk. Are $TCR^{lo}CD4^{lo}$ or $TCR^{lo}CD8^{lo}$ *csk*-deficient cells dependent on MHC molecules for their positive selection? To address this question, $csk^{fl/fl}$*lck-cre* mice were bred onto $\beta 2m^{-/-}A\alpha^{-/-}$ (*MHC*$^-$) background (5, 8). The development of CD4-SP thymocytes and CD4 T cells in $csk^{fl/fl}$*MHC*$^-$*lck-cre* mice largely resembles that seen in $csk^{fl/fl}$*MHC*$^+$ controls (Fig. 3 A). The development of CD8 lineage cells is unaltered compared with $csk^{fl/fl}$*MHC*$^-$ control mice (Fig. 3 A). Despite the absence of MHC molecules, thymocytes in $csk^{fl/fl}$ *MHC*$^-$*lck-cre* mice show typical signs of positive selection. Upregulation of CD5 and CD69 on wild-type DP thymocytes are the first signs of positive selection (26, 27). Expression of both markers is similarly increased in a fraction of DP cells in $csk^{fl/fl}$*MHC*$^-$*lck-cre* mice and $csk^{fl/fl}$*MHC*$^+$ control mice, compared with the $csk^{fl/fl}$*MHC*$^-$ control mice (Fig. 3 B). Also, the thymocyte maturation marker CD24/HSA is downregulated similarly in CD4-SP cells of control and $csk^{fl/fl}$*MHC*$^-$*lck-cre* mice (Fig. 3 B).

Aberrant Positive Selection of csk-deficient Thymocytes Expressing an MHC Class I–restricted Transgenic TCR. To analyze in a more direct way whether the TCR can influence the positive selection and lineage fate of *csk*-deficient DP thymocytes, we introduced TCR transgenes into $csk^{fl/fl}$*lck-cre* mice. For an MHC class II–restricted TCR transgene, we used the DO11.10 TCR transgenic mouse strain, which expresses the DO11.10 TCR that recognizes a chicken ovalbumin–derived peptide in the context of $I\text{-}A^d$ MHC molecules (28). As expected for MHC class II–restricted transgenic TCR, mostly CD4 T cells develop in both control and *csk*-deficient mice. No obvious differences could be detected in the numbers of control and *csk*-deficient thymocyte populations and peripheral T cells (data not shown). Notably, in both thymocytes and peripheral CD4 T cells the expression levels of CD4 as well as the transgenic TCR are reduced when *csk* deletion is achieved

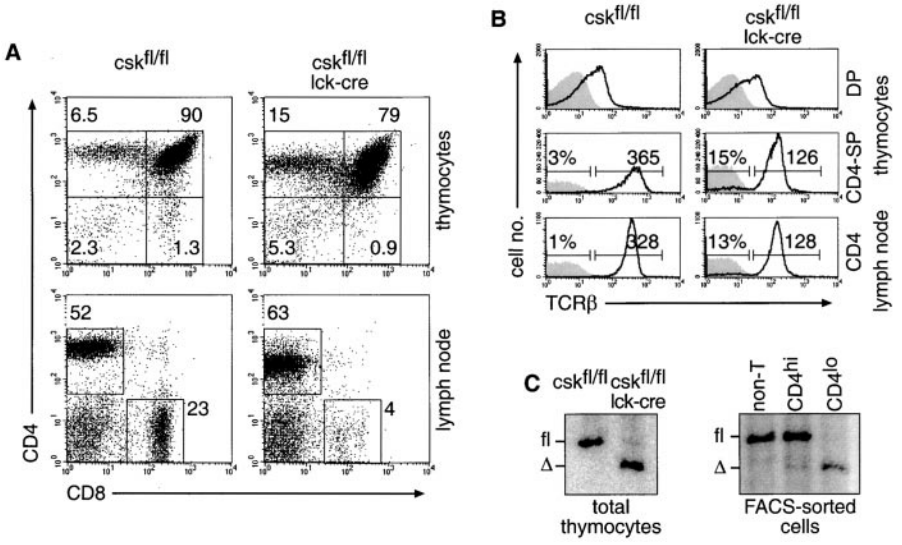

Figure 2. Development of TCR-bearing T cells in the absence of Csk. (A) Surface expression pattern of CD4 and CD8 on thymocytes and lymph node cells isolated from control and $csk^{fl/fl}$*lck-cre* mice, as indicated. Similar data were obtained in more than five independent experiments. (B) Histograms show surface expression of TCR β on thymocyte and lymph node cell populations as gated in A. Percentage numbers indicate the fraction of TCR β^- cells within the gated population. Plain numbers indicate the mean fluorescence intensity of the gated peak. Staining controls are shown as shaded area in all histograms. (C) Southern blot analysis of genomic DNA isolated from total thymocytes, non–T cells, $CD4^{hi}$, or $CD4^{lo}$ T cells of control and $csk^{fl/fl}$*lck-cre* mice, as indicated. Southern blotting was performed as described in the legend to Fig. 1. fl, *loxP*-flanked; Δ, deleted *csk* alleles.

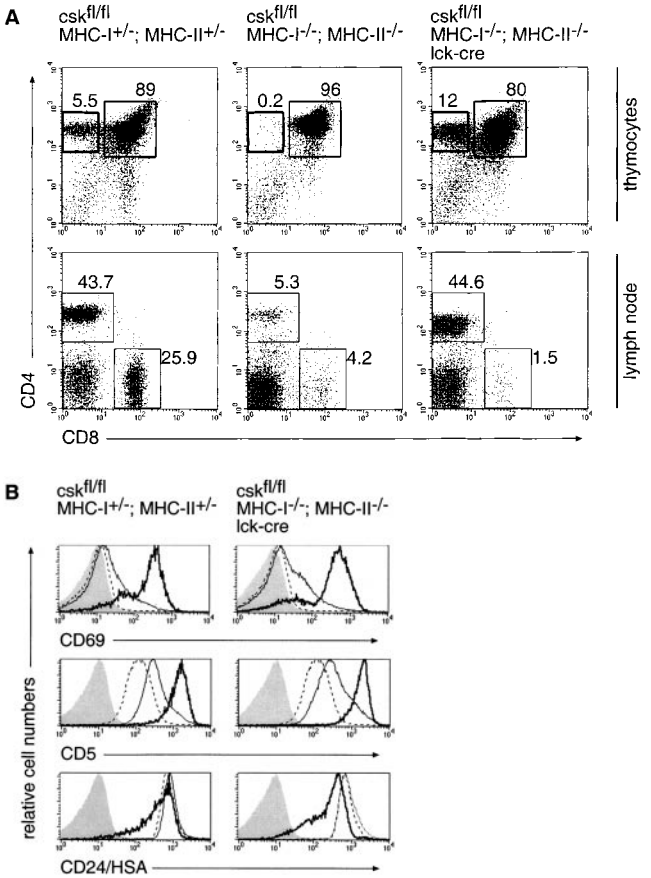

Figure 3. MHC-independent positive selection of *csk*-deficient CD4 T cells. (A) FACS® analysis of CD4 and CD8 surface expression of thymocytes and lymph node cells isolated from control and $csk^{fl/fl}MHC^{-}$*lck-cre* mice, as indicated. Numbers show percentages of live-gated cells. Total thymocyte numbers were $271 \pm 93 \times 10^6$ ($n = 3$), $353 \pm 95 \times 10^6$ ($n = 2$), and $251 \pm 81 \times 10^6$ ($n = 2$) for $csk^{fl/fl}MHC^{+}$, $csk^{fl/fl}MHC^{-}$, and $csk^{fl/fl}MHC^{-}$lck-cre mice, respectively. The number of splenic CD4 cells were $24.3 \pm 10.1 \times 10^6$ ($n = 2$), $4.5 \pm 2.6 \times 10^6$ ($n = 2$), and $15.3 \pm 6.6 \times 10^6$ ($n = 2$) for $csk^{fl/fl}MHC^{+}$, $csk^{fl/fl}MHC^{-}$, and $csk^{fl/fl}MHC^{-}$*lck-cre* mice, respectively. (B) Histograms show surface expression of thymocyte maturation markers based on three-color FACS® analysis, as indicated. DP (thin line) and CD4-SP (thick line) cells were gated as shown in A. CD69 and CD5 expression levels on DP thymocytes of $csk^{fl/fl}MHC^{-}$ mice are shown as dotted line for reference. Staining controls are shown as shaded area in all histograms.

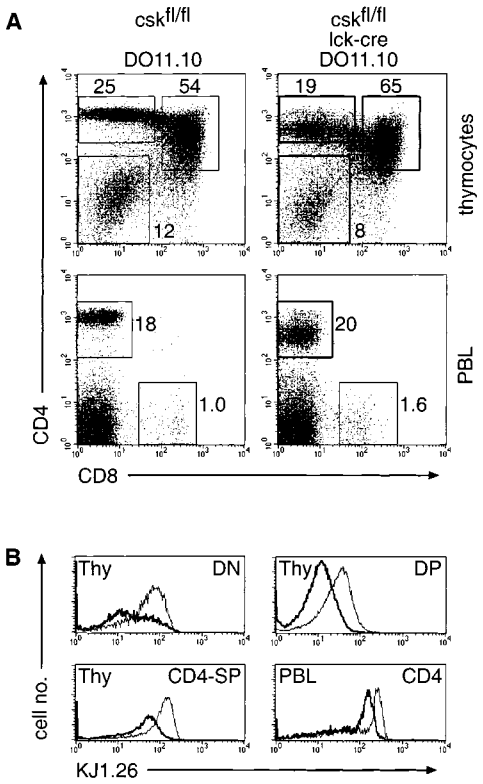

Figure 4. Development of *csk*-deficient thymocytes expressing an MHC class II–restricted transgenic TCR. (A) FACS® analysis of CD4 and CD8 surface expression on thymocytes and PBLs isolated from control $csk^{fl/fl}$*DO11.10-TCRtg* (left) and $csk^{fl/fl}$*lck-cre DO11.10-TCRtg* mice (right) expressing I-A^d. Numbers show percentages of live-gated cells. Total cell numbers of thymi are depicted at the top. (B) Overlay histograms show surface expression of the transgenic TCR chain, as detected with the KJ1.26 antibody on thymocyte and PBL populations as indicated. DN, DP, CD4-SP, and CD4 cells were gated as shown in A on $csk^{fl/fl}$*DO11.10-TCRtg* (thin line) or $csk^{fl/fl}$*lck-cre DO11.10-TCRtg* mice (thick line). Similar results were obtained in two independent experiments.

by the *lck-cre* transgene (Fig 4, A and B). For an MHC class I–restricted TCR transgene, we used the HY-TCR transgenic mouse strain. $Csk^{fl/fl}$*lck-creHY-TCRtg* mice were bred on H-$2D^b$ or H-$2D^d$ background. The HY-TCR is specific for the male HY antigen presented by H-$2D^b$ molecules (29). In female mice, HY-TCR transgenic cells are positively selected by an unknown peptide and only CD8 lineage T cells express both transgenic TCR α and TCR β chains (30; Fig. 5, A and B). The analysis of positive selection of DP thymocytes in female mice expressing transgenic TCR and H-$2D^b$ MHC molecules reveals a strong reduction in the percentage of CD8-SP thymocytes in the absence of Csk compared with control HY-TCR transgenic mice (Fig. 5 A, top panels). At the same time, the percentage of DP cells is reduced whereas CD4-SP and DN cell fractions are increased about twofold. Despite reduced numbers of CD8-SP thymocytes in $csk^{fl/fl}$*lck-creHY-TCRtg* mice, a sizable number of peripheral CD8 T cells exists in these mice (Fig. 4 A). As evident from the expression of the transgenic TCR α chain on these CD8 T cells, positive selection of CD8 T cells is not completely abrogated (Fig. 5 B). In normal female HY-TCR transgenic mice, all CD4 cells express an endogenous TCR α chain which forms an MHC class II–restricted TCR in combination with the transgenic TCR β chain (30). Therefore, the transgenic TCR α chain is not expressed on CD4 T cells in these mice. The analysis of surface expression of transgenic TCR on *csk*-deficient CD4 T lineage cells reveals that in contrast to control mice, a large fraction of CD4-SP thymocytes and peripheral CD4 T cells express the transgenic TCR α chain (Fig. 5 B). Similar to the phenotype of $csk^{fl/fl}$ *lck-cre* mice, surface expression levels of CD4 and CD8 are reduced on thymocytes and peripheral T cells of $csk^{fl/fl}$*lck-creHY-TCRtg* mice (Fig. 5 A). Similar to the DO11.10 TCR transgene, expression from a multicopy transgenic TCR locus does not compensate for the reduction of ex-

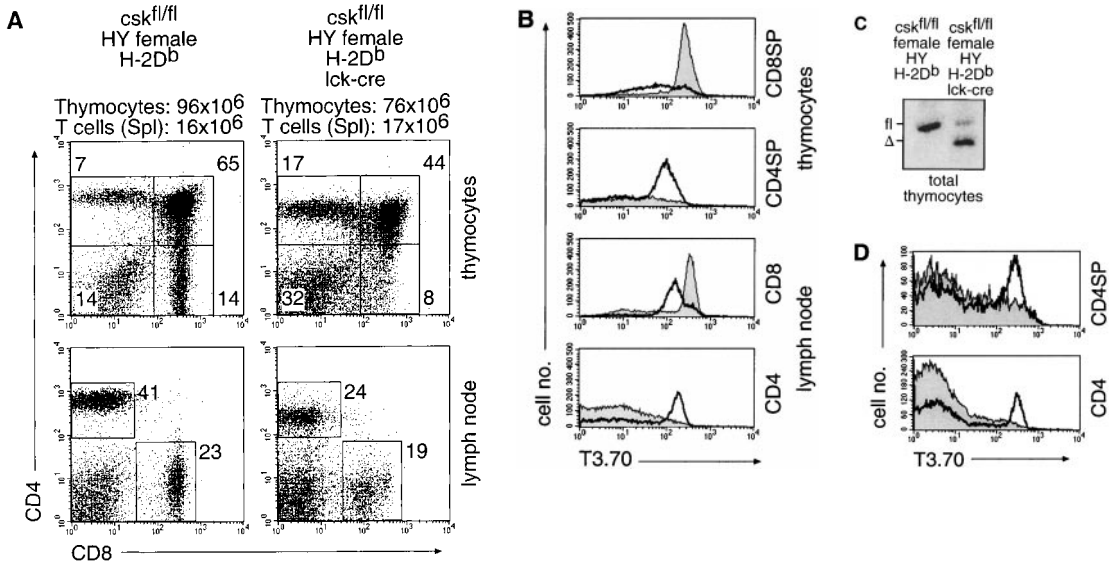

Figure 5. Aberrant positive selection of *csk*-deficient thymocytes expressing an MHC class I–restricted transgenic TCR. (A) FACS® analysis of CD4 and CD8 surface expression on thymocytes and lymph node cells isolated from female control $csk^{fl/fl}$*HY-TCRtg* (left) and $csk^{fl/fl}$*lck-cre HY-TCRtg* mice (right) expressing H-2D^b. Numbers show percentages of live-gated cells. Total thymocyte and splenic (Spl) T cell numbers are depicted at the top. (B) Overlay histograms show surface expression of the transgenic TCR α chain, as detected with the T3.70 antibody on thymocyte and lymph node cell populations of $csk^{fl/fl}$*HY-TCRtg* mice (shaded area) or $csk^{fl/fl}$*lck-cre HY-TCRtg* mice (thick line) on H-2D^b background, as indicated. CD8-SP, CD4-SP, CD8, and CD4 cells were gated as shown in A. The numbers of T3.70$^+$ CD4 T cells were 0.14×10^6 and 2.51×10^6 for $csk^{fl/fl}$*HY-TCRtg* and $csk^{fl/fl}$*lck-cre HY-TCRtg* mice, respectively. Data are representative of five experiments. (C) Southern blot analysis of genomic DNA isolated from total thymocytes of control and $csk^{fl/fl}$*lck-cre HY-TCRtg* mice on H-2D^b background, as indicated. Southern blotting was performed as described in the legend to Fig. 1. fl, loxP-flanked; Δ, deleted csk alleles. (D) Overlay histograms showing TCR α chain expression on thymocytes (CD4-SP) and T cells (CD4) of $csk^{fl/fl}$*HY-TCRtg* mice (shaded area) or $csk^{fl/fl}$*lck-cre HY-TCRtg* mice (thick line) on H-2D^d background. Thymocyte numbers were 68×10^6 and 73×10^6 for $csk^{fl/fl}$*HY-TCRtg* and $csk^{fl/fl}$*lck-cre HY-TCRtg* mice, respectively. The numbers of T3.70$^+$ CD4 T cells were 0.07×10^6 and 0.56×10^6 for $csk^{fl/fl}$*HY-TCRtg* and $csk^{fl/fl}$*lck-cre HY-TCRtg* mice, respectively. Similar results were obtained in two independent experiments.

pression of transgenic TCR α and β chains both on thymocytes and peripheral T cells of $csk^{fl/fl}$*lck-creHY-TCRtg* mice (Fig. 5 B, and data not shown). Southern blot analysis of the *csk* locus on genomic DNA of total thymocytes shows efficient *csk* deletion in $csk^{fl/fl}$*lck-creHY-TCRtg* mice.

Finally, the analysis of $csk^{fl/fl}$*lck-creHY-TCRtg* mice on the nonselecting H-2D^d background reveals that even in the absence of the positively selecting H-2D^b MHC molecules, CD4^{lo}TCR^{lo} cells expressing the transgenic TCR α chain can develop (Fig. 5 D). Unlike on H-2D^b background, there is no efficient development of T3.70$^+$ CD8 T cells in $csk^{fl/fl}$*lck-creHY-TCRtg* mice on H-2D^d background (data not shown).

HY-TCR–mediated Negative Selection of Thymocytes Is Functional in the Absence of Csk. The analysis of positive selection of α/β TCR–expressing *csk*-deficient cells suggests that selection signals originating from the expressed TCR have little influence on the fate of *csk*-deficient DP thymocytes. To test whether TCR signaling has any impact at all on selection steps during thymocyte development, we analyzed negative selection of the autoreactive HY-TCR–expressing thymocytes in male $csk^{fl/fl}$*lck-creHY-TCRtg* mice on H-2D^b background. Similar to male control mice, thymocyte numbers are drastically reduced in $csk^{fl/fl}$*lck-creHY-TCRtg* mice compared with female control mice (Figs. 5 A and 6 A). Our unpublished data show that the *lck-cre* transgene leads to deletion of the *loxP*-flanked *csk* gene in up to 25% of DN thymocytes. We also found that cre-mediated deletion of *csk* in 15% of DN is sufficient to generate a full-sized thymus in TCR β or *rag-1*–deficient mice (18; Fig. 1). If negative selection would be defective in *csk*-deficient thymocytes, the small fraction of *csk*-deficient cells detected by Southern blot analysis in total thymocytes of $csk^{fl/fl}$*lck-creHY-TCRtg* mice (Fig. 5 B) should give rise to thymocyte populations similar to those observed in $csk^{fl/fl}$*rag-1*$^{-/-}$*MX-cre* mice (Fig. 1). As no increase in thymocyte numbers or percentages of DP and SP cell populations occurs in male $csk^{fl/fl}$*lck-creHY-TCRtg* mice, negative selection in the HY-TCR system functions normally in the absence of Csk.

The Development of csk-deficient T Lineage Cells Depends on Lck and Fyn. The interpretation of changes in T cell development in *csk*-deficient mice rests on the assumption that the negative role of Csk in the regulation of Src family PTKs activity is the main reason for the observed phenotypes. We have shown previously that the specific activities of Lck and Fyn are increased in thymocytes isolated from $csk^{fl/fl}$*TCR*β$^{-/-}$*MX-cre* mice (18). We first tested whether the activation of Fyn caused by the deletion of *csk* could improve thymocyte development in *lck*-deficient mice, which have strongly reduced thymocyte numbers and impaired positive selection into CD4-SP and CD8-SP cells. When $csk^{fl/fl}$*lck-cre* mice were crossed onto an *lck*$^{-/-}$ background, a moderate twofold increase of thymus cellularity

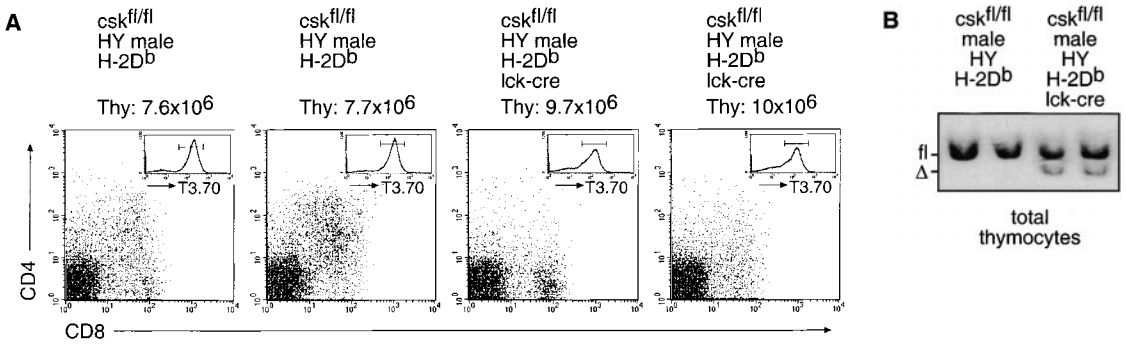

Figure 6. Normal negative selection of *csk*-deficient thymocytes expressing an MHC class I–restricted transgenic TCR. (A) FACS® analysis of CD4, CD8, and T3.70 surface expression on thymocytes isolated from male $csk^{fl/fl}$*HY-TCRtg* and $csk^{fl/fl}$*lck-cre HY-TCRtg* mice on H-2D^b background. Dot plots show T3.70$^+$ thymocytes, gated as indicated in the histogram insert. Total cell numbers of thymi (Thy) are shown at the top. Data are representative of three independent experiments. (B) Southern blot analysis of genomic DNA isolated from total thymocytes of two male control and two male $csk^{fl/fl}$*HY-TCRlck-cre* mice on H-2D^b background, as indicated. Southern blotting was performed as described in the legend to Fig. 1. fl, *loxP*-flanked; Δ, deleted *csk* alleles.

was apparent compared with $lck^{-/-}$ mice. However, thymi of $csk^{fl/fl}lck^{-/-}$*lck-cre* mice were still five to sixfold smaller than *csk*-deficient and control thymi (Fig. 7 A). Notably, a small number of CD4-SP thymocytes and CD4 T cells with reduced expression levels of CD4 and TCR could develop in $csk^{fl/fl}lck^{-/-}$*lck-cre* mice (Fig. 7 A). These data support the notion that the activation of Fyn is responsible for the incomplete block in thymocyte development of $lck^{-/-}$ mice (14). However, with respect to the TCR-independent development of *csk*-deficient T lineage cells, there is no formal proof that not other substrates of Csk could be responsible for that phenotype. To address this question we bred $csk^{fl/fl}$ *lck-cre* mice onto a $fyn^{-/-}lck^{-/-}$ background (13, 14, 31, 32). We then analyzed whether the autonomous development of *csk*-deficient T cells could still occur in $csk^{fl/fl}fyn^{-/-}$ $lck^{-/-}$*lck-cre* mice after deletion of *csk* in DN thymocytes mediated by the *lck-cre* transgene. The absence of both Fyn and Lck normally causes the arrest of thymocyte development early at the DN stage, and the analysis of thymocytes from $csk^{fl/fl}fyn^{-/-}lck^{-/-}$*lck-cre* mice revealed no increase in thymus cellularity and a block at the CD25$^+$CD44$^-$ stage of thymocyte development similar to control $csk^{fl/fl}fyn^{-/-}$ $lck^{-/-}$ mice (Fig. 7 B). Southern blot analysis of total thymocytes shows that *csk* deletion occurred in a small fraction of the mostly DN cells of $csk^{fl/fl}fyn^{-/-}lck^{-/-}$*lck-cre* mice (data not shown). Given that the *lck-cre* transgene leads to the development of TCR-deficient cells after deletion of *csk* in DN thymocytes, the lack of *csk*-deficient T lineage cells in $csk^{fl/fl}fyn^{-/-}lck^{-/-}$*lck-cre* mice shows that Csk substrates or interacting proteins other than Lck and Fyn are not by themselves sufficient to cause the TCR-independent development of T lineage cells in the absence of Csk.

Discussion

We used the conditional inactivation of *csk* in T lineage cells to address the question whether the activation of Src family PTKs Lck and Fyn is sufficient to support all steps in α/β T cell development that are normally controlled by pre-TCR or TCR engagement. It was of particular interest to understand if engagement of the α/β TCR can influence the fate of *csk*-deficient thymocytes, which in principle can also develop in the complete absence of TCR molecules.

The phenotype caused by conditional *csk* deletion in *rag-1*–deficient mice shows that TCR$^-$ T lineage cells, as evident by CD90/Thy-1 and CD5 expression, can develop in the absence of Csk (Fig. 1). This phenotype is similar to the previously reported pre-TCR, α/β TCR–independent development of α/β T lineage cells in $csk^{fl/fl}TCR\beta^{-/-}$*MX-cre* mice (18). Therefore, the possibility of any critical contribution from the γ/δ TCR to the α/β TCR–independent development of α/β T lineage cells in the absence of Csk can be excluded.

Based on the lack of α/β T cell development of $csk^{fl/fl}$ $fyn^{-/-}lck^{-/-}$*lck-cre* mice, it can also be excluded that Csk substrates or interacting proteins other than Lck and Fyn are sufficient for the autonomous T cell development in the absence of Csk (Fig. 6). Lck and Fyn are absolutely required for the development of thymocytes past the CD25$^+$CD44$^-$ DN stage, even in the absence of Csk. This does not exclude that Csk may regulate other proteins or may have additional functions earlier in T cell development before the proximal *lck* promoter becomes active (33, 34).

Given that the TCR is dispensable for T cell development in the absence of Csk, we wanted to understand whether engagement of the TCR or coreceptors on *csk*-deficient thymocytes influences their developmental fate. Using $csk^{fl/fl}$ mice in combination with the *lck-cre* transgene, we could analyze the development of *csk*-deficient thymocytes in the presence of the TCR. Similar to $csk^{fl/fl}$ $TCR\beta^{-/-}$*MX-cre* mice and $csk^{fl/fl}rag\text{-}1^{-/-}$*MX-cre* mice, the number of CD8 T cells is reduced both in the thymus and in peripheral lymphoid organs of $csk^{fl/fl}$*lck-cre* mice, whereas the number of CD4-SP thymocytes is consistently increased in the absence of Csk compared with controls (Figs. 1 A and 2 A). This bias for commitment to the CD4 T lineage could be the result of increased or extended activity of Lck and Fyn in the absence of Csk. Indeed, we have shown previously that the specific activity of Lck and

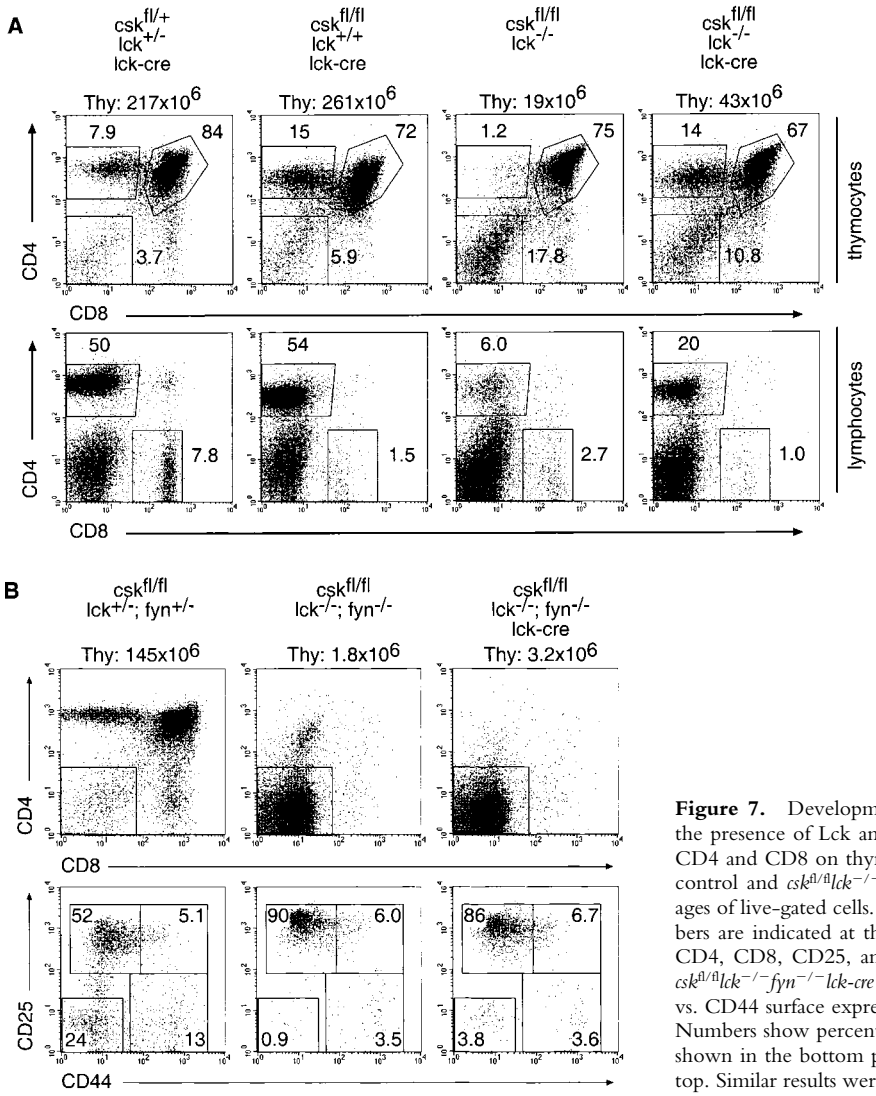

Figure 7. Development of *csk*-deficient T lineage cells is dependent on the presence of Lck and Fyn. (A) FACS® analysis of surface expression of CD4 and CD8 on thymocytes and lymph node cells isolated from various control and $csk^{fl/fl}lck^{-/-}$*lck-cre* mice, as indicated. Numbers show percentages of live-gated cells. The total thymocyte (Thy) and splenic T cell numbers are indicated at the top. (B) FACS® analysis of surface expression of CD4, CD8, CD25, and CD44 on thymocytes isolated from control and $csk^{fl/fl}lck^{-/-}fyn^{-/-}$*lck-cre* mice, as indicated. The bottom panel shows CD25 vs. CD44 surface expression on DN cells gated as shown in the top panels. Numbers show percentages of live-gated cells. Similar numbers of dots are shown in the bottom panel. Total thymocyte numbers are depicted at the top. Similar results were obtained in three independent experiments.

Fyn are increased in *csk*-deficient thymocytes (18). This could be interpreted by the cell as a strong TCR-derived signal, which according to various recent reports favors development of CD4 lineage cells (35–39).

Development of CD4 T cells in the absence of Csk can occur without TCR/MHC interaction. Three independent results of our experiments support this conclusion. First, TCR-deficient T cells are present in *csk*-deficient *rag-1*$^{-/-}$ or TCR $\beta^{-/-}$ mice (reference 1; Fig. 1). Second, TCR-expressing T cells develop in the absence of MHC class I and MHC class II (Fig. 3). And third, transgenic TCR-expressing T cells develop in the absence of the positively selecting MHC molecule (Fig. 5 B). Despite the independence of TCR/MHC interaction, in all of these cases, the typical changes in the expression of thymocyte maturation markers (CD69, CD5, and CD24/HSA) that normally accompany positive selection are observed (Figs. 1 and 3, and data not shown). It is intriguing that even in the absence of TCR engagement, CD69 and CD5 maturation markers are expressed on a similar fraction of DP cells, as in controls. This could indicate that the deletion of *csk* initiates a predetermined developmental program that includes the changes in maturation marker expression at the appropriate time. Alternatively, after the deletion of *csk* in DP thymocytes, a gradual decline of Csk protein levels reaches thresholds at which Lck and Fyn become activated only in a small fraction of cells at a given time. This activation of Lck and Fyn may mimic a "true" TCR-derived signal and lead to the changes in maturation marker expression.

We investigated directly whether engagement of the TCR influences the development of *csk*-deficient thymocytes by the analysis of development of T cells expressing a transgenic TCR. Although development of TCR transgenic thymocytes in an MHC class II–restricted DO11.10 system appears largely normal in the absence of Csk, the MHC class I–restricted HY-TCR system showed very unusual features. Similar to the situation in nontransgenic *csk*-deficient mice, positive selection in $csk^{fl/fl}$*lck-creHY-TCRtg* mice produces mostly CD4-SP thymocytes

in contrast to the *csk*$^{fl/fl}$*HY-TCRtg* controls. Also different from control mice, the *csk*-deficient CD4-SP as well as peripheral CD4 cells in *csk*$^{fl/fl}$*lck-creHY-TCRtg* mice express both the transgenic TCR α and β chains (Fig. 4 B). Positive selection of these unusual CD4 lineage cells is unlikely mediated by their MHC class I–restricted transgenic TCR, but rather appears to be uncoupled from TCR engagement as seen for example in *csk*$^{fl/fl}$*MHC*$^{-}$*lck-cre* mice (Fig. 3). This interpretation is consistent with the recently published ability of activated Lck to redirect the positive selection of thymocytes expressing a different MHC class I–restricted TCR transgene from the CD8 into the CD4 lineage (38). Notably, in that study a TCR transgene was used that can function independently of the CD8 coreceptor. Thus, positive selection and commitment to the CD4 lineage by activated Lck may also be TCR and MHC independent, as in the absence of Csk.

Despite the apparent uncoupling of TCR engagement and positive selection into the CD4 T lineage, two results of the analysis of *csk*$^{fl/fl}$*lck-creHY-TCRtg* mice point to the fact that signals derived from the TCR of *csk*-deficient thymocytes can influence their developmental fate. First, CD8 T cells bearing the transgenic TCR do develop in *csk*$^{fl/fl}$*lck-creHY-TCRtg* mice. This occurs at reduced efficiency compared with *csk*$^{fl/fl}$*HY-TCRtg* control mice; however, the frequency of TCR transgenic *csk*-deficient CD8 T cells is significantly higher than that of CD8 T cells in *csk*$^{fl/fl}$*lck-cre* mice (compare Fig. 5, A and B, and Fig. 2 A). Therefore, appropriate positive selection of *csk*-deficient thymocytes into the correct T cell lineage can occur, albeit at reduced efficiency. Second, the analysis of male *csk*$^{fl/fl}$*lck-creHY-TCRtg* mice on H-2D^{b} MHC background males clearly shows that TCR-mediated negative selection in the HY-TCR system in *csk*-deficient thymocytes is not impaired (Fig. 5). The reduced number of HY-TCR expressing CD8-SP thymocytes in female *csk*$^{fl/fl}$*lck-creHY-TCRtg* mice on H-2D^{b} MHC background may result from a deviation of positive selection into the CD4 T lineage due to stronger TCR-derived signal in the absence of Csk. Further analysis of thymocyte development in the absence of Csk in different TCR transgenic systems is required to confirm the differential role of Csk-mediated regulation of Lck and Fyn in positive and negative selection.

The surface expression of TCR as well as CD4 and CD8 coreceptors is significantly reduced on *csk*-deficient compared with control thymocytes and peripheral T cells. This phenotype is observed in all mice analyzed in this study, including those expressing a transgene-encoded TCR. The correlation between low receptor expression levels and *csk* deficiency is established by Southern blot analysis of CD4^{lo} and CD4^{hi} cells (Fig. 2 C). Reduction of TCR expression levels in the absence of Csk could be the consequence of Lck activation in these cells. On mature T cells, the ligation of TCR by MHC/peptide ligands and presumable activation of Lck leads to the endocytosis and lysosomal degradation of TCRs (40). Also, expression of the constitutively active Tyr505Phe mutant of Lck in mature T cells causes downregulation of surface TCR and degradation in a lysosomal compartment (41). Mice expressing a transgenic TCR and constitutively active Lck transgene show reduced TCR surface expression on thymocytes compared with only TCR transgenic control mice (42). Little is known about the regulation of CD4 and CD8 surface expression. However, both positive selection of DP thymocytes, which presumably activates Src family PTKs, and stimulation of mature T cells is accompanied by the reduction of coreceptor surface expression (43–46). Downregulation of TCR and coreceptors may also in some way be coordinated; however, the low expression level of CD4 on TCR-deficient T lineage cells in *csk*$^{fl/fl}$*TCR*β$^{-/-}$*MX-cre* and *csk*$^{fl/fl}$rag-*1*$^{-/-}$*MX-cre* mice argues against a tight coordination.

The reduced TCR and coreceptor expression levels seen in the absence of *csk* do not impair the function of mature T cells as defined by analysis of Ca^{2+} flux and proliferation in response to TCR cross-linking (data not shown, unpublished observations). It remains to be shown whether the reduced TCR and coreceptor expression levels represent a cell autonomous adaptation to the defective regulation of Lck- and Fyn-mediated signaling from the TCR and coreceptors.

In conclusion, our data show that the Csk-mediated regulation of Lck and Fyn during T cell development is essential for the establishment of developmental checkpoints that require signals from the pre-TCR and TCR to be faithfully transmitted to the developing thymocyte. It remains to be addressed whether the *csk*-deficient T cells populating peripheral lymphoid organs are dependent on TCR-mediated survival signals.

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