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Patty A. Trobridge, Katherine A. Forbush, and Steven D. Levin

Considerable evidence supports a role for the Src family protein tyrosine kinase Lck in regulating multiple aspects of thymocyte development. In this report, we establish that early events in T lymphopoiesis are restored to Lck-deficient mice by provision of a transgene encoding a version of Lck that cannot interact with the coreceptors CD4 and CD8. In addition, we demonstrate that later events in thymocyte development, specifically, the processes of positive and negative selection, are compromised in mice where the only Lck available cannot associate with either CD4 or CD8. We conclude that not only is Lck activity required for positive and negative selection, but that that activity must be coupled to the CD4 and CD8 coreceptors. The Journal of Immunology, 2001, 166: 809 – 818.

The protein tyrosine kinase Lck has been identified as an important signaling molecule in T lymphocytes. Cell lines that lack Lck exhibit diminished TCR signaling capacity (1–3), whereas those that overexpress an activated form of Lck respond more vigorously to Ag receptor stimulation (4, 5). In addition, Lck interacts with the coreceptors CD4 and CD8 (6–9), and this coupling is thought to contribute to Lck signaling function because these coreceptors are colocalized with the TCR during Ag recognition by virtue of their binding to nonpolymorphic determinants on the MHC class II and class I molecules, respectively. The interaction of Lck with CD4 and CD8 is dependent on two cysteine residues in Lck (C20 in CD4 and C23 in CD8) and two cysteines in the cytoplasmic domains of CD4 and CD8α (C418 and C420 in CD4 and C200 and C202 in CD8; Refs. 8, 9). Formation of the Lck-coreceptor complex is mediated by the coordination of a Zn$^{2+}$ ion between the cysteines, and alteration or chemical modification of the amino acid side chains of these residues prevents assembly of the complex (10, 11).

Lck has been shown to be critical for the normal development of T lymphocytes (12–15). It is expressed in the earliest thymic immigrants and in all subsequent T lineage cells (16). Previous work has clearly demonstrated that Lck is an important signal-transducing element from the pre-TCR complex that governs allelic exclusion at the TCR-β locus and regulates the proliferation associated with the transition from an immature CD4$^+$/CD8$^-$ precursor (double negative, DN)$^3$ to a more mature CD4$^+$/CD8$^-$ (double positive, DP) thymocyte (12–15, 17). Mice that lack the Lck protein because of a targeted disruption of the gene have reduced thymus cellularity (∼5–10% of normal) with essentially normal numbers of DN, but greatly reduced numbers of DP cells (14). Thymic cellularity is further reduced in mice that lack both Lck and the related Src family kinase Fyn (18) or in mice that overexpress a transgene encoding a catalytically inactive form of Lck (15). Mice lacking both Fyn and Lck and those that express the dominant negative form of Lck fail to develop any DP thymocytes, although the number of DN cells is normal. These results have been interpreted to mean that in Lck$^{-/-}$ mice, Fyn is capable of functionally compensating for Lck, but to only a limited extent. Moreover, some evidence suggests that this early regulation of thymopoiesis by Lck may be independent of its ability to interact with the CD4 and CD8 coreceptors (13).

The perturbations of thymocyte development at the DN to DP transition in mice in which Lck level or activity has been altered have largely precluded an analysis of the role of Lck in the processes of positive and negative selection that take place in DP thymocytes before their transition to mature CD4$^+$/CD8$^-$ or CD8$^+$/CD4$^+$ (single positive, SP) thymocytes. However, it is worth noting that some CD3$^+$ DP thymocytes are generated in Lck$^{-/-}$ mice, and yet there are virtually no mature SP thymocytes and very few peripheral T lymphocytes generated (14). One possible explanation for this result is that Lck activity is required to avoid the “death by neglect” that occurs in the absence of a positive selection signal. In addition, it has been reported that overexpression of a catalytically inactive Lck protein (with transcription under control of the Lck distal promoter, which comes on somewhat later in thymocyte development) compromises both positive and negative selection (19). Taken together, these data suggest that Lck activity is required for progression of DP thymocytes to the mature SP stage.

Although numerous studies support the view that Lck signals are required to promote thymocyte maturation, experiments to assess whether Lck must be physically coupled to the coreceptors to elicit these effects have provided more ambiguous results. Mutated forms of the CD4 or CD8 coreceptors that are unable to bind to Lck can nonetheless promote development of both helper and cytotoxic T cells, suggesting that the Lck-coreceptor interaction may not be critical for coreceptor function at the DP to SP transition (20, 21). However, it should be noted that these experiments relied on dramatic overexpression of the mutant coreceptors to restore development of the lineage under investigation. Moreover, it has also been noted that overexpression of wild-type CD4 in the thymocytes of transgenic mice titrates Lck away from CD8. When CD4 overexpression, which essentially strips all Lck away from the protein tyrosine kinase Lck has been identified as an important signaling molecule in T lymphocytes. Cell lines that lack Lck exhibit diminished TCR signaling capacity (1–3), whereas those that overexpress an activated form of Lck respond more vigorously to Ag receptor stimulation (4, 5). In addition, Lck interacts with the coreceptors CD4 and CD8 (6–9), and this coupling is thought to contribute to Lck signaling function because these coreceptors are colocalized with the TCR during Ag recognition by virtue of their binding to nonpolymorphic determinants on the MHC class II and class I molecules, respectively. The interaction of Lck with CD4 and CD8 is dependent on two cysteine residues in Lck (C20 in CD4 and C23 in CD8) and two cysteines in the cytoplasmic domains of CD4 and CD8α (C418 and C420 in CD4 and C200 and C202 in CD8; Refs. 8, 9). Formation of the Lck-coreceptor complex is mediated by the coordination of a Zn$^{2+}$ ion between the cysteines, and alteration or chemical modification of the amino acid side chains of these residues prevents assembly of the complex (10, 11).

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CD8, is superimposed on a class I-restricted TCR transgene, both positive and negative selection are inhibited (22), suggesting that CD8-Lck interaction is required for positive and negative selection.

To test directly whether the interaction of Lck with the coreceptors was required for generation of mature SP thymocytes, we have used transgenic mice that express either a CD4/CD8 interaction-competent form of Lck, or one that is incapable of this association. Mice containing these transgenes were then bred to Lck-deficient mice so that the only source of Lck was derived from the transgene product. We show here that both of the Lck transgenes restored early aspects of thymocyte development, but only the association-competent form permits the development of normal mature peripheral T lymphocytes. In fact, while provision of the association-deficient transgene produce few peripheral T cells, which exhibit the same phenotypic abnormalities observed in T cells from Lck<sup>−/−</sup> mice. Examination of the effects of these transgenes superimposed on the TCR transgene recognizing the male-specific HY Ag confirms that both positive and negative selection are compromised if Lck cannot interact with the coreceptor molecules. Taken together, our data demonstrate that the interaction of Lck with the CD4 and/or CD8 coreceptors is absolutely required for these later stage developmental processes.

Materials and Methods

Transgene constructs

The LGF and LGCA transgene expression constructs have been previously described (12, 13, 23). Briefly, the LGF construct includes 1.1 kb of the Lck proximal promoter with the entire Lck structural gene including all of the coding sequence, but with the codons for cysteine residues 20 and 23 have also been changed to alanine codons as described (13).

Mice

Lck<sup>−/−</sup> mice (14) that had been bred more than 10 generations to C57BL/6 mice, the LGF/2954 mice (12, 23), and HY TCR-transgenic mice (24) were provided by Dr. Roger Perlmutter (Merck Research Laboratories, Rahway, NJ). Three new lines of mice expressing the LGCA transgene (13) were generated for this research and designated 36038, 36039, and 36040 (denoted as LGCA38, LGCA39, and LGCA40 in the text). All mice were maintained under specific pathogen-free conditions in the animal facility at the University of Washington.

Flow cytometry

Mice were sacrificed by cervical dislocation, and thymi and spleens were removed in HBSS plus 3% FCS, and processed as described (13, 15). Cell suspensions were stained as described with Abs to CD4 (anti-CD4 conjugated to PE, Caltag, South San Francisco, CA), CD8 (anti-CD8 conjugated to FITC (Caltag) or to Tri-Color (PharMingen, San Diego, CA)), and CD3 (anti-CD3 conjugated to either biotin or FITC (PharMingen), DN subsets were fractionated by staining with anti-CD4 and anti-CD8 Abs (both conjugated to PE, Caltag and PharMingen, respectively), anti-CD25 conjugated to FITC (PharMingen), and anti-CD44 conjugated to Tri-Color (a gift from Dr. Andrew Farr, University of Washington). HY TCR-transgenic mice were stained for CD4 and CD8 as above, plus with the T3.70 Ab that recognizes the HY epitope Ag receptor complex (25). This Ab was conjugated to digoxigenin, and staining was detected using a secondary Ab against digoxigenin conjugated to FITC. Both of these reagents were provided by Dr. Farr. In addition, a biotinylated Ab against the TCR β-chain was also used (F23.2); this was provided by Drs. Catherine McMahen and Pamela Fink (University of Washington). Anti-CD24 conjugated to FITC was obtained from PharMingen. Biotinylated Abs were detected by streptavidin conjugated to PE, FITC, or Tri-Color (also provided by Dr. Fink). Stained samples were run on a FACSscan flow cytometer, and results were analyzed using CellQuest Software (Becton Dickinson, Mountain View, CA).

Immunoblot analysis

Lysates were generated from thymocytes by resuspending cells in TNT (25 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM PMSF) buffer as described (13, 15) at 10<sup>6</sup> cells/ml for 20 min at 4°C. Lysates were clarified by centrifugation at 12,000 rpm in a microfuge, and soluble material was transferred to fresh tubes. CD4 and CD8 immunoprecipitations (IPs) were performed using 10 μg of anti-CD4 Ab GK1.5 (PharMingen) plus 50 μl of a 30% slurry of protein G-agarose (Pharmacia, Piscataway, NJ), 100 μl of lysate (10<sup>7</sup> cell equivalents) in a total volume of 500 μl of TNT. IPs were rocked overnight at 4°C, and agarose beads were washed twice in 1 ml each of TNT. Pellets were resuspended in SDS sample buffer and boiled, and supernatants were loaded onto 10% acrylamide gels. For assessment of Lck protein levels, 2.5 μl of supernatants were subjected to SDS-PAGE following a method previously characterized and compared with mice expressing a transgene with the wild-type tyrosine at position 505 as described (12, 13, 23). Mice expressing these activated Lck transgenes have been previously characterized and compared with mice expressing a transgene with the wild-type tyrosine at position 505 (12, 13, 23). We have found that expression of the activated kinase has effects on thymocyte development that are indistinguishable qualitatively from those seen in mice that overexpress the wild-type kinase, although the activated version is somewhat more potent (12, 23). Moreover, expression of the activated kinase does not lead to constitutive production of IL-2 in T cell hybridomas (4); the cell lines still require Ag receptor stimulation to be activated, hence we reasoned that expression of these activated kinases would accurately reflect real effects of Lck and not nonspecific consequences of Lck overexpression. Moreover, because each form of the kinase used in these experiments was activated, any differences observed between transgenic mice could be attributed to whether the protein product could interact with the CD4/CD8 coreceptors. Each of these lines was bred to Lck-deficient mice to generate animals that harbored the transgene and were either Lck<sup>−/−</sup> or Lck<sup>−/−</sup>, and the resultant mice were designated by their transgene and endogenous Lck genotype (e.g., LGF<sup>+</sup>Lck<sup>−/−</sup>, LGF<sup>+</sup>Lck<sup>+/−</sup>, LGCA40<sup>+</sup>Lck<sup>−/−</sup>.

Results

Rationale and generation of transgenic mice

To directly test whether interaction of Lck with the CD4 and CD8 coreceptors was required for various Lck-dependent aspects of thymocyte development, we generated three lines of transgenic mice that had incorporated into their genome an Lck transgene in which the codons for two cysteines required for CD4/CD8 interaction had been changed to alanine codons, LGCA is the same except that the codons for cysteine residues 20 and 23 have also been changed to alanine codons as described (13).

Materials and Methods

In addition, we used an existing line of mice that expressed a transgene encoding a coreceptor association-competent form of Lck (LGF2954, designated LGF in the text; Ref. 12). The protein products encoded by each of these transgenes was an activated form of Lck generated by substitution of a phenylalanine codon for the tyrosine codon at position 505 as described (12, 13, 23). Mice expressing these activated Lck transgenes have been previously characterized and compared with mice expressing a transgene with the wild-type tyrosine at position 505 (12, 13, 23).
LGCA40”/Lck+/−, etc.). By comparing developmental progression of thymocytes in mice lacking an endogenous Lck gene, but bearing either transgene, we hoped to draw conclusions about the importance of Lck-coreceptor association in mediating various aspects of thymocyte development altered in Lck−/− mice.

Expression of both transgenes was driven off of the thymocyte-specific Lck proximal promoter as previously described (12, 13, 23). The Lck proximal promoter has been extensively characterized and is widely used to drive expression of transgenes in thymocytes, and detailed analysis has revealed that it comes on in DN cells and is shut off as a consequence of positive selection in SP thymocytes (27–29). In addition, we have noted that the fidelity of this tissue-specific expression is much improved by the inclusion of Lck genomic structure, suggesting that sequences within the introns of Lck contribute to regulation of the promoter (12, 13).

Hence, by providing Lck transgenes that included the Lck structural gene, we reasoned that transgene expression during this window of thymocyte development would be sufficient to allow an assessment of whether one or both of these transgene products could restore aspects of this progression to genetically Lck-deficient thymocytes.

Both transgene products are expressed, but the LGCA transgene product does not coimmunoprecipitate with CD4 and CD8

We first sought to characterize transgene expression by quantitating levels of transgene-derived Lck protein produced in thymocytes from each line of mice in the absence of endogenous Lck. Thymocyte extracts from each transgenic line on the Lck−/− background were subjected to immunoblot analysis (Fig. 1A and data not shown) and relative protein levels were quantitated as described in Materials and Methods. Based on such analyses we have estimated the amount of transgene product relative to levels of Lck in thymocytes from Lck−/− mice for each line (Fig. 1A, Table I).

Although considerable data has been published concerning the requirements for Lck interaction with the CD4 and CD8 coreceptors (8–11), the inability of Lck with cysteine residues 20 and 23 mutated to interact with these proteins has only been demonstrated in nonlymphoid cells because virtually all T cells express Lck. Therefore, we wanted to formally test thymocyte lysates from each of the lines for interaction of transgene product with coreceptor. Thymocyte lysates from each of the lines were generated and subjected to IP with Abs against either CD4 or CD8. Immune complexes were collected, resolved on SDS gels, and subjected to Lck immunoblot analysis as described in Materials and Methods. As expected, CD4 immunoprecipitates from lysates containing coreceptor association–competent forms of Lck (LGF+ and all Lck−/− mice) gave an Lck-specific band, whereas lysates containing only the LGCA transgene-derived Lck did not (Fig. 1B).

Lck transgenes restore early aspects of thymocyte development to Lck−/− mice independently of coreceptor association potential

Previous work with mice overexpressing Lck transgenes has revealed that overproduction of Lck can have developmental consequences (12, 13, 23; and see below). Because of this, we first examined mice from each of the Lck-transgenic lines on the Lck−/− background to note phenotypic effects that were a consequence of Lck overexpression. Analysis of Lck+/− progeny from the LGCA38 and LGCA39 lines revealed that thymocyte number was similar to Lck−/− mice without a transgene (Fig. 2A), and that development was normal as judged by expression of the CD3, CD4, and CD8 cell surface markers (Figs. 3 and 4B; and data not shown). Based on immunoblot analysis (Fig. 1A, Table I) and consistent with our previous work (12, 13, 23), this data confirmed that these lines expressed low levels of the transgene product. In contrast, examination of the LGCA40 and LGF2954 lines revealed normal development of DN and DP thymocytes, but a paucity of SP thymocytes with few cells expressing high levels of CD3 (Figs. 3 and 4A; and data not shown). Moreover, thymocyte number in each of these lines was somewhat elevated (Fig. 2A). Both of these observations are characteristic of mice expressing higher levels of an Lck transgene regardless of whether the kinase can interact with the CD4/CD8 coreceptors (12, 13, 23). Our previous work has determined that these results reflect excess Lck protein providing a proliferative signal from the pre-TCR that also includes a signal that is interpreted as successful rearrangement of the β locus, even though in many cells β rearrangement has not taken place (12, 13, 17, 23, 30). Importantly, this proliferation and “bypass” of β-chain rearrangement was also observed in mice overexpressing wild-type Lck, indicating it is not just an effect of the activated form of Lck, and it occurs in Lck-transgenic mice independent of coreceptor association potential (12, 13, 17, 30). Moreover, the defect in TCR β rearrangement is not complete, and phenotypically normal, mature T cells appear in the periphery of Lck-transgenic mice and their numbers increase over time (12, 13). We observed similar...
effects in each of these “high expressing” lines on the Lck<sup>+/−</sup> background, reduced numbers of mature SP thymocytes (Fig. 3), but production of normal mature peripheral T cells (Fig. 5), albeit in somewhat reduced numbers (Fig. 2, B and C). Taken together, these results confirm previously published information indicating that expression of Lck transgenes at high levels alters aspects of thymocyte development, but that the cells that do develop, accumulate, and function normally.

To test the efficacy of these transgenes for restoring aspects of normal T cell development to Lck-deficient mice, we analyzed progeny from each line that were genotypically Lck<sup>+/−</sup> and were dependent on the transgene product as the only source of Lck. The ability of each transgene to restore aspects of thymocyte development to Lck<sup>−/−</sup> mice was examined. Thymi from Lck-deficient mice are considerably smaller than normal and show dramatically reduced cellularity (Fig. 2A; Table I; Ref. 14). Each of the Lck-transgenic lines we tested restored some measure of thymic cellularity when superimposed on the Lck<sup>−/−</sup> background, and the restoration of thymocyte number correlated with the level of transgene expression (Figs. 1A and 2A, Table I). We and others (14) have noted that development of DN cells in Lck<sup>−/−</sup> mice is relatively normal and we find the same to be true in Lck<sup>−/−</sup> mice expressing any of the transgenes tested here, at least in terms of the distribution of pro-T cell subsets as defined by expression of CD25 and CD44 in DN cells (Ref. 31; data not shown). However, the paucity of thymocytes in Lck<sup>−/−</sup> mice results from diminished numbers of DP thymocytes and is attributable to a reduction in the proliferation that normally accompanies the DN to DP transition (14, 15). As shown in Figs. 2A and 3, each of the transgenes tested restored DP development in Lck<sup>−/−</sup> mice in a dose-dependent manner that was independent of CD4/CD8 association potential. These results and previous work from our laboratory and others clearly indicate that Lck plays a critical role in the transition of thymocytes from the DN to DP stage (12–15, 18) but that this role is independent of the ability of Lck to interact with the CD4 and CD8 coreceptors (13).

**Mature thymocytes fail to develop in the absence of coreceptor-associated Lck**

Two lines of experimental evidence suggest that Lck activity is essential for the development of mature SP thymocytes. First, Lck<sup>−/−</sup> mice fail to develop SP thymocytes even though the DPs that are generated express surface CD3 (Figs. 3 and 4A; Ref. 14). Second, mice expressing high levels of a catalytically inactive form of Lck in mature thymocytes produce reduced numbers of SP thymocytes (19). Because of this, we evaluated the effects of Lck-coreceptor association on later stages of T cell development, specifically the development of mature SP thymocytes. Analysis of the CD3, CD4, and CD8 profiles from the high-expressing lines (LGF2954 and LGCA40) demonstrated the same reduction in mature SP thymocytes regardless of whether the mice were Lck<sup>+/−</sup> or Lck<sup>−/−</sup> (Fig. 3 and 4A, and data not shown). However, we also noted that the LGCA38 and LGCA39 lines, which produced normal CD3, CD4, and CD8 profiles in Lck<sup>+/−</sup> mice, failed to produce mature CD3<sup>high</sup> SP thymocytes in Lck<sup>−/−</sup> mice (Figs. 3 and 4B, and data not shown). We did note the generation of substantial numbers of CD3<sup>+</sup>CD8<sup>+</sup> thymocytes in LGCA38 Lck<sup>−/−</sup> mice and fewer, but significant numbers of CD3<sup>+</sup>/CD4<sup>+</sup> cells in this line (data not shown). Such cells were occasionally observed in thymocyte preparations from other transgene-positive Lck<sup>−/−</sup> mice (e.g., see Fig. 3, LGF2954 Lck<sup>−/−</sup> profile), but in all cases where they were examined, they were deemed immature by virtue of the fact that they were CD3<sup>+</sup> and CD24<sup>+</sup>. Moreover, they were only observed in peripheral lymphoid organs of LGCA38 Lck<sup>−/−</sup> mice (Fig. 2, B and C, and data not shown). We have not characterized these populations further, except to note that they do not appear in significant numbers in Lck<sup>−/−</sup> mice, in LGCA38 Lck<sup>+/−</sup> mice, or in any of the other lines we have generated.

**Peripheral T cells fail to develop normally in the absence of coreceptor-associated Lck**

The failure to develop mature SP thymocytes in LGCA<sup>+</sup> Lck<sup>+/−</sup>-transgenic mice suggested that interaction of Lck with CD4 and/or CD8 was necessary for this developmental step. Mice that completely lack a functional Lck gene also fail to produce significant numbers of SP thymocytes (14). However, as demonstrated above, other explanations can account for diminished numbers of mature thymocytes, including a failure to rearrange the TCR β locus in a majority of thymocytes in mice that overexpress Lck. In Lck<sup>−/−</sup> mice β locus gene rearrangement appears to be normal, and in fact, nearly all of the thymocytes express surface CD3 (Fig. 4A; Refs. 14, 32). Therefore, alterations in Ag receptor assembly were unlikely to account for the defect in SP production in Lck<sup>−/−</sup> mice. Moreover, although the LGCA38 and LGCA39 lines failed to produce mature thymocytes on the Lck<sup>−/−</sup> background, they produced normal mature thymocytes on the Lck<sup>+/−</sup> background. This observation suggested that Lck-mediated “by-pass” of TCR β-chain gene rearrangement could not account for the failure to develop SP thymocytes in the absence of a functional endogenous Lck gene in the LGCA38 and LGCA39 lines. This was obviously not true in the LGCA40 line, where even on the Lck<sup>−/−</sup> background, the development of SP cells was compromised to the same level as in the LGF2954 line (Fig. 3). However, it has been previously noted that the block in TCR β locus rearrangement is incomplete in “high-expressing” Lck-transgenic mice (e.g., LGF2954) and that substantial numbers of normal peripheral T lymphocytes still emerge (12, 13). Therefore, we reasoned that if coreceptor-coupled Lck kinase activity was important for the

### Table I. Characteristics of Lck-transgenic lines

<table>
<thead>
<tr>
<th>Line</th>
<th>CD4/CD8 Association</th>
<th>Expression Level</th>
<th>Thymocyte No.</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; Splenocyte No.</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt; Splenocyte No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lck&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>−</td>
<td>0</td>
<td>8.4 ± 1.6</td>
<td>3.0 ± 0.3</td>
<td>6.2 ± 1.0</td>
</tr>
<tr>
<td>Lck&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>+</td>
<td>0.5</td>
<td>93 ± 18</td>
<td>20 ± 3.7</td>
<td>10 ± 1.6</td>
</tr>
<tr>
<td>LGCA38</td>
<td>−</td>
<td>0.2 ± 0.1</td>
<td>22 ± 2.1</td>
<td>9.9 ± 1.0</td>
<td>17 ± 2.9</td>
</tr>
<tr>
<td>LGCA39</td>
<td>−</td>
<td>0.3 ± 0.1</td>
<td>47 ± 7.7</td>
<td>5.6 ± 1.0</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>LGCA40</td>
<td>−</td>
<td>2.2 ± 0.3</td>
<td>168 ± 48</td>
<td>4.3 ± 0.8</td>
<td>3.7 ± 1.0</td>
</tr>
<tr>
<td>LGF2954</td>
<td>+</td>
<td>1.8 ± 0.3</td>
<td>195 ± 30</td>
<td>15 ± 2.5</td>
<td>4.0 ± 0.7</td>
</tr>
</tbody>
</table>

* Shown here are the levels of Lck expression for each line of Lck-transgenic mice on the Lck<sup>+/−</sup> background determined as described in Materials and Methods with the mean values from at least three experiments ± SD. In addition, we show the mean thymocyte and CD4<sup>+</sup> and CD8<sup>+</sup> splenocyte numbers (× 10<sup>6</sup>) for each line on the Lck<sup>+/−</sup> background ± SDs. For purposes of comparison, we also provide the same information for Lck<sup>+/−</sup> and Lck<sup>−/−</sup> mice (see also Fig. 2).
emergence of normal, mature peripheral T cells, then LGCA+ Lck−/− mice should produce few normal peripheral T cells and those that do develop should resemble the abnormal T cells produced in Lck−/− mice. To determine whether this was the case, we examined peripheral T lymphocytes in each of our transgenic lines on the Lck−/− background by FACS. Representative FACS profiles documenting the accumulation of CD4 and CD8 SP peripheral T cells for each line with or without an endogenous Lck gene are shown in Fig. 5, and quantitation of our cumulative data for each line of mice is provided in Table I and Fig. 2, B and C. For each line we noted that both mature CD4+ and CD8+ T cells accumulated in the spleens of Lck+/− mice (Fig. 5). However, on the Lck−/− background, only the LGF+ spleens accumulated significant quantities of mature T lymphocytes (Fig. 5). The number of CD4+ splenocytes was compromised in all of the LGCA lines on the Lck−/− background, except for LGCA38 where most of the CD4+ cells are CD3− (see above). Although the number of CD4+ cells produced in the LGF2954 line were reduced relative to Lck+/− mice without a transgene, they were the same as in Lck−/− mice bearing this transgene (Figs. 2B and 5). However, it should be noted that there were generally slightly more CD4+ cells observed in spleens from the LGCA39 and LGCA40 lines on the Lck−/− background (Fig. 2B, Fig. 5, Table I). It is unclear whether this indicates that the LGCA transgene can actually facilitate the development of peripheral T cells to a limited extent, or whether this simply reflects an increase in cells “leaking” through development because there are a greater number of thymocytes produced. In any case it seems clear that in the absence of coreceptor-associated Lck, the development of CD4+ peripheral T cells was compromised.

Examination of the CD8+ T cells in Lck−/− mice with or without a transgene revealed that the number of CD8+ T cells was not substantially reduced (Fig. 2C, Table I). This would appear to suggest that the generation of CD8+ T cells was less dependent on Lck-coreceptor interaction. However, we further noted that the phenotype of the CD8+ T cells produced in Lck−/−-transgenic lines with a coreceptor association-competent transgene product (e.g., LGF2954 Lck−/−) was normal as judged by the level of CD3 and CD8 expression, whereas CD8+ T cells produced in LGCA+ Lck−/−-transgenic mice showed the reduced levels of surface CD3 and CD8 (data not shown) characteristic of Lck−/− mice (14). These data cumulatively support a role for coreceptor-Lck association in the development of normal CD4 and CD8 peripheral T cells.

Mature thymocytes and T cells fail to develop due to a defect in positive selection in the absence of coreceptor-associated Lck

Thymocyte CD4 vs CD8 profiles that demonstrated the failure to develop SP thymocytes (Fig. 3) and normal peripheral T cells (Fig. 5) in LGCA+ Lck−/− mice were reminiscent of profiles observed in TCR-transgenic mice when crossed onto nonselecting backgrounds. This suggested that the reason for failing to develop SPs

**FIGURE 2.** Lck transgenes restore thymocyte number regardless of coreceptor association potential, but mature T cell development requires CD4 and/or CD8 interaction. A, Lck transgenes restore thymocyte numbers in Lck−/− mice. Mean thymocyte numbers × 10^6 ± SEM are plotted for each of the Lck-transgenic lines on both the Lck−/− (left) and Lck+/− background (right). Mouse lines are arranged in increasing order of transgene expression level from left to right. **B,** Only coreceptor association-competent Lck restores development of CD4+ peripheral T cells. The number of CD4+ T cells harvested from the spleens of the various Lck-transgenic mice were calculated by multiplying the percentage of CD4+ cells, based on FACS analysis, times the number of total splenocytes. Values are plotted as mean cell number × 10^6 ± SEM for each of the transgenic lines on the Lck−/− (left) and Lck+/− (right) background. **C,** The number of CD8+ peripheral T cells are similar in all Lck-transgenic lines on the Lck−/− background. The number of CD8+ T cells harvested from the spleens of the various lines of mice were calculated as for CD4+ cells, except that the total splenocyte number was multiplied by the percentage of CD8+ cells. Cell numbers are plotted as for A and B above. All means presented in this figure are derived from analysis of between five and twelve individual mice of each genotype.
Results of such an analysis are shown in Fig. 6. CD4 vs CD8 profiles of thymocytes from Lck-transgenic mice reveal developmental defects induced by transgene overexpression or the inability of Lck to interact with coreceptors. Depicted are representative FACs plots showing the distribution of thymocytes expressing CD4 (y-axis) and CD8 (x-axis) from mice of the indicated transgenic lines on either the Lck+/− (left) or Lck−/− (right) background. Numbers represent the percentage of cells found in each quadrant. Similar results were obtained in analysis of between five and eight mice of each genotype.

FIGURE 3. CD4 vs CD8 profiles of thymocytes from Lck-transgenic mice reveal developmental defects induced by transgene overexpression or the inability of Lck to interact with coreceptors. Depicted are representative FACs plots showing the distribution of thymocytes expressing CD4 (y-axis) and CD8 (x-axis) from mice of the indicated transgenic lines on either the Lck+/− (left) or Lck−/− (right) background. Numbers represent the percentage of cells found in each quadrant. Similar results were obtained in analysis of between five and eight mice of each genotype.

was a defect in positive selection. To test this directly and to compare effects on positive selection between the LGF and LGCA transgenes, we bred HY TCR-transgenic mice to the LGCA40 and LGF2954 lines on either a Lck+/− or Lck−/− background. These two lines were chosen for comparison because they expressed nearly equivalent levels of Lck transgene product (Fig. 1A). The MHC class I-restricted HY TCR is positively selected in female mice of the H-2b haplotype (33, 34), and positively selected cells can be identified as CD8 SP cells that also stain with the T3.70 Ab (25), which recognizes the clonotypic Ag receptor complex. Hence, female mice from the resultant litters were analyzed for production of mature CD8+ peripheral T cells and found that these cells only express a transgene product that does not mediate CD4 or CD8 association, is a defect in the process of positive selection.

FIGURE 4. CD3 expression is abnormal on thymocytes from Lck-transgenic mice. A, LGF2954 and LGCA40 lines produce few CD3+ thymocytes. Thymocytes from mice of the indicated strains were stained for CD3 and analyzed by FACs. Histogram overlays of total thymocytes are presented, including a profile illustrating the elevated level of CD3 expression in thymocytes from an Lck−/− mouse for purposes of comparison. B, Thymocytes from LGCA39 Lck+/− mice have a normal profile of CD3+ cells, whereas LGCA39 Lck−/−-derived thymocytes have no mature CD3+ cells. Shown here are single parameter histogram overlays of CD3 profiles from representative examples of the indicated mice. Similar results were obtained from six (Lck+/−) and seven (Lck−/−) mice from the LGCA39 line.

profiles from total thymocytes illustrate that although provision of a TCR transgene restored the development of both CD4 and CD8 SP thymocytes in LGF2954 Lck−/− mice, it failed to do so in either LGCA40 Lck−/− (Fig. 6, top row) or in Lck−/− mice (data not shown). This effect was even more obvious when thymocytes were gated on T3.70+ cells where, in Lck+/− and LGF2954+ Lck−/− mice, most of the SP thymocytes that develop were CD8 SPs (Fig. 6, second row). These cells were lacking in mice from the LGCA40 Lck−/− line (Fig. 6, second row). In accord with previous reports, we also noted somewhat diminished levels of Ag receptor and CD8 expression on thymocytes from HY+ females from the LGF2954 line (35, 36). Although the CD8 SP cells that developed in the LGF2954 Lck−/− mice expressed somewhat lower levels of CD8 than those that lacked an Lck transgene, these cells were clearly being positively selected as judged by the down-regulation of CD24 in a substantial percentage of the CD4+/CD8− thymocytes from both Lck transgene-negative Lck−/− and LGF2954 Lck−/− mice (Fig. 6, third row). As a final assessment of successful positive selection, we monitored the appearance of T3.70+/CD8+ peripheral T cells and found that these cells only appeared in mice that expressed coreceptor interaction-competent forms of Lck in the thymus (Fig. 6, bottom row, and data not shown) but not in LGCA40 Lck−/− (Fig. 6, bottom row) or Lck−/− mice (data not shown). From these experiments, we conclude that the reason for the failure to develop mature SP thymocytes and peripheral T cells in mice that lack Lck, or in those that only express a transgene product that does not mediate CD4 or CD8 association, is a defect in the process of positive selection.
FIGURE 5. CD4 vs CD8 profiles of peripheral T cells from Lck-transgenic mice demonstrate a requirement for coreceptor-Lck interaction for normal T cell development. Total splenocytes were stained for CD4 and CD8 and analyzed by flow cytometry. The transgenic line represented is indicated to the left of the profiles with plots from the Lck+/− background (left) and those from the Lck−/− background (right). Numbers indicate the percentages of CD4+ and CD8+ cells in each profile with CD4 plotted along the y-axis and CD8 along the x-axis. Profiles from nontransgenic Lck+/− and Lck−/− mice are shown for purposes of comparison. Plots shown are representative examples of between five and 12 mice of each genotype.

Negative selection is compromised in the absence of coreceptor-associated Lck

A particular advantage of the HY TCR-transgenic system is that both positive and negative selection can be studied within the same line of mice (24, 25, 33). Male mice bearing the HY TCR transgene negatively select against the potentially autoreactive CD8+ T3.70b population. Studies with a dominant negative form of Lck had previously suggested a role for this kinase in negative selection (19). To investigate the necessity of Lck-coreceptor interaction in this process, we analyzed male mice from the same crosses that had previously suggested a role for this kinase in negative selection (19). To test this directly, we bred the LGCA40 and LGF2954 lines of transgenic mice to restore development of normal SP thymocytes in otherwise Lck-deficient mice, even though these cells did develop in mice that were transgene positive and Lck+/− (Fig. 3), and despite the fact that these two lines on the Lck+/− background restored coreceptor-independent Lck functions (Figs. 2 and 3). In fact, analysis of the CD3 and CD4 vs CD8 profiles from LGCA38 or LGCA39 Lck+/− mice were reminiscent of profiles obtained from mice bearing TCR transgenes of defined specificity and restriction that have been bred onto “nonselecting” backgrounds (Figs. 3 and 5). Because of this, we hypothesized that the defect in SP development was due to a failure of positive selection, whereby immature thymocytes receive a signal that prevents them from entering the SP compartment.

To test this directly, we bred the LGCA40 and LGF2954 Lck−/− mice with HY TCR-transgenic mice. This TCR is normally positively selected in female mice on the H-2Db molecule (33, 34). These two lines were chosen because they expressed nearly the same level of Lck transgene product (Fig. 1A, Table I), and because Lck overexpression compromises rearrangement of the TCR β locus to a similar degree in both (Fig. 4A). Hence, providing this TCR transgene served a dual function by allowing us to analyze thymocyte selection, and by providing a rearranged TCR β gene, which previous work has shown is the major defect in thymocytes from mice expressing high levels of an Lck transgene (12, 13, 17). In fact, providing a rearranged TCR β-chain restores normal thymocyte development patterns to the LGF2954 Lck−/− transgenic line revealed that thymocytes expressed lower levels of the Ag receptor on their surface regardless of whether they were Lck+ or Lck− (Fig. 7B and data not shown). It is possible that this accounts for the 2- to 3-fold increase in T3.70b/CD8+ cells compared with Lck−/− (data not shown)- or LGCA40 Lck−/− (Fig. 7A)-derived thymocytes. Collectively, these data support a role for coreceptor-associated Lck in regulating negative selection in the thymus.

Discussion

We have used a transgenic system to investigate the role of Lck interaction with the CD4 and CD8 coreceptors in thymocyte development. Our previous work suggested that effects of Lck transgene expression on early thymocyte development were independent of the capacity of the transgene product to associate with CD4 and CD8 (13). This was not surprising in light of the fact that many of these effects are manifested in immature thymocytes before the coreceptors are expressed. In any case, before this report, these effects were observed in mice with a normal endogenous Lck gene. We definitively address the requirement for Lck-coreceptor association here by demonstrating that providing an Lck transgene to otherwise Lck-deficient mice restores thymocyte numbers and the development of DP thymocytes in a dose-dependent manner regardless of whether the encoded Lck can interact with CD4 or CD8. Expression of these transgens was directed by the Lck proximal promoter, which previous work has shown is active in DN thymocytes and is switched off by positive selection signals at the DP to SP transition (27–29). Hence by using the Lck proximal promoter to direct expression of Lck transgenes in otherwise Lck-deficient thymocytes, we were able to evaluate effects of expression of each form of Lck on thymocyte development from the DN to SP compartments.

We have also identified a unique role for Lck that is critically dependent on interaction with CD4 and/or CD8. Specifically, co-receptor-associated Lck is required for the generation of significant numbers of mature SP thymocytes and normal peripheral T cells. This function was perhaps best illustrated by the inability of the LGCA38 and LGCA39 lines of transgenic mice to restore development of normal SP thymocytes in otherwise Lck−/− mice, even though these cells did develop in mice that were transgene positive and Lck+/− (Fig. 3), and because Lck−/− mice are shown for purposes of comparison. Plots shown are representative examples of between five and 12 mice of each genotype. The Journal of Immunology
Our results contrast with those of other investigators who have noted that providing Lck interaction-deficient versions of CD4 or CD8 does not normally be selected by sustaining a TCR signal through sustained TCR engagement. Additional experiments will be required to confirm this.

Finally, we were able to investigate the role of Lck-coreceptor interaction in the process of negative selection, whereby potentially autoreactive thymocytes are eliminated before leaving the thymus. In normal HY males, thymic cellularity is dramatically reduced due to this elimination, with very few thymocytes expressing high levels of CD8 and the clonotypic Ag receptor (24, 25). We see this same effect in LGCA40 Lck<sup>+</sup> mice (Fig. 7A), which indicates that the LGCA transgene on the Lck<sup>+</sup> background does not adversely affect negative selection. In other words, excess Lck kinase activity by itself, in the absence of coreceptor coupling, does not alter negative selection. However, expression of the LGF transgene on either the Lck<sup>+</sup> (data not shown) or Lck<sup>-/-</sup> (Fig. 7A) background does allow the development of slightly more CD8<sup>+</sup> peripheral T cells, as seen by the appearance of mature CD8<sup>+</sup> thymocytes and peripheral T cells expressing the clonotypic Ag receptor (24, 25).

In our experiments, positive selection of a class I-restricted TCR took place when the Lck transgenic product could interact with coreceptors (LGF2954 line) even though the levels of CD8 and Ag receptor were lower than normal. In contrast, we could see no evidence of positive selection in thymocytes from mice where the only Lck available could not interact with CD8, despite the fact that the development of CD8<sup>+</sup> cells appears to be less affected by Lck deficiency than the development of CD4<sup>+</sup> cells (Fig. 5 and Ref. 14). Thus, we suggest that positive selection of thymocytes is dependent on a coreceptor-Lck signal. However, it is possible that the requirement for this signal can be at least partially overcome by sustained TCR engagement. Additional experiments will be required to confirm this.
of the Lck transgene, and these differences were attributed to decreased levels of surface TCR in the presence of excess Lck kinase activity (35). Interestingly, we make similar observations here—mice expressing the LGF transgene have diminished levels of surface TCR expression in the thymus, whereas those expressing the LGCA transgene do not (Fig. 7B). It is probable that this diminished TCR expression accounts for the slight increase in CD8+/T3.70+ cells we observed in LGF2954 male mice of both the Lck+/− and Lck−/− genotype. In any case, the important result is the elevated percentage of CD8+/T3.70+ thymocytes noted in both Lck−/− and LGCA40 Lck−/− mice (Fig. 7A), suggesting that negative selection is compromised in the absence of coreceptor-associated Lck. However, it should also be mentioned that this does not translate into a generation of autoreactive T cells in peripheral lymphoid organs, and in fact these mice produce virtually no T3.70−/CD8−/CD4− thymocytes or peripheral T cells (data not shown). In contrast, male mice that have any form of Lck that can associate with CD4 and/or CD8 produced substantial numbers of T3.70−/CD8−/CD4− peripheral T cells (data not shown), but these lymphocytes expressed low levels of the CD8 coreceptor and were functionally anergic, failing to respond to the male HY Ag both in vivo and in vitro (Ref. 25; P.A.T. and S.D.L., unpublished results). Presumably, these cells have somehow escaped negative selection and “death by neglect,” but have left the thymus and been rendered anergic by peripheral tolerance mechanisms. We suggest that the reason such cells are not observed in Lck−/− or LGCA40 Lck−/− mice is the superimposition of a defect in positive selection in these mice. Although they may escape negative selection, they cannot escape “death by neglect,” which would normally be averted by a positive selection signal that is dependent on Lck-coreceptor association. Other possible explanations exist, but we feel this to be most likely.

The use of transgenic and knockout mice has allowed us to begin to understand the multifaceted role of Lck in T lymphocyte development. This Src family kinase has been established as an important regulator of the DN to DP transition by virtue of its provision of a differentiative signal that triggers expression of the CD4 and CD8 coreceptors, a proliferative signal that leads to dramatic increases in thymic cellularity, and an allelic exclusion signal. All of these functions are independent of the ability of Lck to interact with CD4 or CD8. In addition, we have established here that Lck regulates both positive and negative selection of thymocytes, and that this function depends on coreceptor association. Presumably these defects represent a failure to activate one or more downstream signaling pathways. It has been suggested that activation of the extracellular signal-related kinase-mitogen-activated protein kinase pathway conveys a positive selection signal, whereas activation of the c-Jun N-terminal kinase- and/or p38-mitogen-activated protein kinase pathways delivers negative selection signals (38–40). Although the experiments we have presented do not directly address this, we now have the tools to perform experiments that will enhance our understanding of the
biochemical basis for these two processes. Such information will provide yet another piece to the puzzle that will ultimately reveal the full range of Lck function in the development of T lymphocytes.

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