A CD8/Lck Transgene Is Able to Drive Thymocyte Differentiation

Ruben C. Fragoso, Saiju Pyarajan, Hanna Yoko Irie and Steven J. Burakoff

*J Immunol* 2006; 177:6007-6017; doi: 10.4049/jimmunol.177.9.6007

http://www.jimmunol.org/content/177/9/6007

**References**

This article *cites 44 articles*, 21 of which you can access for free at:

http://www.jimmunol.org/content/177/9/6007.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2006 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
A CD8/Lck Transgene Is Able to Drive Thymocyte Differentiation

Ruben C. Fragoso,2* Saiju Pyarajan,2† Hanna Yoko Irie,* and Steven J. Burakoff3*†

Efficient development of thymocytes requires participation of a CD8 or CD4 coreceptor in the TCR:MHC interaction. Both CD8 and CD4 coreceptor cytoplasmic domains associate with Lck. In this study, we attempted to delineate the role of CD8α-associated Lck in driving CD8 single positive (SP) thymocyte development. We used a chimeric molecule encoding the extracellular and transmembrane domains of CD8α fused to full-length Lck. In mice deficient for CD8α and transgenic for 2C, a MHC class I-restricted TCR, robust reconstitution of CD8 SP thymocytes occurred both centrally and peripherally. The reconstituted CD8 SP population was phenotypically and functionally comparable to 2C wild-type counterparts expressing endogenous CD8α. A CD8α/Lck kinase-dead chimera also resulted in reconstitution of CD8 SP thymocytes. Our results suggest that CD8α-associated Lck is sufficient to drive CD8 SP thymocyte development. Furthermore, this CD8 SP development may not necessarily depend on Lck kinase activity. The Journal of Immunology, 2006, 177: 6007–6017.

In normal mice the bulk of T cells found in the vasculature, lymph nodes, and spleen express an αβ-TCR with either a CD4 coreceptor or a CD8αβ heterodimeric coreceptor. These T cells, despite being mutually exclusive for one coreceptor, are positively selected in the thymus from a common double positive (CD4+CD8+) precursor population. Positive selection results in the maturation of single positive (SP) T cells with an optimal TCR:MHC affinity. Double positive (DP) thymocytes with too high or no affinity undergo apoptosis (negative selection and death by neglect, respectively). Absence of CD4 or CD8 results in inefficient development of helper T cells and CTLs, respectively (1, 2).

Although critical molecules involved in development have been identified, the nature of signals leading to lineage commitment is not well understood and several models have been proposed (reviewed in Refs. 3, 4). Lck, a member of the Src kinase family, is an important player throughout thymocyte development (reviewed in Ref. 5), and both the CD4 coreceptor and the CD8 coreceptor are known to bind Lck (6–9). A pair of dicysteines, one on Lck and the other on the coreceptor cytoplasmic tail, mediate the coreceptor-Lck association. Zn2+ ions have been shown to promote this association (10). Also, substantially more Lck is known to associate with CD4 than with CD8. This difference is hypothesized to result in differences in signal intensities that can be discerned by the developing thymocyte and lead to lineage commitment, known as the strength of signaling model (3). Mice expressing either CD8α or CD4 cytoplasmic tailless coreceptors have inefficient development of their respective lineage (11, 12). Presumably, this is due to the inability to involve Lck in the CD3 complex during positive selection. Increasing the expression of wild-type CD4 but not tailless CD4 diminished the CD8 SP thymocyte population in H-Y TCR transgenic mice. It is proposed that as wild-type CD4 expression increases there is less Lck available to bind CD8 (13). The CD8 β-chain has been shown to impart a 5-fold increase in CD8 SP thymocytes when present (14–16) and compromise their development when absent (17–19). It is suggested that the cytoplasmic domain of the CD8 β-chain is integral in enhancing Lck association with CD8α. Mice expressing a mutant Lck that cannot associate with coreceptors have defects in development of both types of SP thymocytes (20). However, it has also been shown that a mutant CD8α coreceptor chain incapable of binding Lck can still mediate development of CD8 SP thymocytes (21). It has also been shown that both coreceptors bind the adapter protein called the linker for activation of T cells (LAT) (22). However, LAT preferentially associates more with CD8 relative to CD4. It was shown for CD8 that the coreceptor dicysteine motif accounts for about two-thirds of LAT binding, whereas some other aspect of the CD8α tail accounts for the remainder.

In this study we addressed the role of CD8-associated Lck by generating a chimeric coreceptor, CD8α-Lck, where the CD8α cytoplasmic domain was replaced by full-length Lck. This allowed us to focus on the Lck signaling requirements of CD8 at the expense of other CD8α cytoplasmic domain binding proteins. The construct also allowed us to differentiate between cytoplasmic Lck and CD8-associated Lck. Furthermore, the chimeric transgene is substantially underexpressed, thereby avoiding aberrant effects due to coreceptor and Lck overexpression, which can bias both development and lineage commitment (23). The CD8α-Lck transgene was analyzed in mice deficient for endogenous CD8α and transgenic for the MHC class I-restricted 2C TCR. The CD8α-Lck transgene reconstituted a CD8 SP population comparable to that found in 2C mice. Interestingly, expression of a kinase-inactive CD8α-Lck coreceptor also led to the reconstitution of the CD8 SP population, suggesting that CD8-associated Lck may rely on some function other than its kinase activity for thymocyte development.
Materials and Methods

Constructs

The CD8α-Lck construct was generated by using the CD8α extracellular and transmembrane domains ligated to full-length Lck. The construct preparation strategy introduced three extra amino acids at the junction of CD8α and Lck ligation as follows: 5′ CD8α - 5′YHRS-ARA-GCCVC-3′ -Lck 3′. The generated construct was placed under the human CD2 regulatory 5′ and 3′ elements using the CD2 minigene vector elements (a gift from Dr. D. Kioussis, National Institute for Medical Research, London, U.K.). The mutant kinase construct was generated from the wild-type chimeric construct. A PCR fragment introducing the appropriate mutation was generated and substituted into the wild-type chimeric construct. The mutation was the equivalent of a lysine to arginine mutation at position 273 of wild-type Lck. DNA constructs were verified by sequencing.

Mice

Microinjection of the wild-type chimeric construct into the sv129 background for generating transgenic founder lines was performed with help from Dr. D. Littman (Skirball Institute of Biomolecular Medicine, New York, NY). Selected mice with germline transmission and protein expression were crossed onto a 2C CD8α−/− background. 2C CD8α−/− mice were generated by crossing C57BL/6 CD8α−/− mice (obtained from Dr. T. Mak, Ontario Cancer Institute, Toronto, Canada) with mice transgenic for the MHC class I-restricted TCR, 2C (obtained from Dr. D. Loh, Hoffmann-La Roche). Mice described as 2C CD8α−/− Tg59 had been backcrossed onto the C57BL/6 background eight or nine times. Mice described as 2C CD8α−/− Tg39 had been backcrossed onto a C57BL/6 background at least three times. Microinjection of the mutant chimeric construct into the C57BL/6 background was performed at the Brigham and Women’s Hospital Transgenic Facility (Boston, MA). As before, the resulting founder mouse was crossed onto a 2C CD8−/− background. C57BL/6 mice used in parallel for these experiments were siblings of 2C mice not expressing the 2C TCR transgene. All mice were housed in the Animal Resource Facility at the Dana-Farber Cancer Institute (Boston, MA).

Thymocyte and lymph node cell isolation

Thymocytes were harvested from mice 5–6 wk of age and lymph nodes were harvested from mice that were at least 12 wk of age. Lymph node harvest included axillary, cervical, mesenteric, inguinal, and iliac lymph nodes. In all cases, single cell suspensions were generated by grinding the tissues with frosted slides and then filtering through a sterile nylon mesh. Single cell suspensions were briefly treated with red blood cell lysing buffer (Sigma-Aldrich) for 1 min on ice. Prepared single cell suspensions were then counted and used for the various experiments.

Abs and FACS analyses

Anti-Lck Ab (clone 3A5) used for Western blotting was obtained from BD Pharmingen and included the following: anti-Lck Ab (clone 3A5) used for Western blotting was obtained from BD Pharmingen. Anti-Lck Ab (clone 3A5) used for Western blotting was obtained from BD Pharmingen. Abs and FACS analyses were then counted and used for the various experiments.

Proliferation assays

Harvested lymph node cells were analyzed by FACS (BD Biosciences) using anti-CD8 PE, anti-CD4 Cy, and CD2-streptavidin-FITC. The proliferation capacity was then assessed by incubating 100 × 103 1B2]+ lymph node cells in with 200 µl of cell culture medium and in the presence of the p2Ca peptide at its indicated concentration. Proliferation assays were performed in triplicate in 96-well, round-bottom plates (Corning) using RPMI 1640 medium (Mediatech) supplemented with heat-inactivated FBS (10% final; Sigma-Aldrich), penicillin-streptomycin (100 IU/ml to 100 µg/ml final; Mediatech), l-glutamine (2 mM final; Mediatech), HEPES (pH 7.4) (10 mM final), and 2-ME. T2-L4 is a TAP-deficient cell line expressing the murine L4 molecule. The p2Ca peptide (LSPFPFDL) is known to be presented by the L4 molecule and forms a ligand for the 2C TCR (24). Cell culture was performed at 37°C under a humidified 5% CO2 atmosphere. At the 42-h time point 0.8 µCi of [1H]thymidine was added, and at the 48-h time point the plates were harvested.

Cytokine production assays

Lymph node cell preparation for cytokine production capacity was performed in the same fashion as the proliferation assays. After the 48-h incubation, 100 µl of supernatant was assayed by ELSA to determine the cytokine produced. The following Abs obtained from BD Pharmingen were used: IL-2 capture and IL-2 detection (JES-1A12 and JES6-5H4, respectively) and IFN-γ capture and IFN-γ detection (R4-6A2 and XM1G1.2, respectively).

Results

CD8α-Lck reconstitutes a CD8 SP thymocyte population

To study the role of Lck directly associated with the CD8 coreceptor in thymocyte development, transgenic murine lines were generated that express the extracellular and transmembrane domains of CD8α fused to the full-length Lck. The CD8α-Lck chimeric construct was driven by a murine CD2 promoter. The two murine lines generated are referred to as Tg59 and Tg39. We first analyzed the expression levels of the transgenic thymocytes by Western blotting. Blotting of whole thymocyte lysates with anti-Lck Ab revealed that only the transgenic animals show a 90-kDa band of the size expected for the CD8α-Lck transgene (Fig. 1A). We then analyzed by FACS the surface expression of CD8 on thymocytes. Because we used the CD2 promoter to drive the transgene, the expression pattern of the transgenic would follow the CD2 gene expression. We reasoned that surface expression of the transgenic CD8α-Lck at the DP thymocyte stage just as thymocytes undergo negative selection would be most vital for survival of these thymocytes. Comparison of C57BL/6 (wild type) and the CD8α-Lck transgenic thymocytes revealed an ~100-fold difference in expression levels of CD8α (Fig. 1B). FACS analysis also showed very similar expression levels of CD8α and CD8β at the critical DP stage as shown in Fig. 1C. To differentiate between the bona fide CD8 cells from other thymocytes that would express the transgene, we used CD8β expression to follow the various CD8+ subpopulations in the thymus and periphery.

2C CD8α−/− and 2C mice have distinct phenotypic profiles with respect to CD3 expression in the thymus (Fig. 2). Most 2C wild-type thymocytes are CD3ehigh. In 2C CD8α−/− mice the majority are CD3elow and those that are CD3ehigh correspond to CD4 SP and double negative (DN; CD4+CD8−) thymocytes, a population known to arise independently of MHC interactions in TCR transgenic animals (25). The CD3e profiles are consistent with the reported 1B2 profiles (26) and highlight the need for CD8 in driving development toward the relatively more mature CD3ehigh thymocytes. Additionally, compared with C57BL/6 mice, the CD3elow peak in 2C CD8α−/− mice is narrower and at the “high end” of CD3elow expression (Fig. 2, histograms). Although this finding could result from CD3 being limiting when associating with the transgenic expression of 2C TCR in those thymocytes, a similar result was found for CD2 and CD5 and cannot be similarly explained. Collectively, the expression pattern of these markers suggests that 2C CD8α−/− thymocytes are receiving signals via their TCRs but are too inefficient to drive them through development.

Although Tg59 thymocyte histograms show a robust shift from the CD3elow peak to the CD3ehigh peak, no shift was readily apparent for Tg39 (Fig. 2, histograms). However, the corresponding CD3e-gated CD8β profiles demonstrate that in both lines there are CD8β SP populations that are CD3ehigh. The expression
having lower CD8+ thymocytes were triple stained with CD8-PE, CD4 Cy, and CD3e FITC. The remaining CD3e- cells were analyzed using standard protocols. For FACS analysis, thy-CD8 shows a novel band at the expected molecular mass of 90 kDa (Kd) for the

FIGURE 1. A chimeric CD8α-Lck transgene is expressed in significant-
antly lower levels in thymocytes of transgenic mice compared with en-
dogenous Lck and CD8α. A, Western blot analysis for Lck expression
shows a novel band at the expected molecular mass of 90 kDa (Kd) for the
CD8α-Lck chimera. This band is absent in thymocytes obtained from mice
not carrying the transgene. Western blot analysis was performed on whole
thymocyte cell lysates using standard protocols. For FACS analysis, thy-
mocytes were triple stained with CD8β PE, CD4 Cy, and CD3e FITC. The
DP thymocytes from the different mice were analyzed using standard pro-
tocols. B, a 100-fold difference in the expression level of CD8α is shown
between the C57BL/6 wild type and the Tg59 animal. C, comparable ex-
pression levels of CD8α and CD8β on the Tg59 DP thymocytes are shown.

of CD8β, expected only on thymocytes entering the CD8 SP line-
geage, identifies reconstituted thymocytes. Tg59 robustly demon-
strates two such populations, a CD8β SP population and another
having lower CD8β expression with natural boundaries extending
from CD4+ to CD4−. These populations are labeled A’ and B’, respectively (Fig. 2, CD3ehigh dot plots). Analogous population in
2C mice are labeled A and B. The remaining CD3elow populations
for Tg59 and Tg39 are similar to their 2C CD8α−/− counterparts.
Phenotypically, Tg59 and Tg39 appear to be intermediates of 2C
wild-type and 2C CD8α−/− mice, with Tg59 more like 2C wild
type and Tg39 more like 2C CD8α−/−.

The 2C TCR (1B2) and CD3e histograms (Fig. 2) were similar
in each of the 2C TCR-expressing mice. Additionally, the CD8β
by CD4 distributions for 1B2low (not shown) and 1B2high were
essentially the same as their CD3e counterparts. One difference,
however, is that most CD3ehighCD4+ thymocytes are not
1B2highCD4+. Rather, this population tends to be 1B2−/low CD4+.
This has been demonstrated previously for CD4 SP T cells from
2C mice (27) and holds true for 2C CD8α−/− and both of the
transgenic lines. Unlike the CD4 SP thymocytes, reconstituted
CD8β-expressing populations are largely 1B2high. This finding
suggests that the CD8α-Lck is specifically promoting the recon-
stitution of CD8β SP thymocytes bearing the 2C TCR.

In 2C mice, the CD3e low dot plot shows a small population that
resembles C57BL/6 DP thymocytes as indicated by an arrow in
Fig. 2. The remaining large fraction of DP thymocytes is CD3ehigh
and CD8βlow when compared with the CD8β SP population within
the same dot plot or with that in C57BL/6 mice. The natural
boundaries for this CD3ehighCD8βlow population appear to be con-
tiguous from CD4+ to CD4− levels. This phenotype is consistent
with thymocytes undergoing selection and is probably an interme-
diate population. An analogous population is also reconstituted
in Tg59.

Comparable numbers of total and CD8 SP thymocytes
reconstituted

Adolescent 2C mice possess a rather small thymus on the order of
20 × 106 thymocytes (25), in contrast to C57BL/6 mice that have
more than five times as many thymocytes. 2C CD8α−/− mice,
however, have an intermediate number of thymocytes. Tg59 mice
showed the same thymic cellularity as 2C mice (Table I) in the
range of 20 × 106 thymocytes. FACS analysis demonstrated that
the proportion of thymocytes that are CD8β SP is comparable to
that found in 2C mice (Table I). The range for Tg59 mice was
3.2–12.0% and for 2C mice it was 6.6–11.4%. This indicates that
Tg59 expression levels are sufficient to recapitulate not only thy-
mic cellularity but also the absolute number of CD8 SP thymo-
cytes seen in 2C wild-type mice.

Reconstituted thymocytes are phenotypically similar to their 2C
wild-type counterparts

Tg59 mice shows the reconstitution of two CD3ehighCD8β+ thy-
mic populations that have analogous populations in 2C mice as
discussed for Fig. 2, indicating that CD8α-Lck has an impact on
positive selection and development. However, CD8α-Lck is dif-
ferent from endogenous CD8 in that each coreceptor interaction
necessarily involves Lck at the expense of other CD8α binding
proteins. With this in mind, we analyzed surface markers in Tg59
and Tg39 mice to see whether development occurred in a pheno-
typically comparable manner to 2C wild-type thymocytes de-
scribed previously (28). The results for Tg59 are detailed here, but
comparable results were obtained for Tg39 also.

First we analyzed the surface expression of CD2 and CD5, the
levels of which are increased on those DP thymocytes that undergo
selection and maturation (29–31). For both of these markers,
C57BL/6 mice showed a biphasic curve (Fig. 3). As was expected
for both surface markers in C57BL/6 mice, “high” expression cor-
related largely with a SP phenotype. Interestingly, CD2 and CD5
CD8-associated Lck drives CD8 SP differentiation

Histogram plots for 2C CD8α<sup>−/−</sup> thymocytes were uniformly monophasic curves with an expression level at the intermediate high-low phenotype for both CD2 and CD5. This suggests that these thymocytes are largely a homogeneous population receiving signals via their 2C TCR but lack the necessary coreceptor signal to efficiently drive development, as suggested previously when discussing CD3e expression.

2C wild-type thymocytes uniformly expressed high levels of CD2. The CD2 histogram profile of Tg59 shows a broader peak. The CD2<sup>high</sup> gate for Tg59 contains nearly all of the A<sup>+</sup> and B<sup>+</sup> thymocytes present, unlike the 2C wild type. This finding is consistent with the CD3<sup>e</sup> histograms in Fig. 2, where there was a comparably large CD3<sup>e<sub>high</sub></sup> population. This indicates that in Tg59 a sizable proportion of the thymocytes are DP and phenotypically similar to 2C CD8α<sup>−/−</sup> DP thymocytes. That may explain why Tg59 profiles tend to be intermediate to 2C wild type and 2C CD8α<sup>−/−</sup>.

A similar analysis revealed that unlike CD2, a smaller proportion of the reconstituted A<sup>+</sup> and B<sup>+</sup> populations fall into the CD5<sup>high</sup> expression gate but still parallel their counterpart A and B populations in 2C wild type. A possible explanation may involve

---

**Table I. Analysis of thymic cellularity and CD8 SP reconstitution**

<table>
<thead>
<tr>
<th>Background</th>
<th>CD8&lt;sup&gt;β&lt;sub&gt;high&lt;/sub&gt;&lt;/sup&gt;/CD4&lt;sup&gt;+&lt;/sup&gt; (%)</th>
<th>Total Thymocytes (× 10&lt;sup&gt;5&lt;/sup&gt;)</th>
<th>CD8&lt;sup&gt;β&lt;sub&gt;high&lt;/sub&gt;&lt;/sup&gt;/CD4&lt;sup&gt;+&lt;/sup&gt; (%)</th>
<th>Total Thymocytes (× 10&lt;sup&gt;5&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>2.3</td>
<td>138.0</td>
<td>2.3</td>
<td>72.0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>2.8</td>
<td>144.0</td>
<td>2.8</td>
<td>82.0</td>
</tr>
<tr>
<td>2C wild type</td>
<td>7.5</td>
<td>14.5</td>
<td>7.5</td>
<td>24.5</td>
</tr>
<tr>
<td>2C wild type</td>
<td>6.6</td>
<td>43.0</td>
<td>6.6</td>
<td>21.5</td>
</tr>
<tr>
<td>2C wild type</td>
<td>11.4</td>
<td>20.5</td>
<td>11.4</td>
<td>48.5</td>
</tr>
<tr>
<td>2C wild type</td>
<td>8.2</td>
<td>25.5</td>
<td>8.2</td>
<td>21.0</td>
</tr>
</tbody>
</table>

<sup>*C57BL/6, 2C CD8α<sup>−/−</sup>, 2C wild-type, and CD8α<sup>−/−</sup> Tg59 mice were analyzed for thymic cellularity by FACS for the percentage representation of CD8β SP T cells. In the latter two mice, this population corresponds to populations A<sup>+</sup> and A<sup>+</sup> as shown in Fig. 1. The thymocytes were assessed on CD8β PE by CD4 Cy dot plot. Both 2C CD8α<sup>−/−</sup> Tg59 and 2C wild-type mice have comparable total thymocyte numbers and percentages of thymocytes that are phenotypically CD8 SP thymocytes (CD8<sup>β<sub>high</sub></sup>/CD4<sup>+</sup>). The numbers are representative of 4- to 6-wk-old mice.</sup>
the higher level of CD5 expression on CD4 SP thymocytes relative to CD8 SP thymocytes (32, 33). Clearly, the Tg59 reconstituted thymocytes demonstrate the same phenotype for the markers CD2 and CD5 as their 2C wild-type counterparts.

Expression of CD24 (heat-stable Ag) and CD69 were also consistent between Tg59 and 2C wild-type thymocytes (Fig. 4). CD24<sup>low</sup> is associated with a mature phenotype (34, 35) corresponding to that of SP thymocytes in C57BL/6 mice (Fig. 4). In the case of Tg59, the CD24<sup>low</sup> gate includes nearly the entire A<sub>11032</sub>/H11032 population, which is lacking in the CD24<sup>high</sup> gate. The B<sub>11032</sub>/H11032 population, however, was mainly CD24<sup>high</sup> with a small proportion being CD24<sup>low</sup>. Again, these populations parallel their 2C wild-type thymocyte counterparts. The early activation Ag CD69 also helps to phenotypically categorize these thymocytes (36, 37) and helps identify thymocytes that have recently engaged their TCR. Again, the analogous Tg59 and 2C wild-type populations showed similar profiles. The histogram (Fig. 4) is monophasic for 2C transgenic mice. With respect to the 2C CD8<sub>H9251</sub>/H11002 curve, both Tg59 and 2C wild-type populations showed similar profiles. The histogram (Fig. 4) is monophasic for 2C transgenic mice. With respect to the 2C CD8<sub>H9251</sub>/H11002 curve, both Tg59 and 2C wild-type populations showed similar profiles. The histogram (Fig. 4) is monophasic for 2C transgenic mice. With respect to the 2C CD8<sub>H9251</sub>/H11002 curve, both Tg59 and 2C wild-type populations showed similar profiles.

In summary, a phenotypic analysis of thymocytes expressing Tg59 demonstrates the reconstitution of two distinct thymocyte populations bearing CD8<sup>+</sup> expression. The first population, A', is largely characterized as CD8<sup>+</sup>CD4<sup>-</sup>CD3<sup>high</sup>CD2<sup>high</sup>CD5<sup>high</sup>CD24<sup>low</sup>, with a substantial proportion being CD69<sup>-</sup>. Population B' is largely characterized as CD8<sup>low</sup>CD4<sup>+</sup>CD3<sup>high</sup>CD2<sup>high</sup>CD5<sup>high</sup>CD24<sup>high</sup>. These phenotypes are consistent for a mature SP and an intermediate thymocyte phenotype, respectively. These phenotypes are comparable to the analogous A and B populations in 2C mice.

Peripheral reconstitution for CD8α-Lck transgenic T cells is equivalent to 2C mice

The peripheral lymph node compartment of CD8 SP T cells for Tg59 and 2C mice were similar for phenotype and total number. In 2C mice there was a large CD8<sup>+</sup>SP population and a modest CD4 SP population. Phenotypically, the CD8α-Lck reconstituted CD8 SP population had a similar profile of expression of CD3<sup>high</sup> and 1B2<sup>low</sup> as in 2C wild-type mice (Fig. 5) and of CD24<sup>low</sup> expression (data not shown). Lymph node cell numbers were similar between Tg59, 2C CD8α<sup>−/−</sup>, and 2C mice (Table II). However, for 2C CD8α<sup>−/−</sup> mice the proportion of 1B2<sup>high</sup> cells was less but there was a large 1B2<sup>−/low</sup>CD3<sup>high</sup>CD4 SP population (Fig. 5). The total numbers of CD8 SP T cells were comparable between...
Overall, lymph node profiles for Tg59 mice were similar to those of 2C mice. Taken together, the data suggest that the CD8-Lck chimeric protein is able to reconstitute a CD8 SP population that efficiently accumulates in lymph nodes and is phenotypically mature. Collectively, the phenotype of the reconstituted CD8 SP T cells is CD3$^+$H9255$^+$H11001$^+$1B2$^+$H11001$^+$CD24$^{\text{low}}$CD62L$^+$CD44$^+$, as is the case for 2C CD8$^+$ SP T cells.

Reconstituted CD8 SP T cells are functionally competent

Because the chimeric coreceptor is qualitatively and quantitatively different from the endogenous CD8 coreceptor, it is possible that the reconstituted cells did not undergo the appropriate program to include functional activity despite expressing the appropriate phenotypic surface markers. To determine whether there were any gross functional abnormalities, lymph node cells from Tg59, 2C, and 2C CD8$^{\text{a/b+}}$ Tg59 thymocytes have a larger proportion of their thymocytes in the CD69$^{\text{hi}}$ (CD69 hi) gate relative to 2C CD8$^{\text{a/b+}}$ thymocytes. Interestingly, the histogram plots are monophasic for these three backgrounds and slightly shifted to the right for 2C wild-type and 2C CD8$^{\text{a/b+}}$ Tg59. Analysis for CD24 and CD69 was performed as already discussed (see Figs. 2 and 3). The numbers at the bottom right indicate the percentage of CD8 SP cells of the total gated population.

FIGURE 4. Analysis of the expression of the maturation marker CD24 indicates that the CD8$^+$ SP population (population A) in 2C CD8$^{\text{a/b+}}$ Tg59 is uniformly CD24$^{\text{low}}$ (CD24 lo), similar to the analogous population in 2C wild-type thymocytes. The other pair of analogous populations, B and B', are largely CD24$^{\text{hi}}$ (CD24 hi). Analysis of the expression of the early activation marker CD69 demonstrates that 2C wild-type and 2C CD8$^{\text{a/b+}}$ Tg59 thymocytes have a larger proportion of their thymocytes in the CD69$^{\text{hi}}$ (CD69 hi) gate relative to 2C CD8$^{\text{a/b+}}$ thymocytes. Interestingly, the histogram plots are monophasic for these three backgrounds and slightly shifted to the right for 2C wild-type and 2C CD8$^{\text{a/b+}}$ Tg59. Analysis for CD24 and CD69 was performed as already discussed (see Figs. 2 and 3). The numbers at the bottom right indicate the percentage of CD8 SP cells of the total gated population.

Tg59 and 2C. Overall, lymph node profiles for Tg59 mice were similar to those of 2C mice.

Taken together, the data suggest that the CD8-Lck chimeric protein is able to reconstitute a CD8 SP population that efficiently accumulates in lymph nodes and is phenotypically mature. Collectively, the phenotype of the reconstituted CD8 SP T cells is CD3$^+$1B2$^+$CD24$^{\text{low}}$CD62L$^+$CD44$^+$, as is the case for 2C CD8 SP T cells.

Expression of a kinase inactive coreceptor also leads to reconstitution

With the CD8$^{\text{a/b+}}$-Lck coreceptor we focused on the direct association between Lck and CD8 in thymocyte development. Lck has multiple domains that include protein adapter domains (i.e., Src homology (SH) 2 and SH3) and a kinase domain. To further delineate the Lck function and define the specific activity that might be required in thymocyte development, we generated a murine line expressing an Lck kinase-inactive CD8$^{\text{a/b+}}$-Lck mutant coreceptor, Tg(K273R). Side by side comparison of C57BL/6 wild type, 2C CD8$^{\text{a/b+}}$ Tg59, and Tg(K273R) revealed that CD8 SP thymocytes are reconstituted in the Tg(K273R) animal to the same extent as the Tg39 animal (Fig. 8A). We decided to determine whether there was any correlation between the extent of CD8 SP reconstitution in these different transgenic animals and their surface CD8 coreceptor expression. We analyzed the expression levels of the different transgenic coreceptors on the surface of thymocytes and mature T cells by FACS. Although we did not observe any CD8$^+$ population in the 2C CD8$^{\text{a/b+}}$ mice, an analogous population of...
cells had surface expression of CD8α and CD8β in all of the transgenic mice analyzed. Because a CD2 promoter drives our CD8α-Lck transgene, most types of thymocytes would be expressing the transgene following the CD2 expression profile. Therefore, to estimate the level of expression of the transgene in CD8 SP cells, we calculated the median fluorescence intensity (MFI) values of CD8β on DP thymocytes and mature T cells in the different mouse lines studied. As shown in Fig. 8B, the MFI values of cells expressing CD8β on the surface were significantly lower on the cells from 2C CD8α−/− and the transgenic mice expressing the CD8α-Lck chimera when compared with the wild-type C57BL/6 animals. Among the different transgenic mice, Tg59 had the highest expression level of CD8β when compared with Tg39 and Tg(K273R), a CD8α-Lck kinase inactive chimera.

Table II. Comparable development of CD8 SP T cells

<table>
<thead>
<tr>
<th>Background</th>
<th>Sex</th>
<th>Age (wk)</th>
<th>Total Lymph Node Cells (× 10⁶)</th>
<th>1B2&lt;sup&gt;hi&lt;/sup&gt; Lymph Node Cells (%)</th>
<th>CD8 SP 1B2&lt;sup&gt;hi&lt;/sup&gt; Lymph Node Cells (%)</th>
<th>Total 1B2&lt;sup&gt;hi&lt;/sup&gt; Lymph Node Cells (× 10⁶)</th>
<th>Total CD8 SP 1B2&lt;sup&gt;hi&lt;/sup&gt; Lymph Node Cells (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C wild type</td>
<td>F</td>
<td>13.0</td>
<td>45.0</td>
<td>59.1</td>
<td>36.9</td>
<td>26.6</td>
<td>16.6</td>
</tr>
<tr>
<td>2C CD8α&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>F</td>
<td>14.4</td>
<td>49.0</td>
<td>46.6</td>
<td>56.6</td>
<td>31.3</td>
<td>22.8</td>
</tr>
<tr>
<td>2C CD8α&lt;sup&gt;−/−&lt;/sup&gt; Tg59</td>
<td>F</td>
<td>14.4</td>
<td>45.5</td>
<td>68.8</td>
<td>43.7</td>
<td>24.8</td>
<td>16.4</td>
</tr>
<tr>
<td>2C wild type</td>
<td>F</td>
<td>14.6</td>
<td>37.5</td>
<td>66.0</td>
<td>43.7</td>
<td>25.2</td>
<td>16.4</td>
</tr>
<tr>
<td>2C CD8α&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>F</td>
<td>15.7</td>
<td>28.0</td>
<td>49.0</td>
<td>13.7</td>
<td>13.7</td>
<td>13.7</td>
</tr>
<tr>
<td>2C CD8α&lt;sup&gt;−/−&lt;/sup&gt; Tg59</td>
<td>F</td>
<td>15.7</td>
<td>24.0</td>
<td>70.1</td>
<td>54.5</td>
<td>16.8</td>
<td>13.1</td>
</tr>
<tr>
<td>2C wild type</td>
<td>M</td>
<td>15.4</td>
<td>26.5</td>
<td>69.3</td>
<td>38.9</td>
<td>19.8</td>
<td>11.1</td>
</tr>
<tr>
<td>2C CD8α&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>M</td>
<td>16.6</td>
<td>25.5</td>
<td>56.1</td>
<td>14.3</td>
<td>7.2</td>
<td>13.3</td>
</tr>
<tr>
<td>2C CD8α&lt;sup&gt;−/−&lt;/sup&gt; Tg59</td>
<td>M</td>
<td>16.6</td>
<td>24.0</td>
<td>54.3</td>
<td>47.2</td>
<td>13.0</td>
<td>13.3</td>
</tr>
</tbody>
</table>

* Lymph node cells from age- and sex-matched 2C wild-type, CD8α<sup>−/−</sup>, and 2C CD8α<sup>−/−</sup> Tg59 mice were isolated, counted, and then analyzed by FACS using 1B2 biotin with streptavidin-FITC, CD8β PE, and CD4 Cy. 1B2 histogram curves and 1B2 by CD8β dot plots were used to gate and quantitate the indicated cell populations. The total CD8 SP T cells present in 2C CD8α<sup>−/−</sup> Tg59 and 2C wild-type mice appear comparable within each experiment.
In the mice expressing the Tg(K273R) transgene a CD8 SP population was reconstituted that represented 1–6% of CD3 positive thymocytes (Fig. 8C). The shift from CD3low to CD3high (Fig. 8C; histograms) was similar to the shift observed in Tg39 and not as apparent as in Tg59. Also, in these mice most of the CD8β-expressing population are largely IB2high, indicating that the 2C CD8 SP cells are being specifically reconstituted despite the lack of kinase activity by CD8 coreceptor-associated Lck. Analysis of the phenotypic profile for thymocyte population from Tg(K273R) mice (Fig. 8C) showed that it was the same as that of the Tg39 population already described, characterized as CD8β−CD4−CD3ehighCD25highCD5highCD24low with a proportion being CD69+. Reconstitution of the CD8 SP cells could be detected in the thymus as well as in the peripheral T cell populations of the spleen, blood, and lymph nodes (Fig. 8 and data not shown).

Discussion

In this study we focused specifically on the association between CD8 and Lck for driving CD8 SP T cell development by replacing the CD8α tail with full-length Lck. The chimeric protein was assessed in mice deficient for endogenous CD8α and transgenic for the MHC class I-restricted TCR, 2C. This provided a homogeneous CD8α-Lck:CD8β coreceptor on DP thymocytes that allowed us to determine whether Lck binding to the CD8α tail alone is sufficient for reconstituting CD8 SP T cells in both a phenotypically and functionally competent manner. Interestingly, robust reconstitution of two thymocyte populations was observed (A and A’ in Fig. 2). Thymocytes and lymph node cells were assayed and found to be comparable to their counterparts in mice with wild-type endogenous CD8 expression. These results suggest that CD8 association with Lck is sufficient to mediate development of CD8 SP T cells.

The impact of the CD8α-Lck coreceptor on thymocyte development is most apparent in Tg59 and most notable by the shift of thymocytes from the less mature CD3e low peak to the more mature CD3e high peak (Fig. 2). The CD8β by CD4 dot plots clearly show that the CD3e high population consists of two distinct populations, both of which express CD8β, consistent with thymocytes auditioning for selection and developing into the CD8 SP thymocyte lineage. Further, each population has essentially the same phenotype as its 2C wild-type counterparts developing with endogenous CD8; i.e., the level of CD3e and 2C expression is equivalent and both populations are largely CD2high and CD5high. This phenotype is expected on DP thymocytes undergoing selection or on SP thymocytes that are completing or have just completed development. However, both of these populations show a clear difference in CD24 expression. The population that is clearly CD8β SP is CD24low, but the second population is largely CD24high. The reconstituted CD24high CD8β SP population represents mature thymocytes, whereas the reconstituted CD24high CD8β SP population likely represents intermediate thymocytes auditioning for selection. If this population is indeed an intermediate population, then the CD8α-Lck chimeric coreceptor is driving development along
the same intermediates as endogenous CD8 and as such, is able to recapitulate phenotypic intermediates of normal development. The counterpart population in 2C mice has previously been shown to possess a phenotype consistent with an intermediate phenotype, similar to our results presented here, including the CD8βlow expression level (28).

We also detect a small population (noted in Fig. 2) that is phenotypically consistent with DP thymocytes in that the CD8 expression is higher (comparable to C57BL/6 DP thymocytes) and CD3εlowCD24high, with some members being CD2low and CD5low. If this population represents DP thymocytes not auditioning for selection, it is curious to note that the population is very small. One possibility is suggested by our 2C CD8α−/− thymocyte data. In these mice the majority of CD3εlow thymocytes appear to be a homogeneous population poised to undergo selection but lacking the coreceptor signal to do so. In 2C mice the CD8 coreceptor may be efficiently driving DP thymocytes into selection, and the cells accumulate as intermediate phenotypes. These intermediate cells, or at least a significant portion, are likely undergoing positive selection because CD8 SP T cells are generated. As such, a significant portion of this population could also be undergoing negative selection, which may in part explain why these mice have such small thymi. It has been shown in 2C mice that a two-fold increase in CD8 coreceptor expression can alter selection from positive to negative in the otherwise positively selecting Kβ background (38). A similar mechanism may be occurring in Tg59 mice. In this case, the expression level of CD8α-Lck appears to provide the requisite activity to promote the efficient and robust development of the intermediate thymocyte population. Again, the reconstituted intermediate population may be undergoing positive selection, leading to the formation of a CD8 SP population and negative selection, partly explaining the small thymi in these animals as well.

The reconstitution of both CD8 SP and intermediate thymocyte populations is likely the result of Lck rather than just the presence of the CD8 extracellular domain. In an earlier study where a CD8α tailless coreceptor chain was expressed in CD8α−/− mice at levels comparable to those in endogenous CD8, development was severely impaired (11). Further, when these transgenic mice expressed the 2C TCR, reconstitution did not occur at significant levels. The expression levels for both Tg59 and Tg39 are significantly lower than that for mice expressing endogenous levels of CD8, and yet reconstitution occurs to significant levels in both the thymus and the periphery. Because tailless CD8α has been shown to be inefficient and low expression levels of transgene Tg59 and Tg39 are sufficient, it can be concluded that Lck is important in efficient development of CD8 SP thymocytes.

An interesting aspect of the CD8α-Lck coreceptor is that, because Lck has replaced the CD8α tail, it might be expected to be more "CD4 like." Engagement of the chimeric coreceptor would involve Lck in the TCR-MHC interaction but not LAT, similar to what might be expected for CD4 but not CD8. Various reports aimed at altering Lck association with coreceptors show alteration of thymocyte development in the direction hypothesized by the strength of signaling model (39–41). It has been shown that expression of a constitutively activated Lck promotes SP development and preferential development of CD4 SP over CD8 SP thymocytes with increasing levels of expression (23). This occurs at the expense of DP thymocytes and is independent of MHC class I or class II expression. It is clear in our studies that CD8α-Lck is promoting the development of CD8 SP thymocytes bearing the 2C TCR and not CD4 SP thymocytes bearing the 2C TCR. In fact, the CD4 SP T cells represent a small percentage and are largely 1B2−/−.

**FIGURE 8.** The expression of a kinase-inactive mutant CD8α-Lck chain also leads to the reconstitution of a CD8 SP population. A. It is shown that the population profiles of the reconstituted thymocytes are phenotypically equivalent to those already discussed for 2C wild-type (WT) and Tg39 mice. The percentage of CD8 SP cells in the gated population is given on the top left corner of the dot plots. B. A comparison of the MFI values of surface CD8β expression is shown. MFI was calculated from pooled groups of animals, with each group having a minimum of four individuals. C. The phenotypic profile of the thymocytes and LN T cells from the Tg(K273R) animal is shown. The analysis is done on fresh thymocytes or lymph nodes from age-matched siblings. The overall phenotypic pattern and level of CD8α-Lck expression is similar to that of 2C CD8α−/− Tg39. The gates indicated within each histogram were generated by using the histogram plots from C57BL/6 thymocytes stained in parallel as in the previous figures. The abbreviations hi and lo represent high and low, respectively.
in 2C mice. This finding indicates that CD8α-Lck is providing specific signals leading to the development of CD8 SP thymocytes. An explanation of this paradox might be that the absolute levels of coreceptor-associated Lck during TCR-MHC interaction might be the lineage-determining factor. Low expression levels of Tg59 and Tg39 by virtue of being ligated to CD8 might involve the same amount of Lck as wild-type CD8 during coreceptor engagement.

To better understand how CD8-associated Lck might be involved in the DP to CD8 SP developmental transition, we generated a murine line expressing a kinase-inactive CD8α-Lck coreceptor. The expression level of this transgene was similar to that of Tg39. Interestingly, it was also able to reconstitute those thymocyte populations observed in Tg59 and Tg39 in a phenotypically equivalent manner. This result was surprising, because the accepted model for coreceptor-associated Lck in TCR signaling involves its kinase activity in phosphorylating CD3 ITAMs. Here, Lck may be effecting development via an adapter function that can occur through the Lck SH2 and/or SH3 domains or by having phosphotyrosine residues that can be bound by other proteins.

The notion that Lck adapter domains (i.e., SH2 and SH3) can contribute to thymocyte development has been explored previously. A chimeric protein consisting of Lck adapter domains ligated to the Fyn catalytic domain in Lck<sup>−/−</sup> mice and driven by the Lck proximal promoter could reconstitute both thymocyte numbers and a robust DP population (42). However, a SP population was not fully reconstituted. Interestingly, the CD8 SP population did not appear to be as affected as the CD4 SP population. The converse chimeric protein of Fyn N-terminal domains ligated to a catalytically active Lck domain could not reconstitute thymocyte numbers but was capable of reconstituting some DN to DP transitions and some DP to SP transitions. These results suggest that the adapter domains of Lck may be more critical than its kinase domain in efficiently promoting a thymocyte developmental transition. It may be that CD8 SP thymocytes rely on Lck-associated adapter function for more efficient development from the DP pool.

In our studies, the adapter function of CD8α-Lck is promoting the efficient development of CD8 SP thymocytes where at low levels of expression the adapter domains may be much more efficient than the kinase domain in exerting their biological effects. In any case, because we did not generate mice with varying levels of the CD8α-Lck kinase-dead transgene we must remain cautious in our statement regarding the role of Lck adapter function.

The exact role of Lck in CD8 development has been less clear than that for CD4. Initial experiments with Lck<sup>−/−</sup> mice showed a marked reduction in total thymocyte numbers but nonetheless were leaky, with CD8 SP being much less affected than CD4 SP (43, 44). This has raised the question as to whether the CD8 delineation is strictly dependent on Lck expression or whether there could be other molecules or mechanisms involved.

Although we cannot formally exclude the possibility that endogenous Lck binding to CD8α-Lck may recruit Lck kinase activity, we were unable to detect any coimmunoprecipitation of endogenous Lck with a CD8α-Lck immunoprecipitation (data not shown). Another interesting example of a role for kinase dead Lck is provided by the studies of Hernandez-Hoyos et al. (39), where Lck with kinase-active and kinase-dead domains were expressed under the control of the distal Lck promoter. In these studies active Lck promoted thymocytes with MHC class I-restricted TCRs to the CD4 lineage, and dead Lck promoted thymocytes with MHC class II-restricted-TCRs to the CD8 lineage. These results support the strength of signaling model. However, one could speculate that providing kinase-dead Lck was decreasing the Lck activity associated with CD4 coreceptors and in essence increasing the adapter function associated with it, thereby promoting CD8 lineage development. Vice versa, introducing active Lck competes with LAT and endogenous Lck binding to CD8 such that a threshold of Lck kinase activity is achieved that predominates over its adapter function and promotes CD4 lineage development.

In summary, our data provide evidence that the association of Lck to CD8 is sufficient to mediate CD8 SP thymocyte development and, interestingly, that it can proceed independently of the associated Lck kinase activity. A possible mechanism for endogenous CD8 might involve an integral Lck adapter function that, at low coreceptor association levels, predominates over its kinase activity to efficiently promote CD8 SP thymocyte development. If indeed only low levels of Lck association to CD8 are required, LAT association may be critical in that it limits Lck association and enables the adapter function aspect of Lck to predominate. Because the exact role of LAT at this developmental point is not known, it would be interesting to see whether a chimeric CD8α-LAT molecule can reconstitute CD8 SP thymocyte development like CD8α-Lck does and at what expression levels. Along these lines, it would be interesting to see whether a CD4-Lck chimeric coreceptor with or without kinase activity expressed at levels comparable to Tg59 can drive development of CD4 SP thymocytes (or are higher levels required?).

**Acknowledgments**

We thank Dr. Dan Littman and Mary Jean Sunshine for help with making the transgenic mice. We also thank Dr. Littman for very helpful discussions and critical reading of the manuscript.

**Disclosures**

The authors have no financial conflict of interest.

**References**


