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Activated p56\(^{\text{Lck}}\) Directs Maturation of Both CD4 and CD8 Single-Positive Thymocytes\(^1\)

Sue J. Sohn,\(^2*\) Katherine A. Forbush,* Xiao Cun Pan,* and Roger M. Perlmutter †

p56\(^{\text{Lck}}\) is a protein tyrosine kinase expressed throughout T cell development. It associates noncovalently with the cytoplasmic domains of the CD4 and CD8 coreceptor molecules and has been implicated in TCR signaling in mature T cells. Its role in early thymocyte differentiation has been demonstrated in vivo, both by targeted gene disruption and by transgene expression. Previously, we showed that expression of a dominant-negative form of p56\(^{\text{Lck}}\) in double-positive thymocytes inhibits positive selection. We now demonstrate that expression of constitutively activated p56\(^{\text{Lck}}\) (p56\(^{\text{Lck}}\)F505) accelerates the transition from the double-positive to the single-positive stage. Importantly, p56\(^{\text{Lck}}\)F505 drives survival and lineage commitment of thymocytes in the absence of TCR engagement by appropriate MHC molecules. These results indicate that activation of p56\(^{\text{Lck}}\) constitutes an early step in conveying maturational signals after TCR ligation by a positively selecting ligand. Our study provides direct in vivo evidence for the role of p56\(^{\text{Lck}}\) in regulating TCR signaling. *The Journal of Immunology*, 2001, 166: 2209–2217.

M aturation of thymocytes from CD4\(^+\)CD8\(^-\) double-positive (DP) to CD4\(^+\) or CD8\(^+\) single-positive (SP) stage requires signals generated by the concerted interaction of surface TCRs and CD4 or CD8 coreceptors and the appropriate MHC molecule (reviewed in Refs. 1 and 2). DP thymocytes that interact with self-MHC with sufficient affinity survive and proceed through final maturation steps (positive selection). T cells that fail to interact or that bind MHC molecules too strongly undergo apoptosis (death by neglect and negative selection, respectively) (reviewed in Ref. 2). The transition from the DP to SP stage is also accompanied by commitment to either the CD4\(^+\) helper or CD8\(^+\) cytotoxic T cell lineage, a decision influenced by the Ag specificity of the TCR and the appropriate MHC-coreceptor match. For example, transgenic mice constitutively expressing MHC class I-restricted TCRs or those expressing MHC class II-restricted TCRs possess mainly CD8\(^+\) or CD4\(^+\) T cells, respectively (3–6). Conversely, mice deficient for MHC class I or class II expression lack CD8\(^+\) or CD4\(^+\) T cells (7–10). Finally, mice lacking CD4 or CD8 coreceptors possess greatly reduced numbers of helper or cytotoxic lineage T cells (11–14).

Although TCRs, coreceptors, and MHC molecules clearly direct the transition from the DP to SP stage, it is unlikely that a “correct” combination of these components sends a strictly instructive signal which simultaneously dictates survival and lineage commitment. Several studies support the notion that lineage commitment occurs first and randomly but that survival depends on sustained, optimal coreceptor/TCR interactions with the MHC molecules (reviewed in Ref. 15). For example, constitutive expression of the CD8 molecule permits maturation of CD4\(^+\) helper T cells (CD4\(^+\)CD8\(^{\text{Tg}}\)) bearing an MHC class I-restricted TCR (16, 17). Conversely, constitutive expression of CD4 molecules rescues CD8\(^+\) cytotoxic T cells bearing a class II-specific TCR (18). Thus, thymocytes expressing class I-restricted TCRs were shown to be positively selected in the absence of CD8 when cultured with peptide variants that artificially improved TCR-MHC interactions (19, 20). These studies indicate that manipulations that change interactions among the TCR, coreceptor, and MHC invoke differential intracellular signals to direct T cell maturation.

Following the TCR \(\alpha (\alpha)\) chain rearrangement, the DP thymocytes undergoing selection express clonotypic \(\alpha\) - and the \(\beta\)-polypeptide chains which comprise a structure that can bind the peptide/MHC complex. This heterodimeric receptor also associates noncovalently with the invariant CD3 subunits and the \(\zeta\) chains, which are required for transducing intracellular signals (21, 22). CD4 and CD8 coreceptors stabilize TCR-MHC interactions and enhance intracellular signaling events triggered by the TCR. Thus, co-cross-linking CD4 and CD3 has been shown to enhance the ability of T cells to flux \(\text{Ca}^{2+}\) relative to CD3 cross-linking alone (23). In contrast, independent cross-linking of CD4 and CD3 decreased the \(\text{Ca}^{2+}\) response. These results indicate that coreceptors can contribute to the overall amplitude and/or quality of signals transmitted by the TCR. Furthermore, expression of a chimeric coreceptor molecule containing the extracellular and transmembrane domains of CD8a and the cytoplasmic domain of the CD4 molecule in F5 TCR (specific for a viral nucleoprotein peptide presented by H-2D\(^{b}\)) transgenic mice promoted the maturation of CD4\(^+\) T cells expressing this class I-restricted TCR (24). These results suggest that the cytoplasmic tail of the CD4 coreceptor can influence the outcome of the thymocyte cell fate decision.

The src family protein tyrosine kinase p56\(^{\text{Lck}}\) is expressed mainly in T lineage cells throughout T cell development (review in Ref. 25). It associates noncovalently with the cytoplasmic tails of the CD4 and CD8 coreceptor molecules and becomes catalytically activated when the coreceptors are cross-linked (26–30). Biochemical evidence suggests that p56\(^{\text{Lck}}\) can also enhance signals mediated through the TCR/CD3 complex (31–33), although a direct physical association between p56\(^{\text{Lck}}\) and TCR/CD3 complex

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\(^{†}\) Abbreviations used in this paper: DP, double-positive; SP, single-positive; DN, double-negative; \(\beta_{m}, \beta_{\text{Lm}}\)-microglobulin; HSA, heat-stable Ag; dLGF, distal promoter driving the lck gene bearing the F505 mutation; RAG, recombine-activating gene.

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has not been established. More importantly, lack of functional p56\(^{lck}\) abrogated mature T cell activation in a mutant Jurkat subclone (JCaM1), providing genetic evidence for the role of p56\(^{lck}\) in TCR signaling (34). In mice, targeted disruption of the \(lck\) gene, or transgenic expression of catalytically inactive p56\(^{lck}\) under the control of the \(lck\) proximal promoter (which drives transgene expression in the CD4\(^{+}\)CD8\(^{+}\) double-negative (DN) and DP thymocytes), interferes with both cellular expansion and allelic exclusion at the TCR \(\beta\)-chain gene locus during the transition from the DN to the DP stage (35, 36). In addition, expression of an activated form of p56\(^{lck}\) (p56\(^{lck}\)F505) under the control of the \(lck\) proximal promoter arrests thymocyte differentiation before the DP stage and at high levels causes transformation and accumulation of immature thymocytes (37, 38).

Data obtained from these studies document the importance of p56\(^{lck}\) in delivering signals from the pre-TCR complex, but they do not provide direct evidence for the role of p56\(^{lck}\) in later stages of T cell development. To study the role of p56\(^{lck}\) in the maturation of SP thymocytes, we have previously used the \(lck\) distal promoter, which is active mainly in mature thymocytes and peripheral T cells, to drive the expression of a dominant-negative form of p56\(^{lck}\) in mice (39). Using the \(lck\) distal promoter circumvents the early developmental block that occurs after expression of a dominant-negative \(lck\) transgene under the control of the proximal promoter. These mice possess reduced numbers of SP thymocytes, indicating that dominant-negative p56\(^{lck}\) inhibits positive selection. However, these results do not resolve whether activation of p56\(^{lck}\) is sufficient to direct survival and/or lineage commitment during the transition from the DP to SP stage. In the present study, we used the \(lck\) distal promoter to drive expression of an activated form of p56\(^{lck}\) (p56\(^{lck}\)F505) in mice. Our data suggest that activation of p56\(^{lck}\) can direct survival and lineage commitment during the final stages of thymocyte maturation. Furthermore, we show that CD4\(^{+}\)CD8\(^{+}\) ratios of developing thymocytes are sensitive to relative levels of p56\(^{lck}\)F505, consistent with the idea that signal “strength” assists in determining the CD4\(^{+}\) vs CD8\(^{+}\) lineage choice.

Materials and Methods

Assembly of dLGF transgene construct and generation of transgenic lines

The \(lck\) gene containing the F505 mutation and a 0.6-kb fragment of the 3’ human growth hormone gene was isolated from a previously reported construct, pLGF (37), by restriction digest with NotI and StuI. The vector containing the \(lck\) distal promoter, pW120 (40), was linearized with BamHI digest, filled in, and was subject to partial digestion with NotI to remove the human growth hormone gene. Finally, we inserted the \(lck\)F505 (including the 3’-human growth hormone gene) into the \(lck\) distal promoter construct by directional ligation. The A-to-T point mutation converting the tyrosine to a phenylalanine, and the joining region of the distal promoter and the \(lck\) gene was verified by nucleotide sequencing.

The transgene DNA was microinjected into fertilized (C57BL/6 × DBA/2)F\(_1\) embryos to generate transgenic founder mice. We determined transgene integration by Southern blot analysis of tail DNA and backcrossed four founders with C57BL/6 animals to establish transgenic lines.

Generation and screening of MHC-deficient dLGF animals

To generate MHC class I-deficient and MHC class II-deficient dLGF animals, we crossed dLGF animals from A16912 line with commercially available 129/\(\beta_{2a}\)-microglobulin (\(\beta_{2a}\))\(^{-/-}\) or \(I-A^{b}\)\(^{-/-}\) mice (Taconic Farms, Germantown, NY). To generate MHC double-deficient dLGF animals, we crossed the dLGF A16809 line animals with \(\beta_{2a}\)\(^{-/-}\)/\(I-A^{b}\)\(^{-/-}\) mice, also purchased from Taconic Farms.

The genotype at the \(\beta_{2a}\) and \(I-A^{b}\) loci was determined by PCR. The primer sequences are as follows. For detection of wild-type \(\beta_{2a}\) locus, the forward primer sequence is 5′-AACT CAG TCG TCA TGC TCC GCT GCC CTC AGA-3′. The reverse primer sequence is 5′-TGG AGG AAC AAC TTG AGG CTT ACC-3′. For detection of wild-type \(I-A^{b}\) locus, the forward primer sequence is 5′-AGC ACC GCG CCG TGA CCG AG-3′ and the reverse primer sequence is 5′-CAG AGG GCA GAG GTG AGA CAG-3′. The absence of amplification product was scored as homozygous disruption in each case.

Analyses of transgene expression by immunoblotting

Transgene expression was determined by immunoblotting total lysates from thymocytes and splenocytes. Briefly, 5 × 10\(^6\) cells were lysed in buffer containing 1% Triton X-100 (50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM PMSEF, 1 mM aminopterin, 1 mM leupeptin, 1 mM Na\(_2\)VO\(_3\),), and the resulting lysates were resolved in 10% SDS-PAGE, transferred onto nitrocellulose, blotted with affinity-purified anti-p56\(^{lck}\) monoclonal reagent (3AS; Santa Cruz Biotechnology, Santa Cruz, CA) at 0.25 \(\mu\)g/ml followed by incubation with HRP-conjugated sheep anti-mouse secondary Ab (Amersham Life Sciences, Arlington Heights, IL) at 1/3000 dilution, and visualized by chemiluminescence. Relative intensity of the signals was determined by densitometric analyses using Molecular Imager system (Bio-Rad Laboratories, Hercules, CA).

Flow cytometry

Thymocytes and splenocytes were stained and analyzed by flow cytometry according to standard protocols. After RBC lysis treatment, 10\(^6\) cells were incubated in staining media (HBSS-BSA at 10 mg/ml) containing appropriate concentrations of fluorochrome-conjugated mAbs on ice, washed, incubated with secondary reagent where required, and fixed in 1% paraformaldehyde (PBS, pH 7.4). Flow cytometry was performed on FACSScan (Becton Dickinson, San Jose, CA) and analyzed using ReproMac software (TrueFacts Software, Seattle, WA). The following monoclonal reagents were purchased from PharMingen (San Diego, CA): biotin-anti-CD3e (145-2C11); biotin-anti-CD69 (H.12F3); biotin-anti-CD4 (H129.19); biotin-anti-heat-stable Ag (HSA); PE-anti CD8a (53-67); FITC-anti-TCR (H57-597), FITC-anti-Qu-2 (1-1-2), PE-anti-CD4 (CT-CD4), FITC-anti-CD8a (CT-CD8a), and Tri-Color-conjugated streptavidin were purchased from Caltag Laboratories (Burlingame, CA).

For intracellular staining of TCR \(\beta\), cells were first stained for surface markers using biotinylated and PE-conjugated monoclonal reagents, respectively, followed by incubation with Tri-Color-streptavidin, fixed in 4% paraformaldehyde (PBS, pH 7.4), and permeabilized in 0.1% saponin solution (PBS (pH 7.4)-1% FCS-0.1% NaN\(_3\)). The cells were then incubated with FITC-anti-TCR diluted in permeabilization buffer and analyzed on FACSScan.

Isolation of thymocyte subpopulations

To isolate DP and CD4\(^{+}\) SP thymocytes for cell cycle analysis, total thymocytes were stained with fluorochrome-conjugated reagents specific for CD4 and CD8 and sorted on FACStar (Becton Dickinson, San Jose, CA). For proliferation assay, the CD4\(^{+}\) and CD8\(^{+}\) T cells were purified from splenocytes by negative selection using magnetic bead separation methods. Briefly, splenocytes were incubated with Ab cocktail containing biotinylated anti-B220, anti-I-A\(^{b}\), anti-CD11b, anti-NK1.1, and anti-CD4, or anti-CD8 monoclonal reagents (PharMingen, San Diego, CA) for 30 min and washed. Then magnetic beads preconjugated with streptavidin (Dynal, Lake Success, NY) were added at an 10:1 bead-target ratio and mixed for 45 min at 4°C. Cells that did not bind the beads were collected after the magnetic separation for experiments described in the text.

Cell cycle analysis

Purified DP and SP thymocytes were stained with propidium iodide as described by Hardy et al. (41) and analyzed by flow cytometry to determine nuclear DNA content. Briefly, sorted cells were washed in PBS, fixed in 95% ethanol, and incubated in staining buffer containing propidium iodide (Calbiochem, San Diego, CA) at 20 mg/ml, RNase A at 1 mg/ml, and 0.01% Nonidet P-40, followed by analysis on FACSScan.

Proliferation assay

Total or purified splenocytes were stimulated in RPMI (supplemented with 10% FCS, i-glutamine, nonessential amino acid, penicillin-streptomycin, and 2-ME) containing various combinations of 145-2C11 ascites, PMA (Calbiochem), recombinant murine IL-2 (Boehringer Mannheim, Indianapolis, IN), and ionomycin (Calbiochem) for 2 days, pulsed with \(^{[3]}H\) thymidine, and harvested on day 3. Proliferation was measured by the cpm incorporated and normalized to the number of mature T cells in each well. For allo-specific responses, indicated numbers of total splenocytes were cultured with irradiated splenocytes from C3H/HeJ animals in the presence or absence of exogenous IL-2. The culture was pulsed on day 4 and harvested on day 5.
Results

Generation of transgenic mouse lines and analyses of transgene expression

To study the role of p56\(^{lck}\) during late stages of thymocyte ontogeny, we generated mice that express an activated form of p56\(^{lck}\) (p56\(^{lck}\)F505) under the control of the lck distal promoter (Fig. 1A). These transgenic mice are herein referred to as dLGF mice. We generated four independent dLGF lines by backcrossing founders onto a C57BL/6 background. As shown in Fig. 1B, immunoblot analyses indicate that the total abundance of p56\(^{lck}\) is increased in thymocytes from transgene-positive mice relative to littermate controls. Densitometric scanning of a representative blot showed that transgene expression increased the total abundance of p56\(^{lck}\); 1.4- to 2.5-fold (lanes 2, 4, 6, and 8) compared with littermate controls (lanes 1, 3, 5, and 7). The overall levels of transgene expression declined in the periphery (Fig. 1B, lanes 9–16), a result different from the known pattern of lck distal promoter activity (40). This discrepancy may be explained by the fact that the proportion of T cells among total splenocytes decreases in the periphery of dLGF mice, as shown in Fig. 2A.

Activated p56\(^{lck}\) increases representation of SP thymocytes

dLGF animals appeared healthy and did not develop tumors of the thymi or peripheral lymphoid organs (up to 5 mo of age). We assessed the effects of transgene expression on thymocyte development by flow cytometric analyses of CD4, CD8, and CD3 expression. As shown in Fig. 2A, thymi from dLGF animals contained substantially increased proportions of cells with mature CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^+\) phenotypes (upper panels). The increase in the percentage of such SP thymocytes was accompanied by a concomitant decrease in the percentage of CD4\(^+\)CD8\(^+\) DP thymocytes (from 87.0% to 34.7%). The increased proportion of SP thymocytes in dLGF animals reflected an increased production of mature T cells as indicated by the increased absolute number of the SP thymocytes. Moreover, the number of SP thymocytes increased as the abundance of p56\(^{lck}\)F505 increased (Fig. 2B). Importantly, the total number of thymocytes remained normal in all dLGF lines, indicating that the expansion was selective for the SP compartment. At a 0.7:1 ratio (p56\(^{lck}\)F505: wild-type p56\(^{lck}\) in A16924 line), p56\(^{lck}\)F505 increased the number of SP thymocytes at least 3-fold relative to that of the control mice. Thus, production of SP thymocytes is extremely sensitive to the level of p56\(^{lck}\) activity.

In contrast to thymocytes, the representation and the number of mature T cells in the periphery of dLGF mice are lower compared with the littermate control (Fig. 2A, lower panels). These data suggest either that p56\(^{lck}\)F505 inhibits migration of mature thymocytes to the periphery or that the presence of p56\(^{lck}\)F505 is detrimental to the survival of peripheral T cells.

In addition to the altered representation of thymocyte subsets, we observed that a large number of both the DP and the SP thymocytes in dLGF animals displayed reduced CD4 and CD8 coreceptor expression on their cell surfaces (Fig. 2A, upper panels). The mechanisms responsible for regulation of coreceptor expression on T cell surfaces are presently unknown. Thymocytes from dLGF animals also display reduced levels of surface CD3 proteins. In control mice, ~10% of thymocytes express high levels of surface CD3 and correspond to SP mature cells (Fig. 2C, left). In contrast, only 2% of thymocytes from dLGF animals express high levels of surface CD3 (right), even though the percentage of SP...
Thymocytes are increased in these animals (10% in control vs. 58% in transgenic mice). Moreover, the percentage of cells arbitrarily defined as CD3<sup>mid</sup> is reduced (48.5% in control vs. 23.7% in Tg animals) with a concomitant increase in the percentage of CD3<sup>−</sup> cells in dLGF animals, suggesting that a large proportion of DP thymocytes fail to up-regulate surface TCR-CD3 expression. Activation of p56<sup>lck</sup> has been previously correlated with decreased TCR-CD3 surface expression in immature and mature T cells. In DN thymocytes, activated p56<sup>lck</sup>, expressed under the control of the lck proximal promoter, inhibits TCR-β chain gene rearrangement and thus prevents production and surface expression of functionally rearranged TCR β-chains (42). In mature T cells, p56<sup>lck</sup>F505 down-regulates surface accumulation of TCR by directing TCR-CD3 complexes to lysosomal compartments for degradation (43). To determine which of these mechanisms may be responsible for the reduced accumulation of surface TCR/CD3 in dLGF animals, we permeabilized thymocytes from control, pLGF, and dLGF animals and stained them for cytoplasmic TCR (Fig. 2D). The percentage of cells containing cytoplasmic TCR β-protein is reduced in the DN thymocytes (Fig. 2D, center) from mice that express high levels of p56<sup>lck</sup>F505 under the control of the lck proximal promoter (pLGF (37)). These results are consistent with the known effects of p56<sup>lck</sup>F505 on TCR β-chain gene rearrangement. In contrast, the percentage of DN cells containing cytoplasmic β-chains remains normal in dLGF animals (Fig. 2D, right), indicating that TCR gene rearrangement is not inhibited. Production of TCRs, judged by the content of intracellular β-chain proteins, is also normal in DP thymocytes from these mice (data not shown). This finding, together with the fact that p56<sup>lck</sup>F505 is not expressed in DN thymocytes (data not shown), is consistent with the idea that TCR-CD3 levels decrease in dLGF animals due to posttranslational degradation rather than diminished synthesis of functional TCR chains.

**p56<sup>lck</sup>F505 does not promote cellular proliferation in dLGF animals**

The finding that p56<sup>lck</sup>F505 increases the proportion and the number of SP thymocytes suggests either that the transition from the DP to the SP stage is enhanced or that thymocytes in the SP compartment undergo proliferation. To address these possibilities, we purified DP and CD4<sup>+</sup> SP thymocytes from control and dLGF animals and measured their DNA content. As shown in Fig. 3, the percentages of cells that are in S + G<sub>2</sub>-M phase are comparable between Tg and control animals in both the DP and the SP compartments, and the percentage distribution of cells in G<sub>1</sub> and in S + G<sub>2</sub>-M phases in our experiments is in agreement with previous reports (44). These results indicate that p56<sup>lck</sup>F505, at expression levels achieved in our system, does not induce spontaneous thymocyte proliferation, arguing against the hypothesis that the increase in SP thymocyte number results from postselection proliferation.

**p56<sup>lck</sup>F505 drives differentiation of SP thymocytes**

The observation that p56<sup>lck</sup>F505 increases the proportion and the number of SP thymocytes, without inducing their proliferation, suggests that activated p56<sup>lck</sup> accelerates the maturation of thymocytes from the DP to the SP stage. In normal animals, maturation through this differentiation step requires the interaction of functional TCRs with appropriate MHC molecules. However, we observed that SP thymocytes in dLGF animals develop efficiently despite the reduced surface levels of CD3, suggesting that p56<sup>lck</sup>F505 might compensate for any loss or attenuation of signals normally triggered by TCR-MHC interaction. To test this possibility, we investigated whether SP thymocytes can develop in...
dLGF mice bred to MHC class I (β2m−/−), MHC class II (I-Aβb−/−), or MHC double-deficient backgrounds. Fig. 4A shows CD4 and CD8 staining profiles of thymocytes from dLGF mice crossed onto an MHC class I−/− background. As expected, non-transgenic (non-Tg) MHC class I−/− mice (lower left) exhibited a profound deficiency of CD8+ SP thymocytes when compared with MHC class I+/− mice (upper left). In contrast, expression of p56lckF505 in MHC class I−/− mice (lower right) reconstitutes the CD8+ SP compartments to levels comparable with that of its MHC class I+/−/dLGF counterpart. These results indicate that p56lckF505 can drive the maturation of CD8+ SP thymocytes in the absence of MHC class I molecules. As in a wild-type background (Fig. 2C), the presence of the dLGF transgene caused decreased levels of surface TCR-CD3 on DP as well as on SP thymocytes in MHC class I−/−/dLGF mice (Fig. 4B, bottom). Thus, in dLGF mice (center and bottom), <10% of total CD8+ SP thymocytes express appreciable levels of TCR-CD3, and the level of TCR-CD3 expression in these cells is significantly reduced compared with MHC class I+/−/non-Tg controls (top). Nonetheless, CD8+ SP thymocytes in MHC class I−/−/dLGF mice up-regulate CD69 as well as do their MHC class I+/−/nontransgenic and MHC class I+/−/dLGF counterparts (Fig. 4C). This suggests that dLGF thymocytes have received an activating signal during the DP-to-SP transition, even in the absence of MHC class I molecules.

Similarly, expression of p56lckF505 in an MHC class II-deficient background drove maturation of CD4+ T cells. As shown in Fig. 5A, MHC class II−/−/dLGF mice possess a large number of CD4+ SP thymocytes (lower right), in contrast to the MHC class II−/−/nontransgenic control (lower left). We also noted that the percent of CD4+ SP thymocytes in MHC class II−/−/dLGF mice was slightly reduced compared with the MHC class II+/−/dLGF control. This result suggests that MHC class II molecules can enhance but are not required for maturation of SP thymocytes directed by p56lckF505. The level of surface TCR/CD3 is again reduced on thymocytes from MHC class II−/−/dLGF mice (data not shown), although CD4+ SP thymocytes clearly up-regulate surface expression of CD69 (data not shown) and the mature T cell marker Qua-2 (Fig. 5B). To test whether CD4+ T cells acquire the ability to respond to activating signals, we measured the ability of CD4+ T cells to proliferate. We purified CD4+ splenic T cells from MHC class II-positive (MHC class II+/+) or -negative (MHC class II−/−) and nontransgenic or dLGF animals and cultured them with H-2k allogeneic stimulators in the absence or presence of exogenous IL-2. The CD4+ cells from MHC class II+/−/nontransgenic control animals proliferate in response to increasing numbers of stimulators in the absence of exogenous IL-2, but those from MHC class II−/−/non-Tg, MHC class II+/−/dLGF, and MHC class II−/−/dLGF animals do not proliferate (Fig. 5C, left). Addition of exogenous IL-2 enhances the overall response but still fails to restore proliferation of T cells from MHC class II+/−/dLGF and MHC class II−/−/dLGF animals to the control level (Fig. 5C, right).
right). The apparent deficit in proliferative responses by MHC class II^{+/−}/dLGFRG and MHC class II^{−/−}/dLGFRG splenocytes may reflect the reduced levels of surface TCR on these cells (data not shown). This hypothesis is supported by the fact that stimulation with ionomycin and PMA, which bypasses TCR ligation, restores proliferation to near normal levels (Fig. 5D).

The data above strongly suggest that p56^{−/−}fS05 compensates for both the lack of appropriate MHC and the reduced TCR/CD3 levels on developing thymocytes, to drive lineage commitment and differentiation of thymocyte in vivo. To determine whether p56^{−/−}fS05 is sufficient for both of these processes, we crossed dLGFRG mice onto an MHC-deficient (class I- and class II-deficient) background. Remarkably, large numbers of both CD4 and CD8 SP thymocytes develop in MHC-deficient dLGFRG mice (Fig. 6A, lower right). Importantly, the overall representation of thymocyte subsets remains unchanged compared with the MHC-positive dLGFRG control (upper right). Moreover, CD4 and CD8 SP thymocytes from MHC-deficient dLGFRG mice down-regulate HSA as efficiently as do their MHC-positive counterparts and better than the MHC-positive

![FIGURE 5](image_url) Expression of the dLGFRG transgene drives differentiation of CD4^{+} SP thymocytes in MHC class II-deficient environment. A, CD4 and CD8 profiles of thymocytes from progeny of the dLGFRG × MHC class II^{-/−} cross are shown. Numbers indicate percentages of thymocytes in corresponding quadrants. The results are representative of four independent experiments. B, The CD4^{+} SP thymocytes from MHC class II^{-/−}/dLGFRG animals express Qa-2. Surface expression of Qa-2 was analyzed by flow cytometry. In each panel, the solid line represents the Qa-2 profile of the CD4^{+} SP thymocytes, and the broken line represents the profile of the DP thymocytes. Percentages of Qa-2- cells are indicated. C, Proliferation of purified CD4^{+} T cells. Purified CD4^{+} splenic T cells were cultured with irradiated splenocytes from C3H (H-2^k) animals. The culture was pulsed with [3H]thymidine on day 4 and harvested on day 5. cpm are shown as the mean of triplicate samples; error bars, SEM. The results from two MHC class II^{-/−}/non-Tg animals (▲), one MHC class II^{-/−}/dLGFRG animal (■), one MHC class II^{-/−}/non-Tg animal (○), and three MHC class II^{-/−}/dLGFRG animals (♦) are shown. D, Purified CD4^{+} T cells were stimulated in culture with media only or with ionomycin and PMA. The culture was pulsed with [3H]thymidine on day 2 and harvested and counted on day 3. The mean of triplicate wells and the SEM are also shown.

![FIGURE 6](image_url) Expression of dLGFRG transgene drives differentiation of SP thymocytes independent of MHC. A, CD4 and CD8 profiles of thymocytes from MHC-positive (MHC^{1+/−}/class I^{1+/−}/class II^{1+/−}) non-Tg (upper left) and dLGFRG (upper right) animals and MHC-deficient (MHC^{−/−}/class I^{−/−}/class II^{−/−}) counterparts (lower panels) are shown. The percentage of cells in each quadrant is also indicated. These profiles represent two independent experiments. B, Expression of HSA was determined by triple-staining thymocytes with fluorochrome-conjugated reagents specific for CD4, CD8, and HSA, followed by flow cytometric analysis. Solid line, HSA profile of indicated CD4^{+} or CD8^{+} SP thymocyte population; broken line, profile of the gated DP thymocytes for each animal. The numbers correspond to the percentage distribution of cells that have down-regulated or maintained high level expression of HSA.
The mean ratio values are plotted against relative total p56 calculated for the four dLGF-transgenic lines and their non-Tg littermates. We observed that the ratio of CD4:CD8 SP thymocytes varied among the four dLGF lines. As depicted in Fig. 7A, the mean CD4:CD8 ratio of individual transgenic lines increases with increasing total p56 abundance (A16924 and A16912 lines) but abruptly returns to the normal ratio at the highest level of transgene expression (A16809 line). The ratios in individual animals from the two intermediate lines (A16912 and A16924) varied significantly but were consistently higher than those in their littermate controls. We hypothesized that quantitative changes in total p56 activity may directly influence the CD4:CD8 ratio of developing thymocytes or that the site of transgene integration indirectly affects the balance of lineage commitment. To test whether alterations in p56 abundance affect the CD4:CD8 ratio, we intercrossed transgenic mice from the A16912 line expressing an intermediate level of p56F505 (and exhibiting increased CD4:CD8 ratios), thus generating animals hemizygous or homozygous for the dLGF transgene. We analyzed three litters of animals from this cross to individually assess the total p56 abundance in thymocytes and to document the CD4:CD8 SP thymocyte ratio. The results, summarized in Fig. 7B, demonstrate that augmented p56F505 levels initially increase the representation of CD4 cells in the SP compartment to a ratio of ~10:1. Thereafter, further increases in p56F505 abundance result in gradual normalization of the CD4:CD8 ratio.

Relative p56<sup>cko</sup> activity determines the ratio of CD4:CD8 SP thymocytes

Our study demonstrates that thymocytes are exquisitely sensitive to changes in p56<sup>cko</sup> activity during the transition from the DP to SP stage. The lck distal promoter-driven expression of p56<sup>cko</sup>F505 at ~2.5- to 3-fold over endogenous wild-type p56<sup>cko</sup> levels causes a 5-fold increase in the number of SP thymocytes (Fig. 1D). This result is in marked contrast to the effects of p56<sup>cko</sup>F505 expression under the control of the lck proximal promoter (pLGF), which blocks generation of mature SP thymocytes (38). Several lines of evidence strongly suggest that activated p56<sup>cko</sup> drives differentiation of mature SP thymocytes in dLGF mice. First, cell cycle analyses indicate that the proportion of proliferating cells in the DP and SP compartments did not increase in dLGF mice (Fig. 3). Thus, massive expansion of SP thymocytes cannot easily explain the increased number and representation of these cells. Second, SP thymocytes from dLGF animals express mature T cell markers: CD69 and Qa-2 are both induced, HSA is down-regulated, and mutually exclusive expression of the CD4 and CD8 coreceptors takes place. These results suggest that the presence of p56<sup>cko</sup>F505 permits an increased number of DP thymocytes to survive and undergo final maturation in the thymus.

Interestingly, surface TCR-CD3 and coreceptor expression is notably diminished in the DP and particularly in the SP compartment in dLGF animals. Our data show that production of TCR-β-chains is normal in these mice. We further considered the possibility that p56<sup>cko</sup>F505, acting as an agent that delivers all aspects of positively selecting signal, may cause premature cessation of the α-chain gene rearrangement in dLGF mice by shutting off the recombiningase-activating gene 1 (RAG1) and RAG2 genes (45–48). However, TCR α-chain production occurs normally in dLGF mice, as judged by the level of endogenous α-chains expressed in dLGF-TCR double-transgenic mice (J. Alberola-Ila, personal communication). Previous studies indicate that activated p56<sup>cko</sup> can down-regulate surface TCR by a posttranslational mechanism. Ab-mediated ligation of coreceptors, which presumably activates p56<sup>cko</sup>, decreases surface TCR expression in DP thymocytes (49, 50), whereas transgenic expression of dominant-negative p56<sup>cko</sup> increases surface TCR expression (39). Subsequently, expression of activated p56<sup>cko</sup> (p56<sup>cko</sup>F505) was correlated with trafficking of TCR-CD3 complexes to lysosomal compartment for degradation (43). Given that TCR-β protein levels are normal in DN thymocytes and that TCR α-chain production is normal, posttranslational degradation is almost certainly responsible for the reduced surface expression of TCRs in dLGF animals. It is also possible that, in the presence of activated p56<sup>cko</sup>, cells expressing fewer TCRs on their surfaces are preferentially selected and survive. However, down-regulation of the TCR clearly takes place in the DP compartment (before selection), indicating that the catalytically active p56<sup>cko</sup> can directly down-modulate surface expression of this receptor. The effects of p56<sup>cko</sup> activation on coreceptor expression remain unclear. However, in vivo administration of anti-CD4 mAbs has been shown to diminish CD4 expression in addition to its effect on surface TCR expression (49), suggesting that p56<sup>cko</sup> may play a role in regulation of coreceptor expression or that expression of coreceptors is in some way coordinately regulated with that of TCR-CD3.

Current models of thymocyte selection posit that the avidity of the combined interactions among the TCR, coreceptor, and the MHC influences the cell fate choice in one of three ways. If the
combined interaction is too weak (or does not occur), cells die by
gn. If the interaction is too strong, cells die by negative
le. In contrast, those cells that interact with intermediate af
The fact that p56<sup>64</sup>F505 increases the number of
Quantitatively, this may mean that p56<sup>64</sup>F505
s increase signal strength to permit survival of cells that would
p56<sup>64</sup>F505 also increases the number of
due to low expression. This was true, for all
cells that possess p56<sup>64</sup>F505 may potentially survive and mature.
The tyrosine-to-phenylalanine mutation renders p56<sup>64</sup> consti
atively active, which has been shown to greatly potentiate Ag re
ceptor-mediated signals in T cells (32). Thus, we tested whether
the p56<sup>64</sup>F505-driven differentiation process required TCR li
ation at all, by breeding dLGFl animals directly onto MHC class I
deficient, or double-deficient background. Our data show that
p56<sup>64</sup>F505 drives both maturation of CD<sup>8</sup> SP thymocytes in the
absence of MHC class I and maturation of CD<sup>4</sup> SP thymocytes
in the absence of MHC class II, implying that constitutively acti
ated p56<sup>64</sup>F505 can direct thymocyte maturation in the absence
of appropriate MHC engagement. It is unlikely that the effect of
p56<sup>64</sup>F505 expression is to promote aberrant selection of SP cells
on mismatched MHC molecule (52) because CD<sup>4+</sup> and CD<sup>8+</sup> SP
thymocytes emerge even in MHC double-deficient dLGFl animals.
These results strengthen our view that p56<sup>64</sup>F505 drives survival
and maturation of thymocytes independent of TCR ligation by
MHC. The ability of activated p56<sup>64</sup> to substitute for pre-TCR-de
pendent signals has been previously demonstrated. In RAG-1
deficient mice crossed with pLGFl-transgenic animals, expression
of p56<sup>64</sup>F505 (under the control of lck proximal promoter) was
sufficient to overcome the block at the DN-to-SP transition (53).
Our results demonstrate that the presence of an activated form of
p56<sup>64</sup> can bypass the requirement for TCR ligation during the
DP-to-SP transition. The phenotype of dLGFl mice is also similar
to the phenotype of mice lacking CsK, a negative regulator of
p56<sup>64</sup> (54). In these mice, the absence of CsK in immature thy
mocytes drove thymocyte differentiation through the DP and SP
stages, independent of TCR ligation. The data from our study im
ply that CsK deficiency leads to hyperactivation (or prolonged ac
tivation) of p56<sup>64</sup>, which in turn drives thymocyte maturation.

Mature T cells that develop in dLGFl animals exit the thymus
and circulate in the periphery but exhibit limited functional ca
pacity. Purified CD<sup>4+</sup> T cells from MHC class II<sup>−/−</sup>/dLGFl animals
proliferated only weakly in response to allogeneic stimulators (Fig.
5C), although stimulation with ionomycin and phorbol ester re
stored the response significantly (Fig. 5D). These results are per
haps not surprising, given that T cells from dLGFl mice possess
substantially decreased levels of surface TCR, and these cells may
remain refractory to TCR engagement. Alternatively, the presen
tence of p56<sup>64</sup>F505 in mature T cells may be interpreted as TCR
engagement and, in the absence of appropriate costimulatory
signal, may result in anergy. The lack of IL-2 production during

T cell activation has been previously correlated with anergy
(55). IL-2 production by purified CD<sup>4+</sup> T cells from MHC class II<sup>−/−</sup>/dLGFl mice was indeed greatly diminished in our exper
iments (data not shown), suggesting that anergy in vivo may at
least partially account for the observed unresponsiveness of
these cells in vitro. Finally, the TCR repertoire in dLGFl mice
may be less sensitive to distinguishing between self and nonself
because the constitutively activated p56<sup>64</sup> drives thymocyte
differentiation regardless of individual TCR specificity. In this
scenario, relative frequencies of self-restricted T cells would be
reduced, accompanied by an increased production of potentially
self-reactive T cells. In support of this hypothesis, CD8<sup>+</sup> cy
totoxic T cells from dLGFl mice exhibited elevated levels of
cross-reactivity toward syngeneic targets in vitro, relative to
non-Tg controls (data not shown).

We also present data that suggest that total p56<sup>64</sup> activity can
fluence the relative representation of CD4<sup>+</sup> vs CD8<sup>+</sup> SP thymocyte
population. Expression of p56<sup>64</sup>F505 increased the repre
sentation of CD4<sup>+</sup> SP thymocytes (Fig. 7A), a trend that was re
verted by intercrossing the dLGFl mice to further increase in the
abundance of transgene protein (Fig. 7B). We speculate that at low
levels, p56<sup>64</sup>F505 promotes differentiation and/or survival of CD4
lineage cells as a result of its preferred interaction with CD4 (28).
At higher levels of p56<sup>64</sup>F505, the CD4:CD8 ratio returns to nor
mal as the p56<sup>64</sup>F505-CD4 interactions become saturated and
p56<sup>64</sup>F505-CD8 interactions increase. Whether p56<sup>64</sup>F505 sends
an instructive signal to direct lineage commitment or rather pro
motes survival of precommitted thymocytes remains unclear. Pre
viously, a model was proposed in which the relative strength of the
signal generated by coreceptor-associated p56<sup>64</sup> determines CD4
and CD8 lineage preference (24). The quantitative interpretation
of this model and our current data is consistent with the observation
that p56<sup>64</sup> interacts with the cytoplasmic tail of the CD4 mole
ule with higher affinity than it does with the CD8 molecule (28). Oth
er studies have indicated that thymocyte lineage commitment occurs
independent of TCR-MHC specificity and even independent of
coreceptors, before selection (17, 19, 20). In our system, the de
ficiency of MHC exerted little effect on the ability of p56<sup>64</sup>F505 to
fluence CD4:CD8 ratios (Figs. 4A, 5A, and 6A), suggesting that
the total activity of p56<sup>64</sup>, rather than coreceptor ligation (by
MHC), determines lineage choice and/or survival. Two recently
published studies further strengthen our view that total p56<sup>64</sup> ac
tivity determines the CD4:CD8 ratios. In one study, the level of
total p56<sup>64</sup> activity was shown to override MHC restriction of a
given TCR in driving the lineage commitment decisions (56). In a
second study, reconstitution of wild-type p56<sup>64</sup> in lck<sup>−/−</sup> mice
resulted in maturation predominantly of the CD4<sup>+</sup> SP population
(57). The observed skewing toward the CD4 lineage in this system
is consistent with our data that increased p56<sup>64</sup> activity initially
favors differentiation of CD4<sup>+</sup> cells (Fig. 2B) and suggests that
the level of activity achieved by the expression of the wild-type p56<sup>64</sup>
must fall within this range. A comprehensive series of experiments
now supports the view that p56<sup>64</sup> is a pivotal regulator of early
thymocyte development entrained by the pre-TCR. Our studies
make plain that the selection processes ordinarily attributed to
TCR signaling later in thymocyte maturation can also be mimicked
by simple activation of p56<sup>64</sup>. This observation encompasses not
only the emergence of SP cells with “mature” cell surface pheno
types but also the relative proportion of thymocytes that display
CD4<sup>+</sup> vs CD8<sup>+</sup> characteristics. It is plausible that the strength of
the p56<sup>64</sup>-derived signal can by itself direct all aspects of lineage
commitment during thymocyte maturation.
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